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RESEARCH ARTICLE

Survival of *E. coli* O157:H7 in Yogurt Incubated until Two Different pH Values and Stored at 4°C [1]

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Summary

In the present study the survival of *Escherichia coli* O157:H7 in yoghurt produced at two different initial pH levels was investigated. Samples were contaminated with two different doses of *E. coli* O157:H7 (10^3 cfu/mL and 10^5 cfu/mL). Yoghurt samples were taken from incubation at pH 4.0 and 4.6. Incubated samples were stored at 4°C for 14 days. In the samples, viable counts of *E. coli* O157:H7, pH and titratable acidity were determined on day 0 and the 1st, 2nd, 4th, 6th, 8th and 14th days of storage. *E. coli* O157:H7 survived in yoghurt produced at pH 4.0 and 4.6 in the both contamination level. The survival of *E. coli* O157:H7 was significantly lower at pH 4.00 than at pH 4.60 ($P<0.001$) and at 10^3 cfu/mL than at 10^5 cfu/mL contamination level ($P<0.001$).

Keywords: *Yoghurt, Escherichia coli O157:H7, pH*

İki Farklı pH Değerine Kadar İnkübe Edilen ve 4°C'de Depolanan Yoğurtlarda *E. coli* O157:H7'nin Canlı Kalma Durumu

Özet

Bu çalışmada iki farklı başlangıç pH'sı ile üretilen yoğurtlarda *Escherichia coli* O157:H7'nin canlı kalma durumu araştırılmıştır. Örnekler iki farklı dozda *E. coli* O157:H7 (10^3 cfu/mL ve 10^5 cfu/mL) ile kontamine edilmiştir. Yoğurt örneklerinin inkübasyonları pH 4.0 ve 4.6'da sonlandırılmış ve örnekler 4°C'de 14 gün depolanmıştır. Örneklerde depolamanın başlangıcında ve 1, 2, 4, 6, 8 ve 14. günlerinde *E. coli* O157:H7 sayılar, pH ve asitlik değerleri belirlenmiştir. *E. coli* O157:H7 pH 4.0 ve 4.6 başlangıç pH'sı ile üretilen her iki örnek grubunda da canlı kalmıştır. Ancak *E. coli* O157:H7 canlılığı 4.0 başlangıç pH'lı örneklerde, 4.6 pH'lı olanlara göre ve 10^3 cfu/mL kontaminasyonlu örneklerde 10^5 cfu/mL kontaminasyonlu olanlardan çok önemli derecede ($P<0.001$) düşük olmuştur.

Anahtar sözcükler: *Yoğurt, Escherichia coli O157:H7, pH*

INTRODUCTION

Yoghurt is a dairy product produced by bacterial fermentation of milk. Fermentation of the milk sugar (lactose) produces lactic acid, which acts on milk protein to give yoghurt its characteristic textural and sensorial properties. It is believed that yoghurt originated in the Middle East, and the evolution of this fermented product through the ages can be attributed to the culinary skills of the nomadic people living in that part of the world [1]. The word is derived from Turkish "*yoğurt*" [2,3]. Yoghurt is one of the most important dairy products and are consumed approximately 2.293.431 tons/year in Turkey [4].

The serotype O157:H7 among *E. coli* strains is highly virulent. This serotype not only associated with a number of highly publicized food-borne outbreaks, but also it is able to survive acidic conditions [5]. *Escherichia coli* O157:H7, a Gram-negative, facultative anaerobe, is a food-borne pathogen well-known in the dairy and food industries [6,7]. It was first recognized as a pathogen in 1982 [6,8] and is considered to be an important causative agent of diarrhoea, hemorrhagic colitis and hemolytic uremic syndrome (HUS) [8,9]. It has been identified as the causative agent of severe enteric illness which can strike suddenly, affect all

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ages, has a low infectious dose and can cause death [10]. Foods of animal origin were reported to be the primary sources of *E. coli* O157:H7 infection [11].

Subsequent outbreaks, which predominantly involve the *E. coli* O157:H7 serotype, have been reported in foods including minced beef, cheese, sprouts, salami, and apple cider [5]. Several authors have demonstrated that *E. coli* O157:H7 can survive in dairy products over several days and weeks: yoghurt and Colby, Romano and Feta cheeses, Cheddar cheese, sour cream, buttermilk, and cheese, goat milk lactic cheeses and Cheddar cheese whey [12-17]. It has been observed that *E. coli* O157:H7 inoculated into yoghurt may remain viable form a few hours at 30-40°C, and up to 1-8 days while refrigerated [18]. The survival of this microorganism for up to several weeks illustrates the potential health risks associated with post-processing contamination of even low levels of *E. coli* O157:H7 in several dairy products [15]. It is reported that there was only 1 outbreak caused by *E. coli* O157:H7 involving yoghurt [19] but possibility of contamination does exist [18].

