Survival Characteristics of *E. coli* O157:H7, *S. typhimurium* and *S. aureus* during Kefir Fermentation

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Abstract

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In this research, the growth and survival of *E. coli* O157:H7, *Salmonella typhimurium* and *Staphylococcus aureus* were investigated during kefir fermentation. Two different levels of inoculation of the strains were conducted; the levels of 10^2 CFU/ml (EC-1, SA-1 and S-1) and 10^3 CFU/ml (EC-2, SA-2 and S-2). At 0, 2, 6, 12, and 24 hours of kefir fermentation at 23 ± 1°C, samples were taken and the counts of *E. coli* O157:H7, *S. typhimurium*, and *S. aureus* were determined. EC-1 grew from 2.29 ± 0.02 log CFU/ml to 4.13 ± 0.18 log CFU/ml whereas EC-2 grew from 3.22 ± 0.04 log CFU/ml to 6.78 ± 0.99 log CFU/ml. Both S-1 and S-2 viable populations grew during the fermentation period, where sample S-1 grew from 2.37 ± 0.20 log CFU/ml to 4.64 ± 0.67 log CFU/ml and sample S-2 grew from 3.52 ± 0.07 log CFU/ml to 5.60 ± 0.10 log CFU/ml. SA-2 strains grew from 3.06 log CFU/ml to 3.64 log CFU/ml, SA-1 strains grew from 2.28 log CFU/ml to 2.66 log CFU/ml. According to the findings, *E. coli* O157:H7, *S. typhimurium,* and *S. aureus* can survive in kefir during fermentation.

Keywords: kefir fermentation; *E.coli* O157:H7; *Salmonella typhimurium*; *Salmonella aureus*

Kefir is a fermented milk product produced by the coculture of certain lactic acid bacteria, acetic acid bacteria, and yeasts. Kefir grains are small, irregularly shaped, yellowish-white particles that resemble miniature caulifowers.

Kefir is used for the production of a refreshing probiotic fermented milk beverage, wellknown to East Europeans, by inoculating milk with kefir grains (Güzel-Seydim *et al*. 2000; Wszolek *et al*. 2001). The acidic fermented milk is slightly carbonated and contains small amounts of alcohol. Kefir differs from the traditional fermented milks (yoghurt) in that it is made only from kefir grains, which are composed of bacteria and yeasts (Marshall & Cole 1985). Kefir beverage contains live microflora from the grains which are removed by filtration after the fermentation process. Kefir grains are conglomerates of lactic acid bacteria (*Lactobacilli, Lactococci*, and *Leuconostoc*), acetic acid bacteria (*Acetobacter aceti*), and yeasts (*Saccharomyces cerevisiae, Candida kefir, Kluyveromyces marxianus*) held together by a matrix of different exoploysaccharides.

Probiotics have been shown to possess inhibitory activity against the growth of pathogenic bacteria such as *Escherichia coli, Listeria monocytogenes, Salmonella* spp., and others (Haris *et al*. 1989; Chateau *et al*. 1993). This inhibition is supposedly due to the production of inhibitory compounds such as bacteriocins, hydrogen peroxide, or organic acids,

as well as competitive adhesion to epithelium. Kefir also contains microorganisms that help to maintain a well-balanced intestinal flora and is known as an antitumour factor (Zubillaga *et al*. 2001).

Kefir is made by fermentation of pasteurised milk. In the case of an inadequate pasteurisation, pathogen microorganisms such as *S. typhimurium*, *E. coli* O157:H7 and *S. aureus* may grow and multiply in milk and kefir. Since milking is realised by hand in open areas, especially in the countryside under poor hygienic conditions, milk can be contaminated by several sources such as diseased animals, milk handlers, and contaminated equipment (Ogwaro *et al*. 2002). It is also known that raw milk is one of the sources of *E. coli* O157:H7. This pathogen has been isolated from raw milk (McDonough *et al*. 1991; Reitisma & Henning 1996). Therefore, the attention has to be extended to the contamination of dairy products.

In this study, the growth and survival of *E. coli* O157:H7, *Salmonella typhimurium*, and *Staphylococcus aureus* strains during kefir fermentation were examined.

MATERIAL AND METHODS

Preparation of kefir samples. Kefir grains were obtained from Microbiology Laboratory, Department of Dairy Technology, Faculty of Agriculture, Ege University, Izmir, Turkey. Sterilised milk (3% v/v fat) was purchased from Pınar Milk Company. *Escherichia coli* O157:H7, *S. typhimurium* (NRLE E.4463), and *S. aureus* strains were provided by the Food Microbiology Laboratory, Department of Food Engineering, Ege University, Izmir.

