



Survival of *Escherichia coli* O157:H7 and *Staphylococcus aureus* during the fermentation and storage of kefir

Merih KIVANC^{1*}, Evrim YAPICI¹

Abstract

The survival of *Escherichia coli* O157: H7 and *Staphylococcus aureus*, which are foodborne pathogens was investigated during fermentation and storage of kefir. The kefir grains were inoculated with 5% of the whole fat UHT cow milk and the *E. coli* O157: H7 and *S. aureus* cultures were added separately to give 10^5 cfu mL⁻¹ and left to incubate at 22 °C for 24 hours at first stage. During the incubation, the bacteria and yeast counts, pH and titratable acidity amounts were determined. In the second stage, *E. coli* O157: H7 and *S. aureus* cultures were separately added to produce 10^5 cfu mL⁻¹ of kefir and was stocked at 5 °C for 3 days. Every day samples were taken and the same tests were done. During fermentation, in all samples the initial while TA was 0.14%, the fermentation was 1.0% and 1.01% at the end. The kefir samples were pH 4.5 at the end of the 3 day period of storage. *S. aureus* was significantly inhibited while *E. coli* was not inhibited during the fermentation process. During storage, *E. coli* O157: H7 and *S. aureus* were inhibited within 2 days. The kefir has the quality of being a healthy product during fermentation and storage.

Keywords: Kefir; *E. coli* O157:H7; *S. aureus*; lactic acid bacteria; yeast.

Practical Application: Kefir is a functional fermented dairy product very useful for health. The consumption is quite high. The life span of two important food born microorganisms within this product has been determined. If *S. aureus* and *E. coli* contamination occurs after fermentation, the bacteria may be inhibited within 48 hours. This is important for post-fermentation contamination.

1 Introduction

Kefir is a fermented milk drink containing a slightly acidic and yeast flavor, naturally carbonated fermented milk with consistently refreshing sweet flavor. It is a milk product prepared with the addition of kefir grains into the milk. Milk is incubated with the kefir grains until lactose is fermented for one day or more at a room temperature. A sour, carbonated and lightly alcoholic beverage is obtained. Kefir grains are composed of a combination of bacteria and yeast in protein, lipid and sugar matrix (Prado et al., 2015). Kefir also contains lactic acid bacteria (LAB) such as *Lactobacillus brevis*, *Lactobacillus plantarum*, *L. paracasei* spp *paracasei*, *L. lactis* spp *lactis*, *L. acidophilus*, *Leuconostoc mesenteroides*, and *Streptococcus* species as well as yeast such as *Kluyveromyces marxianus*, *K. wickerhamii*, *Saccharomyces cerevisiae*, *Pichia angusta*, *P. guilliermondii*, *Candida glabrata*, *C. kefyr* and sometimes acetic acid bacteria (Kivanç & Yapıcı, 2015; Ünal & Arslanoğlu, 2013; Schneedorf & Anfiteatro, 2004; Garrote et al., 2001).

Saccharomyces and *Torula* yeast can control the harmful yeasts by inhibiting them (Farnworth & Mainville, 2003). In addition to the beneficial bacteria and yeasts, kefir also contains essential minerals and essential amino acids for the body. It also abundantly contains calcium and magnesium for healthy nervous system. The phosphorus it contains helps to use carbohydrates, fats

and proteins for cell growth, maintenance and energy in our bodies. Kefir is rich in vitamin B12, vitamin B1 and vitamin K (Bourrie et al., 2016). Kefir has antimicrobial effect on many Gram positive and Gram negative bacteria and yeast. In vitro tests with kefir cell free extracts, the growth of *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Clostridium tyrobutyricum* and *Listeria monocytogenes* was inhibited (Kim et al., 2016; Kivanç & Yapıcı, 2015). In general, the antimicrobial activity of kefir is caused by bacteriocin-like substances such as lactic acid, lactic acid, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde and/or bacteriocin produced by LAB. In addition, kefir has antitumor, anti-inflammatory and hypocholesterolemic effects as well as immunosuppressive properties (Leite et al., 2013; Bourrie et al., 2016).

Many bacteria such as *Lactobacillus*, *Streptococcus*, *Bacteroides*, *Escherichia*, *Bifidobacterium* and *Clostridium* are colonized in the normal gastrointestinal system. *Lactobacillus* is present in the normal microflora of the gastrointestinal system (Marshall, 1993). It was found that probiotic strains of *Lactobacillus* had inhibitory effects on the growth of pathogenic bacteria, had acid and gall resistance, adherence to intestinal epithelial cells and positive effects on the host health (Finlay & Falkow, 1989; Jacobsen et al., 1999).