According to different research pH values of yoghurt produced in Turkey varies between approximately 3.5-4.6 [20-23]. Strains of *E. coli* O157:H7 have been found to be relatively acid-tolerant and the infectious dose can be less than 50 cells [24]. Hence in this study, two different pH values and two different contamination levels were selected. The aim of this study was to evaluate the behavior of *E. coli* O157:H7 in yoghurts produced at two different initial pH values and stored at 4°C during 14 days storage period.

MATERIAL and METHODS

Yoghurt Production

Cow's milk was used to prepare yoghurt samples. Milk was standardized to approximately 3.8% fat and 12% fat-free dry matter content (according to the Turkish Food Codex Fermented Milks Regulation (2009/25) for full-fat yoghurt) [25]. Fat-free dry matter of the milk was standardized with fat-free milk powder (Pinar A.S., Izmir, Turkey) and its fat with cream. Standardized milk was heated to approximately 90°C for 5 min and then cooled to 45°C in a water bath.

Commercial Direct Set Lyophilized starter (DELVO-YOG CY-223-DSL, Delft, The Netherland) including *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* was used as the starter culture and added to standardized milk at levels recommended by the manufacturer.

Inoculated milk was divided into three groups. The first group was not contaminated with *Escherichia coli* O157:H7 and used as the control group. The other two groups were separately inoculated with dilutions of *Escherichia coli* O157:H7 to achieve an initial population of *ca*. 10^3 cfu/mL (low inoculation) and *ca* 10^5 cfu/mL (high inoculation) respectively.

All the samples control and contaminated with different level of *E. coli* O157:H7 distributed into 100 mL sterile glass jars and incubated at 42°C. Fermentation process was continued until the desired acidity (pH 4.6 and pH 4.0) was reached. Fermentation of half of the samples in the groups was finished at pH 4.6 and the other half was finished at pH 4.0. After fermentation, contaminated and the control group yoghurt samples were stored at 4°C for 14 d. In the samples, viable counts of *E. coli* O157:H7, pH and titratable acidity were determined on day zero and on the 1st, 2nd, 4th, 6th, 8th and 14th days of storage.

Preparation of Escherichia coli O157:H7

E. coli O157:H7 was kindly provided by Prof. S. A. Aytaç (Hacettepe University, Faculty of Engineering, Food Engineering Department, Ankara, Turkey) [26]. Culture was transferred into brain-heart infusion (BHI) broth (Oxoid, UK) at 35±2°C for 24 h. Culture grown 24 h was inoculated on cefixime tellurite sorbitol MacConkey agar (CT-SMAC) at 35-37°C for 24 h. White-colorless colonies on CT-SMAC were transferred into modified tryptic soy (MTS) broth (Oxoid, UK) at 37°C for 24 h. Then, serial dilutions were prepared and bacterial counts were determined for using yoghurt samples. *Escherichia coli* O157:H7 involving initial inoculums levels of *ca* 10^3 cfu/mL and *ca* 10^5 cfu/mL was inoculated into yoghurt samples.

Enumeration of Escherichia coli O157:H7

The glass jars were shaken just before sampling and 1 mL samples were diluted (w/v), in duplicate, in sterile peptone water (Oxoid, UK). 0.1 mL portions from appropriate dilutions were surface-plated onto Sorbitol MacConkey agar (SMAC-Oxoid, UK) including Cefixime Tellurite Selective Supplement (CT-Oxoid, UK). The plates were incubated at 35±2°C for 48 h. After incubation, random selected colonies were confirmed by serology with the *E. coli* O157 latex test (Oxoid, UK).

Chemical Analysis

The pH of the yoghurt samples was measured with a HANNA pH meter (HANNA Instruments, Italy). The titratable acidity (TA, as percent lactic acid) was determined according to the method of AOAC [27].

Statistical Analysis

Data obtained from experiments was analyzed using SPSS software version 8.00 [28]. Variance analyses and Tukey's multiple comparison tests were used to determine significance in the determined characteristic between the samples produced at 4.0 and 4.6 initial pH and contaminated with 10^3 and 10^5 cfu/mL *E. coli* O157:H7. Log_{10} transformations were performed on microbial data.

