

Survival of *Escherichia coli* O157:H7, *Listeria monocytogenes* 4b and *Yersinia enterocolitica* O3 in ayran and modified kefir as pre- and postfermentation contaminant

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ABSTRACT: The survival of *Escherichia coli* O157:H7, *Listeria monocytogenes* 4b and *Yersinia enterocolitica* O3 in traditional yogurt and kefir during fermentation, in ayran (a dairy beverage in Turkey), pasteurised (long-life) ayran, modified kefir (salted and diluted kefir) and pasteurised modified kefir during cold storage were investigated. Pasteurised samples were used to monitor the antibacterial effect of natural flora of yogurt and kefir during cold storage. Populations of all the strains were increased during fermentation, and thus pre-fermentation contamination appeared more risky than postfermentation contamination. Pasteurisation appeared not to be disadvantageous an application on the microbiological safety of the samples, nevertheless biological benefits which may come from live microorganisms is lost. While *E. coli* O157:H7 and *L. monocytogenes* 4b survived for up to 21 days in all samples, *Y. enterocolitica* O3 survived only for 14 days in modified kefir. Yogurt microflora appeared to be more suppressive on the pathogens than that of kefir.

Keywords: ayran; modified kefir; *E. coli* O157:H7; *L. monocytogenes* 4b; *Y. enterocolitica* O3

Although ayran, a Turkish dairy beverage made from yogurt it does not have the same compositional status with yogurt or diluted yogurt (Morley, 1978; Hylmar *et al.*, 1979; Eyers, 1980). Industrial ayran and pasteurised ayran is standardised by Turkish Standards Institute (Anonymous, 1982, 1989). Industrial ayran and yogurt technology is same. But total solids ($\geq 8.5\%$), crude fat (1–3%) and NaCl ($\geq 1\%$) content are standardised by addition of salt and water. Commercial yogurt constitutes 27% of total dairy production, and approximately 800 000 tons are consumed yearly in Turkey (Anonymous, 2000). Ayran is probably served at least equal to that of yogurt in Turkey.

Kefir is an acidic and mildly alcoholic fermented milk originating from the Caucasian mountains and commercially produced in Asia, Europe and America. While there is a very limited amount of homemade production, kefir is not produced yet industrially in Turkey. However, scientific interest in kefir and the promotion of its industrial produc-

tion are growing due to its health benefits (Anar, 2000).

Escherichia coli O157:H7, *Listeria monocytogenes* and *Yersinia enterocolitica* are three of the most important foodborne bacterial pathogens and can lead to foodborne diseases though consumption of contaminated milk and fermented milk products including yogurt (Morgan *et al.*, 1993; Mead *et al.*, 1999). Food-manufacturing processes which result in a pH decrease of foods system by organic acids release such as pickling or lactic fermentation, have long been used as the primary mechanisms to enhance the safety and keeping quality of foods (Aleksieva, 1979; Schaack and Marth, 1988; Farrag, 1992; Presser *et al.*, 1997; Pitt *et al.*, 2000).

Interest in cultured dairy products is continuously increasing owing to their nutritional and microbiological quality, and the health promoting potential. The inhibitory effect of organic acids produced by acidifying microorganisms in dairy foods on pathogens and spoilage microorganisms is well

established. While the behavior of many pathogens of concern to food safety has been extensively investigated in acidified dairy foods such as yogurt, a limited number of studies has been carried out on ayran, kefir and long-life versions of these foods to assess their self protective effect against food borne pathogens (Garrote *et al.*, 2000). The objective of the present study was to monitor the behavior of three selected food borne pathogens; *E. coli* O157:H7, *L. monocytogenes* 4b or *Y. enterocolitica* O3 during fermentation of yogurt and kefir and also during 21 days of cold storage (5–7°C) of ayran and modified kefir after deliberate addition of these pathogens to milk of samples. Kefir was modified to mimic the ayran and pasteurised ayran samples after fermentation. Even though kefir has beneficial microorganisms, we pasteurised it to address whether pasteurisation decreased the microbiological safety compared with ayran samples.