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¹Department of Biology, Faculty of Science, Eskişehir Technic University, Eskişehir, Turkey

*Corresponding author: mkivanc@anadolu.edu.tr

Escherichia coli O157:H7 is one of the most dangerous food pathogens. *E. coli* O157:H7 that produces Verotoxin can be converted into non-mucosal ischemia, hemorrhagic colitis and hemorrhagic uremic syndrome (HUS), which can be complicated by severe anemia and renal failure. It can cause major worldwide food and water epidemics (Buchanan & Doyle, 1997; Mead & Griffin, 1998).

Since *S. aureus* can grow in very large environmental conditions, they can frequently contaminate the animals. Contamination may be originated from raw material or caused during production by people and team and equipment used. Staphylococcal food poisoning is one of the most common diseases of foodborne diseases (Hennekinne et al., 2012). Fermented dairy products are the most common products of *S. aureus* food poisoning (Le Loir et al., 2003). Most strains produce one or more enterotoxins, which are the causes of observed gastrointestinal symptoms (Tamarapu et al., 2001).

In the present study, we tried to evaluate the behavior of *E. coli* O157:H7 and *S. aureus* in kefir, which are important for public health during the fermentation and storage of kefir, the fermented dairy product whose benefits has been proven today and determines the life process of pathogenic bacteria in this product.

2 Materials and methods

2.1 Preparation of *E. coli* O157:H7 and *S. aureus* culture

E. coli O157:H7 and *S. aureus* used in the study were obtained from the Faculty of Science Microbiology unit of Anadolu University. *E. coli* O157:H7 was inoculated into Tryptic Soy broth (TSB) and *S. aureus* was inoculated into Brain Heart Infusion broth (BHI) and incubated for 24 hours at 37 °C (Chapman, 2000). Then, *E. coli* O157:H7 was inoculated onto Sorbitol MacConkey agar with cefixime tellurid (CT-SMAC) and *S. aureus* was inoculated onto egg yolk tellurite Baird Paker agar (BPA) and both were incubated at 35 °C for 24 hours; and then colony morphology was examined and Gram staining was performed to check the purity in the microscope (Chapman, 2000; Harrigan & McCance, 1996).

2.2 Kefir production

Kefir grains were obtained from home-made kefir in Eskişehir (Turkey). Kefir grains were washed with sterile distilled water. It was then inoculated into full fat UHT cow milk (3.6% fat content) and incubated at 22 °C for 24 hours. After fermentation, the kefir grains were separated from the kefir with the help of a plastic sieve, washed with sterile distilled water and stored in milk at 4 °C. The study was conducted in two stages: Stage I includes the fermentation process and Stage II contains the storage process. In the first group study, 5% kefir grain was added into full fat UHT cow milk. In the second group, *E. coli* and *S. aureus* cultures were separately added into the whole milk UHT cow milk to be 10⁵ cfu mL⁻¹ separately with 5% kefir grain and left to incubate at 22 °C for 24 hours. During the incubation, 0., 1., 2., 6., 12. and at 24 hours, the samples were taken and analyzed. After fermentation, kefir was washed with sterile distilled water, separated with a plastic sieve and kept in milk at 4 °C.

In the stage II of the study, after Kefir was produced and then the Kefir samples without grains were taken into sterile glass jars. *E. coli* and *S. aureus* cultures were separately added to a group of kefir jar in the amount of 10⁵ cfu mL⁻¹ and all the jars were kept at 5 °C for 3 days. Samples were taken and analyzed on a daily basis. The non-bacterial kefir sample was examined as a control. All the studies were carried out in pair parallel.

2.3 Microbiological analysis

Dilutions were prepared from kefir samples and LAB counts were counted on the MRS agar containing 200 ppm cycloheximide and incubated for 24-48 hours at 35 °C under 10% CO₂ conditions. After incubation, they were counted, different appearing colonies were purified, and then Gram staining and catalase assay were performed. Yeast was counted on the yeast peptone dextrose agar (YDP) and by incubated at 25 °C for 48 hours. *E. coli* was grown on CT-SMAC for 24 hours at 35 °C. The colorless colonies formed on CT-SMAC were evaluated. *S. aureus* was counted on BP Agar and incubated at 37 °C for 48 h. The typical black or gray colonies of BP Agar were evaluated as *S. aureus* (Chapman 2000; Harrigan & McCance, 1996). The colonies with different appearances were purified and examined under the microscope by Gram staining.