Principal Components Analysis (PCA) was also performed to show relationships among groups of variables and between objects [29].

RESULTS

Viable counts of *E. coli* O157:H7 showed a slightly increase in milk during fermentation period at 42°C. Counts of *E. coli* O157:H7 in yoghurt samples produced at two different initial pH, contaminated *ca*. 10^3 or 10^5 cfu/mL pathogen and stored at 4°C were given in *Fig. 1* and *Fig. 2*. During the 14-day storage period, *E. coli* O157:H7 was not found in any uncontaminated yoghurt control samples. In the yoghurt samples produced 4.6 initial pH and contaminated low level *E. coli* O157:H7, counts of *E. coli* O157:H7 decreased from 3.93 $\log_{10}$ cfu/mL to 2.19 $\log_{10}$ cfu/mL on day 0 and 6th respectively. Viable cells number of *E. coli* O157:H7 in these samples decreased to undetectable level after 8 days. In the same samples contaminated with high level pathogens, the counts of *E. coli* O157:H7 reduced to 2.25 $\log_{10}$ cfu/mL on day 14th.

In the present study, *E. coli* O157:H7 numbers in yoghurt produced at initial pH level 4.0 and inoculated with 10^5 cfu/mL *E. coli* O157:H7 decreased to 2.07 $\log_{10}$ cfu/mL after 8 days and, after 14 days viable counts of *E. coli* O157:H7 decreased to undetectable level. In the yoghurt at same pH and with 10^3 cfu/mL contamination level, viable counts of *E. coli* O157:H7 decreased to undetectable level after 4 days.

In all samples produced at 4.0 and 4.6 initial pH and contaminated whit 10^3 and 10^5 cfu/mL *E. coli* O157:H7, differences among the survival of *E. coli* O157:H7 were significant ($P<0.001$). Survival of *E. coli* O157:H7 was affected ($P<0.001$) by initial pH of yoghurt, contamination level of *E. coli* O157:H7 and storage time.

Variances in pH values of yoghurt samples produced in the present study were shown in *Table 1*. The pH values showed a regular variance in samples produced at pH 4.0. The pH of each of the two sample groups contaminated with 10^3 and 10^5 cfu/mL *E. coli* O157:H7 and the control (Day 0 should be excluded as this sample did not contain pathogen) decreased from 4.00 to 3.80 during storage period. The same regular variance in pH levels during storage was observed in the samples produced at pH 4.6. The pH values of these samples decreased from 4.6 to 4.30 at the end of the storage period. The pH values of yoghurt

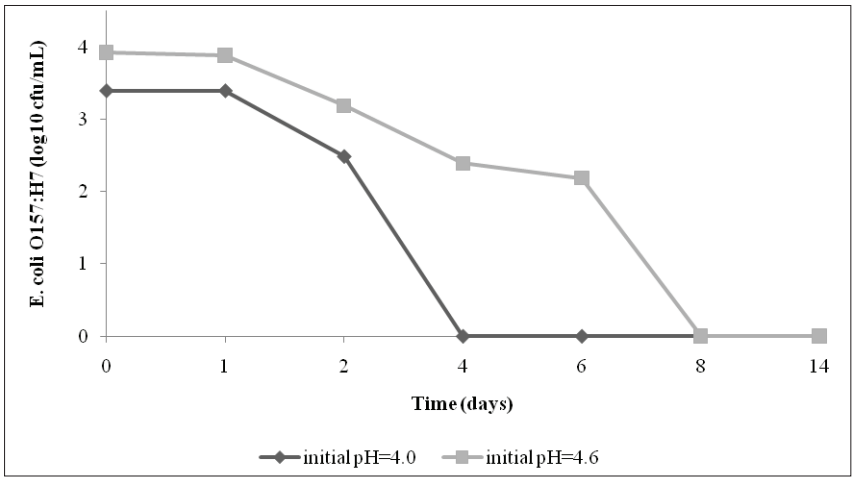

Fig 1. Survival of *E. coli* O157:H7 in yoghurt inoculated with 10^3 cfu/mL *E. coli* O157:H7 during storage at 4ºC

Şekil 1. 10^3 cfu/mL *E. coli* O157:H7 ile kontamine edilen ve 4ºC'de depolanan yoğurtlarda *E. coli* O157:H7'nin canlı kalma durumu