Seven different kefir samples were prepared by inoculating kefir grains into pasteurised milk in the proportion of 5% (w/v). One sample was inoculated with 10^2 CFU/ml of an overnight culture of *E. coli* O157:H7 EC-1. The second sample was inoculated with 10^3 CFU/ml of *E. coli* O157:H7 EC-2. The third (S-1) and fourth (S-2) samples were inoculated with 10^2 CFU/ml and 10^3 CFU/ml of an overnight culture of *Salmonella typhimurium*, respectively. The fifth (SA-1) and sixth (SA-2) kefir samples were inoculated, respectively, with 10^2 CFU/ml and 10^3 CFU/ml of an overnight culture of *Staphylococcus aureus*. The last sample (C) was not inoculated and served as the control sample. Immediately after inoculation, the samples were incubated at 23 ± 1°C for 24 h in an incubator (Memmert, model 400). The samples were then collected after 0, 2, 6, 12, and 24 h and microbiological analysis was performed.

pH measurement. pH of the control sample was measured at the time of sampling. pH-meter (WTW pH537) was used for the measurement.

Microbiological counts. For the enumeration of *E. coli* O157:H7, *Salmonella typhimurium* and *Staphylococcus aureus,* the samples were serially diluted with peptone water (0.1% w/v), and the viable populations were determined by plating 0.1 ml of the diluted samples on Sorbitol Macconkey Agar (Oxoid, CM813), Brilliant Green Agar (Oxoid, CM263), and Baird Parker Agar (Oxoid, CM275), respectively. The samples were then incubated at 37°C for 24–48 hours. At the end of the incubation period, the colonies were counted and their numbers were determined (Baird-Parker 1962; AOAC 2001; Lara *et al*. 2003; Tsegaye & Ashenafi 2005).

Statistical analysis. The mean values and the standard deviation of the data obtained from duplicate experiments were determined. Data were compared by Duncan's multiple range method (SAS 1999).

RESULTS AND DISCUSSION

Acidification is an important measure to control the growth and survival of pathogens and spoilage microorganisms. However, various acidic foods like yoghurt have been implicated in the foodborne outbreaks caused by *E. coli* O157:H7 (Griffin & Tauxe 1991; Uljas & Ingham 1999; McClure 2000; Hsin-Yi & Chou 2001; Gülmez & Güven 2003; Şimsek *et al*. 2007; Bachrouri *et al*. 2006). Since *E. coli* O157:H7 can survive in the pH range between 4.5 and 9.0 (Glass *et al*. 1992; Reinders *et al*. 2001), kefir can act as a medium for its growth and survival. In recent years, acid adaptation and increased resistance of *E. coli* O157:H7 (Cheng & Chou 1999; McIngvale *et al*. 2000) and *Salmonella* spp. (Goverd *et al*. 1979; Foster & Hall 1990; Zhao *et al*. 1993) have been observed by several researchers.

E. coli O157:H7 is known as acid resistant (Zhao *et al*. 1993; Buchanan & Doyle 1997; Guraya *et al*. 1998) and it can survive in fermented dairy products for long periods of time (Dieneen *et al*. 1998; Tsegaye &Ashenafi 2005). Zhao *et al*. (1993) observed the survival of *E. coli* O157:H7 for up to 31 days in refrigerated unpasteurised apple juice at pH 3.9.

As reported by Tsiai and Ingham (1997) and Miller and Kaspar (1994), *E. coli* can survive in acidic foods such as apple cider (pH 3.7), mustard (pH 3.1), and ketchup (pH 3.6). It is also reported that acid adaptation prolonged the survival of some pathogens like *E. coli* O157:H7 and *Salmonella* spp., and may have important implications in terms of food safety (Ackers *et al*. 1998; Hsin-Yi & Chou 2001; Samelis & Sofos 2003).