2.4 Physicochemical analyses

At each sampling time, the pH value of Kefir was measured with a HANNA pH meter (HANNA Instruments, Italy). Titratable acidity (TA, lactic acid in percentage) was determined by titration with 0.1 N NaOH in the presence of phenolphthalein after mixing 25 mL of kefir sample with 10 mL of distilled hot water. Viscosity measurements were performed using a viscometer (Haake Viscotester VT6/7R) at 100 rpm.

All physico-chemical analyzes were performed in four replicates.

3 Results

The pH and titratable acidity values of the Kefir samples during the fermentation and storage period were illustrated in Figures 1, 2, 3 and 4. During fermentation, the pH decreased slowly in all samples (Figure 1). Initially while the pH was 6 in all samples, it eventually decreased to pH 4.5 and 4.6 at the end of fermentation. During storage, the pH did not change significantly. The pH values of kefir were about 4.5 at the end of the storage period (Figure 2). During fermentation, TA% increased after two hours (Figure 3). During storage, TA% value remained the same (Figure 4).

The viscosity (measured in mPa) values of the kefir samples were illustrated in Table 1. The viscosity values increased markedly during fermentation. During the storage of kefir, the viscosity increased until 48th hours and then decreased at 72nd hours.

The changes in the lactic acid bacteria, yeast, *E. coli* and *S. aureus* numbers in kefir fermentation were illustrated in Figure 5. It was found that the lactic acid bacteria and yeast counts were initially 4.74 Log₁₀ cfu mL⁻¹ and 4.58 Log₁₀ cfu mL⁻¹, respectively. At the end of the fermentation process, *E. coli* decreased from

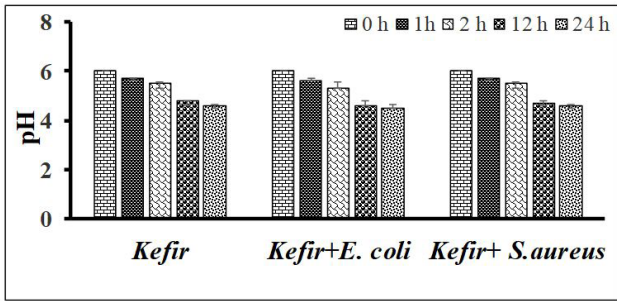


Figure 1. Change of pH values of kefir during the fermentation.

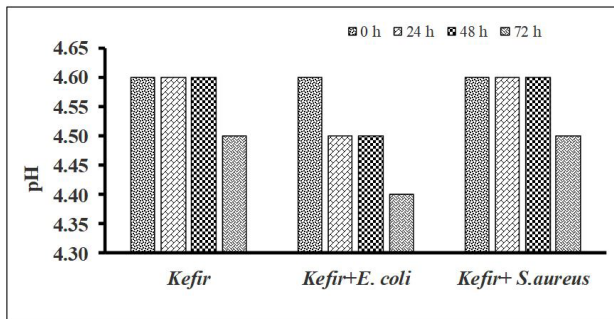


Figure 2. Change of pH values of kefir during the storage 5 °C.

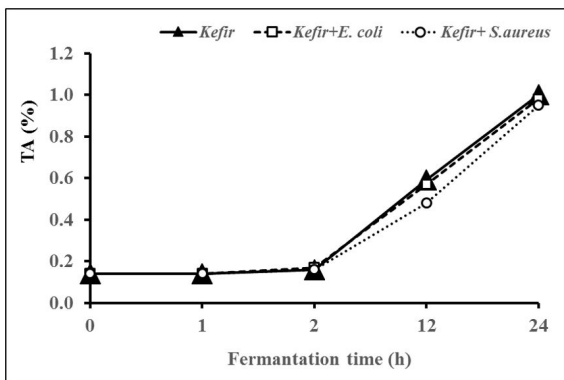


Figure 3. Change of titratable acidity (TA) of kefir during the fermentation.

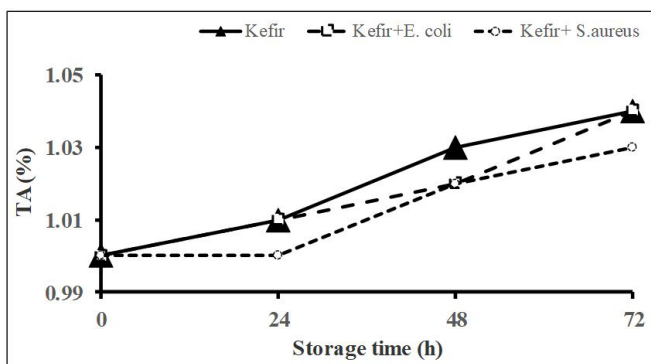


Figure 4. Change of titratable acidity (TA) of kefir during the storage 5 °C.