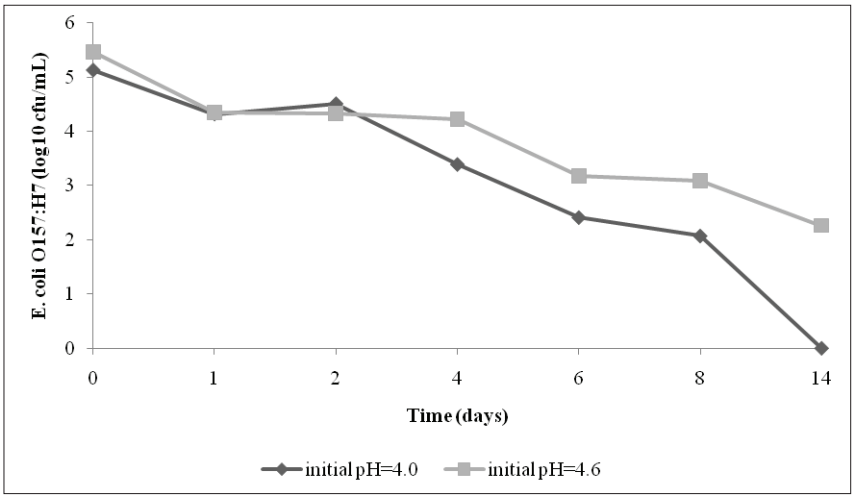

Fig 2. Survival of *E. coli* O157:H7 in yoghurt inoculated with 10^5 cfu/mL *E. coli* O157:H7 during storage at 4ºC

Şekil 2. 10^5 cfu/mL *E. coli* O157:H7 ile kontamine edilen ve 4ºC'de depolanan yoğurtlarda *E. coli* O157:H7'nin canlı kalma durumu

samples were affected (P<0.001) by initial pH of yoghurt and storage time.

Acidity values of all samples increased during storage period *(Table 2)*. In the yoghurt produced at pH 4.6 and contaminated with 0, 10^3 and 10^5 cfu/mL *E. coli* O157:H7, acidity values were determined on day 0 and 14 as 0.76-1.03, 0.85-1.14 and 0.89-1.16, respectively. In the samples produced at pH 4.0 and contaminated by *E. coli* O157:H7 at different levels, (0, 10^3 and 10^5 cfu/mL) on the same days, titratable acidity values were determined as 1.47-1.70, 1.52-1.72 and 1.48-1.80, respectively. The effect of initial pH of yoghurt, contamination level of *E. coli* O157:H7 and storage time was significant (P<0.001) on the titratable acidity values of yoghurt samples.

Results of the principal component analysis (PCA) showed that principal components (PC) 1 and 2 described

Table 1. *Change of pH in yoghurt produced at 4.0 and 4.6 initial pH during storage at 4°C*
Tablo 1*. Başlangıç pH'ları 4.0 ve 4.6 olan ve 4°C'de depolanan yoğurtlarda asitlik değişimleri*

Storage Period (Day)	4.00 Initial pH			4.60 Initial pH		
	Control	Low Inoculation	High Inoculation	Control	Low Inoculation	High Inoculation
0	4.00±0.08	4.00±0.14	4.00±0.09	4.60±0.07	4.60±0.03	4.50±0.01
1	4.00±0.14	4.00±0.21	4.00±0.07	4.50±0.10	4.50±0.07	4.50±0.05
2	3.90±0.07	4.05±0.24	3.90±0.03	4.50±0.01	4.50±0.01	4.50±0.04
4	3.90±0.08	3.90±0.01	3.90±0.00	4.50±0.03	4.40±0.04	4.40±0.03
6	3.90±0.01	3.90±0.03	3.90±0.01	4.40±0.10	4.40±0.01	4.40±0.01
8	3.90±0.03	3.90±0.01	3.80±0.03	4.40±0.01	4.40±0.00	4.30±0.01
14	3.80±0.04	3.80±0.03	3.80±0.07	4.30±0.10	4.30±0.03	4.30±0.07

Table 2. *Change of pH in yoghurt produced at 4.0 and 4.6 initial pH during storage at 4°C*
Tablo 2*. Başlangıç pH'ları 4.0 ve 4.6 olan ve 4°C'de depolanan yoğurtlarda pH değişimleri*