It is reported that *E. coli* O157:H7 could grow and survive at pH 4.5 in a medium adjusted with HCl (Connor & Kotrola 1995). According to Uyttendaele *et al*. (2001), *E. coli* O157:H7 strains of approximately 10^2 CFU/ml, can survive for 5 days in meat, with pH adjusted with 50% lactic acid and fermented at 22°C. The growth and survival of *E. coli* O157:H7 during fermentation of kefir is shown in Table 1. Both EC-1 and EC-2 samples show similar growth patterns during fermentation. EC-1 grew from 2.29 ± 0.02 log CFU/ml to 4.13 ± 0.18 log CFU/ml whereas EC-2 grew from 3.22 ± 0.04 log CFU/ml to 6.78 ± 0.99 log CFU/ ml. Similarly, the populations of *E. coli* O157:H7 strains in both samples were doubled in log units during the same period. During fermentation, pH of kefir decreased from 6.7 to 4.6 (Figure 1). In spite of this decrease in pH, viable populations of *E. coli* O157:H7 strains were increased. As seen in Table 1, the populations of the *E. coli* O157:H7 strains in both EC-1 and EC-2 significantly increased during the fermentation period ($P < 0.05$). This shows that stationary-phase strains of *E. coli* O157:H7 are acid tolerant and can survive during acid fermantation. Stationary-phase cells of *E. coli* O157:H7 are more resistant to acid than log-phase cells. Similiar findings of *E. coli* O157:H7 survival during fermentation of yoghurt were reported by Ogwaro *et al*. (2002). Tsegaye *et al*. (2004) found that *E. coli* O157:H7 strains can survive and grow 1 log unit during the first 24 h of fermentation of green Datta slurry (a traditional Ethopian fermented beverage). Also *E. coli* O157:H7 was reported to grow in TSB at pH levels as low as 2.0 (Koodie & Dhople 2001). Miller and Kaspar (1994) and Roering *et al*. (1999) reported that *E. coli* O157:H7 had survived for several days in apple juice at pH < 3.5. According to Moon *et al*. (2006), *E. coli* O157:H7 was detected in apple juice at pH 3.5 during 10 days.

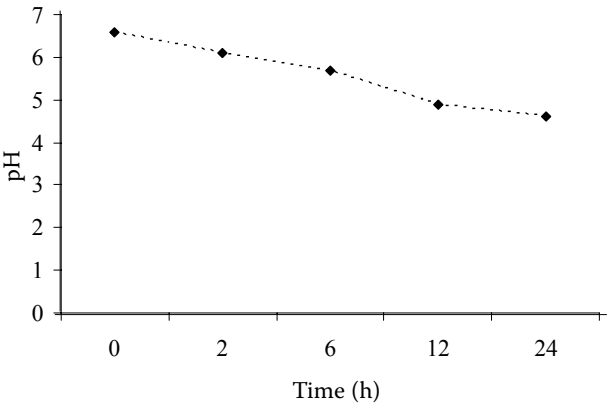

Figure 1. Changes in pH during kefir fermentation (control sample)

Survival of *E. coli* O157:H7 was also reported in fermented sausages (at pH 4.5) for 35 days (Uyttendaele *et al*. 2001), in whey cheeses for 30 days (at pH 5.8) (Govaris *et al*. 2001), in fermented sausages for 8 weeks at pH 4.4 (Glass *et al*. 1992), and in skim milk for 35 days at pH 4.7–5.4 (Guraya *et al*. 1998). Marek *et al*. (2004) revealed that *E. coli* O157:H7 can survive in pasteurised and unpasteurised whey samples up to 21 days and indicated the potential risk of persistance of *E. coli* O157:H7 in whey in the event of contamination with this pathogen. Also Lekkas *et al*. (2006) re-

Table 1. Survival of *E. coli* O157:H7, *Salmonella* spp. and *Staphylococcus aureus* during fermentation of kefir (log CFU/ml)

Time (h)	EC-1	EC-2	S-1	S-2	SA-1L	SA-2
0	2.29 ± 0.02^{c}	$3.22 \pm 0.04^{*c}$	2.37 ± 0.20^{c}	$3.52 \pm 0.07^{*b}$	2.28 ± 0.03^{d}	$3.06 \pm 0.05^{*,d}$
2	$2.87 \pm 0.60^{b,c}$	3.40 ± 0.14^{c}	2.50 ± 0.28^{c}	$4.53 \pm 1.34^{a,b}$	2.46 ± 0.03^{c}	3.25 ± 0.03^{c}
6	$3.38 \pm 0.08^{a,b}$	$4.05 \pm 0.11^{b,c}$	$3.36 \pm 0.51^{b,c}$	$4.91 \pm 0.82^{a,b}$	2.60 ± 0.02^{b}	3.44 ± 0.02^{b}
12	3.77 ± 0.4^{a}	4.64 ± 0.15^{b}	$4.13 \pm 0.03^{a,b}$	5.57 ± 0.09^{a}	$2.64 \pm 0.02^{a,b}$	3.52 ± 0.01^{b}
24	4.13 ± 0.18^{a}	6.78 ± 0.99^{a}	4.64 ± 0.67^{a}	5.60 ± 0.10^{a}	2.66 ± 0.02^{a}	3.64 ± 0.02^{a}
	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$