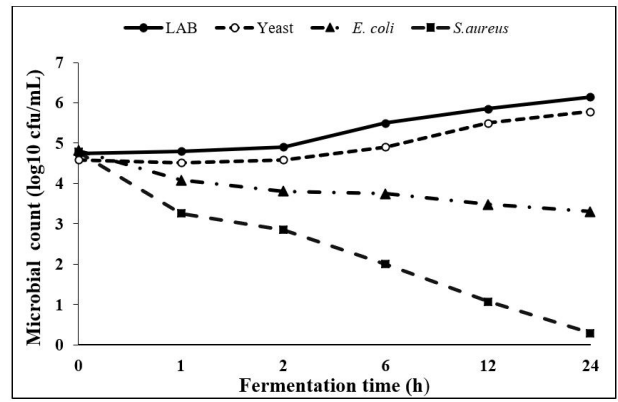


Figure 5. Change of bacteria and yeast count of kefir during the fermentation.

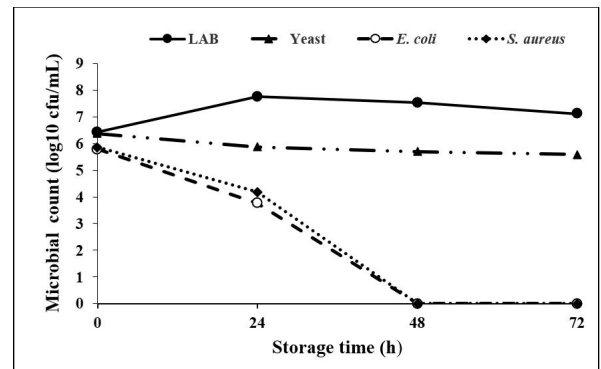


Figure 6. Change of bacteria and yeast count of kefir during the storage 5 °C.

Table 1. Changes in physicochemical values in the kefir samples (mPa).

	Fermentation during (h)					During the storage (h)			
	0	1	2	12	24	0	24	48	72
Viscosity (mPas)	236	237	243	352	466	467	636	612	598

4.82 Log₁₀ cfu mL⁻¹ to 3.30 Log₁₀ cfu mL⁻¹ and *S. aureus* count decreased from 4.78 Log₁₀ cfu mL⁻¹ to 0.30 Log₁₀ cfu mL⁻¹.

During storage, while there was no significant change in yeast and LAB counts, *E. coli* and *S. aureus* counts dropped significantly (Figure 6).

4 Discussion

During fermentation, the pH slowly decreased in all kefir samples. Yoo et al. (2013) obtained similar results in the kefir they produced with different methods. The researchers reported that pH dropped slowly during fermentation. At the end of fermentation, they found that the pH changed from 3.99 to 4.52. In all the samples, while TA was initially 0.14%, the fermentation was 1.00% and 1.01% in the end. There was no significant difference in bacterial inoculated samples either. Similar results were obtained from Yoo et al. (2013). In another

study, Özdeştan & Üren (2010) reported that pH values changed between 4.11 and 4.53 in the kefir samples and the TA values varied between 0.65% and 1.05%.

During storage, the pH did not change significantly. TA% value remained the same. Irigoyen et al. (2005) reported that the pH value of kefir was about 4.5. The fact that TA% values of kefir did not drop too much during storage could be because the number of lactic acid bacteria did not increase or in fact, decreased slightly during storage. The pH of kefir may not have changed much due to the yeast in kefir. Collar (1996) found that yeast and lactic acid bacteria produced lactic acid and lactic acid bacteria more slowly than the lactic acid bacteria.

The viscosity values increased markedly during fermentation. During the storage of kefir, viscosity increased until 48th hours and then decreased at 72nd hours. The studies conducted by various researchers reported an increase in viscosity during storage of yoghurt samples (Abrahamsen & Holmen, 1980). The increase in viscosity of kefir is related to the total solids content of kefir, the degree of hydrolysis of proteins, the exopolysaccharide (EPS) production capacity and acid production capacity of microorganisms in kefir (Tamime & Robinson, 2007).