Storage Period (Day)	4.00 Initial pH			4.60 Initial pH		
	Control	Low Inoculation	High Inoculation	Control	Low Inoculation	High Inoculation
0	1.47±0.01	1.52±0.03	1.48±0.03	0.76±0.01	0.85±0.01	0.89±0.03
1	1.49±0.04	1.54±0.01	1.49±0.03	0.82±0.00	0.89±0.01	0.93±0.03
2	1.52±0.06	1.58±0.03	1.59±0.11	0.83±0.03	0.93±0.03	0.94±0.01
4	1.55±0.03	1.59±0.01	1.63±0.03	0.84±0.01	0.95±0.01	1.09±0.03
6	1.57±0.01	1.60±0.04	1.71±0.01	0.85±0.03	0.99±0.01	1.11±0.07
8	1.58±0.03	1.64±0.03	1.76±0.06	0.89±0.01	1.02±0.01	1.15±0.11
14	1.70±0.04	1.72±0.04	1.80±0.14	1.03±0.03	1.14±0.03	1.16±0.01

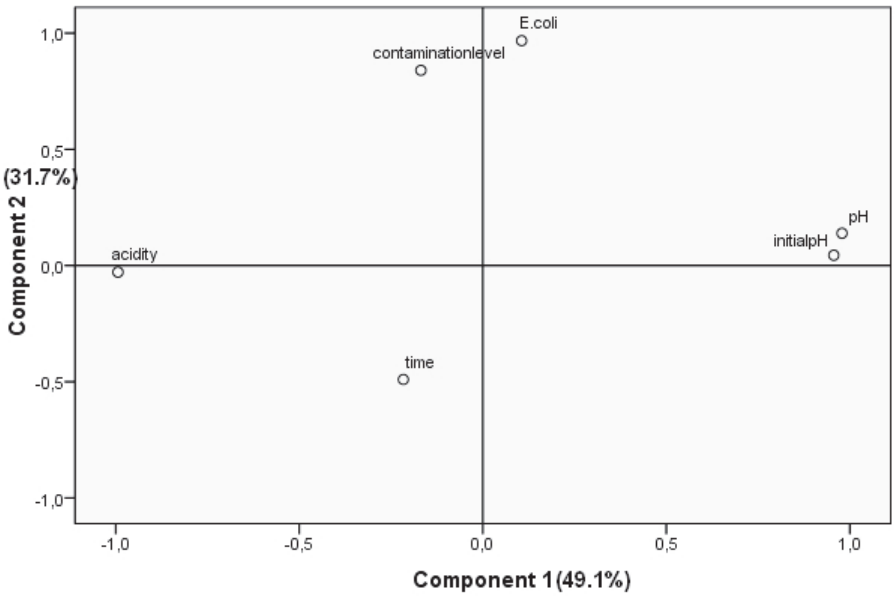

Fig 3. Principal component biplot of counts of *E. coli* O157:H7, initial pH, storage time, pH, titratable acidity and contamination level of *E. coli* O157:H7 in yoghurt

Şekil 3. Yoğurtta *E. coli* O157:H7 canlılığı, başlangıç pH'sı, depolama süresi, pH, asitlik ve *E. coli* O157:H7'nin kontaminasyon seviyelerine ait Principal Component analizi

had about 80.9% of the total variation of the events: 49.1% PC1 and 31.7% PC2 *(Fig. 3)*. PC1 was heavily loaded on initial pH, pH and titratable acidity of yoghurt samples; whereas PC 2 was loaded on contamination level of *E. coli* O157:H7, storage time and counts of *E. coli* O157:H7. The PCA analysis presented a strong positive correlation between initial pH and pH and a strong negative correlation between initial pH and titratable acidity of samples. The PCA results showed that there was a strong positive correlation between contamination levels of *E. coli* O157:H7 and counts of *E. coli* O157:H7 and there was a strong negative correlation between storage time and counts of *E. coli* O157:H7. Results also showed that there was low positive relationship between the pH and the counts of *E. coli* O157:H7 and there was high negative relationship between the pH and titratable acidity *(Fig. 3)*.

DISCUSSION

Viable counts of *E. coli* O157:H7 showed a slight increase in milk during fermentation period at 42°C. Similar result reported by Bachrouri et al.[30]. On contrary, Massa et al.[18] stated that the viable counts of *E. coli* O157:H7 which were inoculated into milk and yoghurt did not change during fermentation at 42°C. Chang et al.[31] stated that *E. coli* O157:H7 grow in skim milk fermented with either *L. delbrueckii* spp. *bulgaricus* or *L. casei* ssp. *casei*.