*Data represent the mean values of two independent experiments and their standard deviations

ported the survival of *E. coli* O157:H7 in Galotyri cheese at pH 3.7. Tsegaye and Ashenafi (2005) investigated the survival characterstics of *E. coli* O157:H7 in Ergo and Ayib, traditional fermented dairy beverages. In the study, it was revealed that during Ayib processing *E. coli* O157:H7 in souring milk increased by 3 log units in 24 hours. Also in Ayib production *E. coli* O157:H7 could survive for 7 days at pH 3.8–3.9.

Guraya *et al*. (1998), found that *E. coli* O157:H7 can survive after a period of a week in non-fat yoghurt. Bachrouri *et al*. (2006) studied the survival of *E. coli* O157:H7 in milk and during refrigeration of home-made yoghurt. They indicated that the results of their study were of high public health significance, since the survival of *E. coli* O157:H7 was long enough to represent a hazard for the consumer. *E. coli* O157:H7 survival was also reported in various tomato products (4.2–4.8 pH) and it was indicated that the survival was notably prolonged at refrigeration temperatures (Eribo & Ashenafi 2003). In another study supporting ours, Rozand *et al*. (2005), indicated that *E. coli* O157:H7 survives the lactic cheese manufacturing process, and that the presence of low numbers of *E. coli* O157:H7 in milk destined for the production of raw milk lactic cheeses can constitute a threat to the consumer.

The growth and survival of *Salmonella typhimurium* during fermentation of kefir are given in Table 1. Both S-1 and S-2 viable populations grew during the fermentation period, where the sample S-1 grew from 2.37 ± 0.20 log CFU/ml to 4.64 ± 0.67 log CFU/ml and the sample S-2 grew from 3.52 ± 0.07 log CFU/ml to 5.60 ± 0.10 log CFU/ml. S-1 and S-2 *S. typhimurium* strains showed resistance to increasing acidity (final pH was 4.6) and grew approximately 2 log units. The fermentation time was found statistically significant with the population of *S. typhimurium* in both kefir samples ($P < 0.05$). Also according to the findings of Roering *et al*. (1999), *S. typhimurium* strains of 10^7 CFU/ml can survive well in apple cider (pH 3.3–3.5) for 21 days at 4°C.

Staphylococcus aureus is considered to be the second most common pathogen causing outbreaks of food poisoning, outnumbered only by *Salmonella* spp. (Lara *et al*. 2003). *Staphylococcus aureus* was demonstrated in dairy products (Martin & Marshall 1995; Iurlina & Fritz 2004). Enterotoxins involved in food poisoning are produced by approximately one-third of the coagulase positive strains of *S. aureus* (Portocarrero *et al*. 2002). Enterotoxin A and Enterotoxin D of *Staphylococcus* are associated with many foodborne diseases.

The growth and survival of *Staphylococcus aureus* strains in the samples SA-1 and SA-2 are given in Table 1. In both SA-1 and SA-2 samples, *Staphylococcus aureus* strians survived and showed similar growth patterns during fermentation. The SA-2 strains grew from 3.06 log CFU/ml to 3.64 log CFU ml, while the SA-1 strains from 2.28 log CFU/ml to 2.66 log CFU/ml, respectively. Growth populations of the strains were found less than 1 log unit, but as concerns the statistical analysis, the fermentation period was found significant for the growth of *Staphylococcus aureus* strains ($P < 0.05$).

CONCLUSION

The results of microbiological analysis revealed that *Salmonella typhimurium*, *E. coli* 0157:H7, and *S. aureus* can survive and multiply during fermentation of kefir. Especially *E. coli* O157:H7 and *Salmonella typhimurium* doubled in log units. Since these pathogens are resistant to acidic conditions, in the case of the contamination of milk used for kefir, they can presumably survive and cause foodborne ilnesses. To prevent the foodborne diseases caused by contaminated kefir, stricter preventive measures should be taken reaching from the dairy farm to the manufacturing unit. For that, the diseased animals should not be used for milking and the equipment for kefir making should be cleaned and sanitised. Milk must be pasteurised or boiled before the production, and the storage of milk should be realised under refrigeration.

With respect to the food safety and hygiene management, pathogen reduction systems like HACCP can be adopted to manufacturing plants to assure microbiological safety.

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