During fermentation, the LAB counts increased from 4.74 Log₁₀ cfu mL⁻¹ to 6.44 Log₁₀ cfu mL⁻¹ and yeast counts increased from 4.58 Log₁₀ cfu mL⁻¹ to 5.78 Log₁₀ cfu mL⁻¹. Similar to the results of this study, Beukes et al. (2001) reported an average of 8 log₁₀ cfu mL⁻¹ LAB counts in traditional South African fermented milk. Yoo et al. (2013) found that the number of LAB increased during fermentation of kefir, the LAB number increased from 4.29 Log₁₀ cfu mL⁻¹ to 8.52 Log₁₀ cfu mL⁻¹, and then their development slowed down. These values are higher than those in our study. This difference may be due to differences in experimental conditions such as different fermentation temperatures as well as to the amount of initial culture. Leite et al. (2013) reported that the added culture for kefir increased significantly in the first 12 hours and then reached the value of 7.8 Log₁₀ cfu mL⁻¹ in 24 hours. The number of LABs during storage did not change significantly. Similar results were reported in Yoo et al. (2013) as well. Kang et al. (2013) reported that the number of LABs did not change much during storage for 9 days.

During fermentation, the yeast counts increased gradually. The yeast counts did not change during storage. It was reported that the traditional fermented dairy products with a high number of yeasts were related to traditional containers used for the fermentation of dairy products. In South Africa, earthenware pots were reported to contain yeast (Kebede et al., 2007).

While *E. coli* was not significantly inhibited, *S. aureus* was inhibited at high rates during the fermentation process. In the storage process, pathogen bacteria were inhibited within 2 days. No pathogenic bacteria could be counted on the day 3 of storage.

E. coli was not inhibited during the fermentation process because it was resistant to acidic conditions (Figure 6). However, the number of hydrogen peroxide produced by the LAB dropped. It was found that the most important inhibition effects of LAB at 5 °C was hydrogen peroxide produced (Yap & Gilliland, 2000).

The inhibition of *E. coli* during storage of kefir may be due to the hydrogen peroxide produced by LAB in kefir. Kıvanç & Yapıcı (2015) reported that the LAB isolated from kefir produced hydrogen peroxide between 0.01 and 0.69 mg mL⁻¹. Similarly, the bacteriocin produced by LAB in the inhibition of *E. coli* or bacteriocin substances may have been effective. In addition, considering that low temperature invigorates *E. coli* O157: H7 (Clavero & Beuchat, 1996), its inhibition in 48 hours during storage is important.

Acidic medium as a result of lactic acid fermentation of the LAB in kefir is one of the most important factors in the inhibition of *S. aureus* and also has inhibitory effect on the LAB-produced hydrogen peroxide *S. aureus* (Charlier et al., 2009). However, LAB may also have inhibitory properties on *S. aureus*. LAB can also synthesize inhibitory bacteriocin and similar substances on *S. aureus* (Cotter et al., 2005). Kıvanç & Yapıcı (2015) reported that *L. plantarum*, *Lactobacillus pentosus* and *Lactobacillus paracasei* spp *paracasei* strains isolated from kefir samples inhibited the neutralized cell-free filtrates of *S. aureus*. The researchers in question reported that lactic acid bacteria isolated from kefir produced lactic acid in 8.68-1.12 mg mL⁻¹ (Kıvanç & Yapıcı, 2015). The rate of LAB/*S. aureus* was effective on the inhibition of *S. aureus* as well (Charlier et al., 2009).

Dineen et al. (1998) reported that *E. coli* and *S. aureus* survived during the yogurt fermentation process and that the presence of these bacteria in yogurt was indicative of a contamination during yogurt production. Farrag (1992) reported that although the acidity and pH levels of kefir samples were not different, the populations of pathogenic microorganisms in these samples decreased at various levels during storage at low temperatures.

Unlike our findings, Gülmez & Güven (2003) reported that *E. coli* O157: H7 survived in the pasteurized kefir and modified kefir for 21 days at 5-7°C. This may be due to the fact that the natural flora was damaged in the pasteurized and modified kefir. It was reported that food stuffs between the kefir microbes and test strains caused a bacteriostatic effect on *E. coli* due to competition and / or substances that might be seen in the early stages of milk fermentation (Garrote et al., 2000). Fermentation and storage conditions in fermented dairy products affect the development of pathogenic microorganisms in these products (Ogwaro et al., 2002). In general, Kefir samples have inhibitory effects on *E. coli* and *S. aureus* and it was reported that this result may be important since it may be too low after contamination of *E. coli* and *S. aureus* infectious dose (Buchanan & Doyle, 1997).

5 Conclusion

The *E. coli* contamination prior to kefir fermentation may be of importance for the public health. *S. aureus* contamination, on the other hand, is of no great importance since *S. aureus* is inhibited during the fermentation process. If *S. aureus* and *E. coli* contamination occurs after fermentation, the bacteria may be inhibited within 48 hours. This is important for post-fermentation contamination. Especially if *E. coli* contamination is assumed to be at low concentration, kefir is a safe fermentation drink. However, the production and storage conditions need to be paid attention to.

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