Bachrouri et al.[30] examined the survival of *E. coli* O157:H7 during milk fermentation at 43°C and storage of home-made yoghurt (initial pH = 4.6) at refrigeration temperatures (2, 4, or 8°C). They reported that *E. coli* O157:H7 counts decreased undetectable level after 21 d at 2 or 4°C and after 7 d stored at 8°C. Guraya et al.[32] reported that, in traditional yoghurts inoculated with 10^5 cfu/g *E. coli* O157:H7 after production, *E. coli* O157:H7 survived at pH 4.65 for 35 days, whereas it survived at pH 4.17 for 7 days. Kasımoğlu and Akgün [33] studied the survival of *E. coli* O157:H7 in traditional and acidophilus yoghurts which were inoculated at levels of different doses (10^2, 10^4 and 10^6 cfu/mL) of *E. coli* O157:H7. They determined that the elimination times of *E. coli* O157:H7 in traditional yoghurts were 48 h for 10^2 and 10^4 cfu/mL contamination levels and 72 h for 10^6 cfu/mL contamination level. In their study, elimination times of *E. coli* O157:H7 were determined as 3 h for 10^2 cfu/mL, and as 48 h for 10^4 and 10^6 cfu/mL in acidophilus yoghurt.

Similarly, in our study, it was determined that in yoghurts inoculated with 10^5 cfu/mL *E. coli* O157:H7 and an initial pH of 4.6, the number of *E. coli* O157:H7 viable counts decreased to 2.6 $\log_{10}$ cfu/mL as a result of storage period and survived even on the 14th day. *E coli* O157:H7 in the yoghurt samples inoculated with 10^3 cfu/mL pathogen and produced 4.6 initial pH, were not found after 8 day.

Dineen et al.[15] found that the survival time of *E. coli* O157:H7 either inoculated into traditional yoghurt (pH 4.0) or into acidophilus yoghurt (pH 4.0), at the post-processing stage, was either 14 or 6 days, respectively. Akdemir-Evrendilek [22] noticed that *E. coli* O157:H7 in the plain yoghurt, yoghurt drink and fresh-salted yoghurt inoculated with 10^6-10^7cfu/mL *E. coli* O157:H7, survived for 45 days at 4 and 22°C. Bachrouri et al.[34] studied the behavior of *E. coli* O157:H7 strains during the storage of plain live yoghurt at 4°C. The researchers reported that *E. coli* O157:H7 counts decreased from 4.4 log cfu/g to 3.6 log cfu/g after 72 h and was not detected after 312 h. In these samples the pH values ranged from 3.94 to 4.11 during the storage.

E. coli O157:H7, with an optimum growth at pH around 7.0, can grow in the pH range between 4.5 and 9.0 [35]. It has been reported that this pathogen can survive in some acidic foods such as sweet pickle (pH 2.8) [36], yoghurt (pH 4.5) [18] and mayonnaise (pH 3.65) [37]. Yoghurt has always been considered intrinsically safe because of its acidic nature. However the resistance of these pathogens to low pH enables them to survive in high acidic food such as yoghurt until they are consumed [15,38-41].

Massa et al.[18] investigated the survival of *E. coli* O157:H7 in traditional and bifidus yoghurt at contamination levels of 10^3 and 10^7 cfu/mL. In this research, it was determined that pH values of traditionally-produced yoghurt samples contaminated with low level pathogen changed between 6.6 and 4.5, while it changed between 6.6 and 4.4 in the samples contaminated with high level pathogen in the 0th hour and 7th day. Kasımoğlu and Akgün [33] studied traditional and acidophilus yoghurts which were stored at 4°C. They controlled the pH variances of samples, which they contaminated with *E. coli* O157:H7 at levels of 10^3, 10^5 and 10^7 cfu/mL and they found that during the refrigerated storage time of traditional yoghurt samples in all treatments, pH dropped from 4.6 to 4.2 and 3.96 at 24, 48 and 72 h, respectively. A similar result was also reported by Gülmez and Güven [42]. In the present study also, it was determined that pH values showed similar, regular variances at both contamination levels.

In this study it was found that both the initial pH values of the yoghurt samples and contamination level of the pathogen were significant ($P<0.001$) factors on the survival of *E. coli* O157:H7. The results of the present study show that *E. coli* O157:H7 survived in yoghurt during fermentation and storage period even at low pH. Although yoghurt is produced from heat-treated milk, potential contamination after heat treatment might negatively affect the reliability of the product and present a significant health risk to consumers. Consequently, high-acidic foods such as yoghurt should not be considered as safe. Because some pathogen such as *E. coli* O157:H7 are resistant to acidic condition and can survive for up to several weeks in several dairy products. According to

results obtained from our study it can be said that post-fermentation contamination and contamination level at storage period are important risk factors regarding the safety of the yoghurt.

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