MATERIAL AND METHODS

Bacterial strains and media

E. coli 937 (serovar. O157:H7) was kindly provided by Dr. Y. Ozbas (University of Hacettepe, Ankara, Turkey), *L. monocytogenes* SLCC 4013 (serovar. 4b) by Munich Ludwig Maximilians University (Munich, Germany) and *Y. enterocolitica* KUEN846-23O3 (serovar. O3) by Culture Collection Center of Istanbul University, Istanbul, Turkey. Each strain was maintained on tryptic soy agar with 0.6% yeast extract (Difco, Detroit, MI, USA) slants at $4 \pm 1^\circ\text{C}$ with monthly transfer. Before use, each organism was activated by inoculation of tryptic soy broth supplemented with 0.6% yeast extract (Difco, Detroit, MI, USA) and incubation for 24 h at 37°C , 30°C or 25°C for *E. coli* O157:H7, *L. monocytogenes* 4b and *Y. enterocolitica* O3, respectively. Cells from the broth culture were harvested by centrifugation at $10\,000 \times g$ for 10 min at $4 \pm 1^\circ\text{C}$. The cell pellets from individual strains were washed and resuspended in 5 ml of 0.85% saline solution and inoculations were made with the addition of 100 μl from the 2nd tenfold serial dilution tube of suspended strains to each separate sample. Inoculation levels were determined by direct plating from both serial dilutions of suspensions and inoculated samples readily after the inoculations.

Modified *E. coli* broth with novobiocin (mEC+n) was used as the basal enrichment medium and

sorbitol MacConkey (SMAC) agar was used as the selective plating medium for enumeration of *E. coli* O157:H7. Listeria enrichment broth (LEB) and Oxford medium base with Oxford antimicrobial supplement (Listeria selective agar, LSA) were used for enrichment and plating of *L. monocytogenes* 4b, respectively. Tryptone soya broth and Yersinia selective agar (CIN agar) with yersinia antimicrobial supplement were used for enrichment and plating of *Y. enterocolitica* O3, respectively. All media and supplements were purchased from Difco (Detroit, MI, USA).

Sample preparations

Industrially produced ultra high temperature (UHT) milk (Pinar A.S., Izmir, Turkey) was used to prepare experimental samples. Two volumes of two liters milk were heated at 85°C for 30 min, and immediately cooled to inoculation temperatures in an ice bath. One of the two volume milks was cultured at $30 \pm 1^\circ\text{C}$ with fresh kefir (5.0% v/v) made by using kefir grains according to Marshall and Cole (1985). The other volume was cultured at $42 \pm 1^\circ\text{C}$ with fresh yogurt (5.0% v/v) made by using commercial lyophilised yogurt culture (*Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Chr. Hensens, Little Island, Cork). Each volume of cultured milks was divided into two batches of four equal portions (200 ml) each. Three portions of each batch were artificially contaminated by inoculation with a 100- μl suspension *Escherichia coli* O157:H7, *Listeria monocytogenes* 4b, or *Yersinia enterocolitica* O3. The fourth portion was not contaminated to serve as a control. Sample preparations used in the present study is shown in Table 1.

Microbiological analysis

All procedures were applied according to the FDA-Bacteriological Analytical Manual (Hitchins *et al.*, 1998). Samples were serially (10-fold) diluted in sterile phosphate buffer (pH 7.0). Subsequently, 1 ml aliquots or 50 μl aliquots of each sample were poured or spread plated onto selective agar media in duplicates. Enrichment procedure was used when no colonies were detected in the lowest dilution by direct plating methods. For the enrichment procedure, a 25 ml sample was enriched in 225 ml of the selective enrichment broth. Enrichment and

Table 1. Sample preparation procedures

Ayran	Modified kefir
MILK (200 ml) cultured with yogurt (5%) was inoculated with pathogenic strains and fermented at $42 \pm 1^\circ\text{C}$ for 4–6 h until the pH is attained to 4.5 ± 1	MILK (200 ml) cultured with kefir drink (5%) was inoculated with pathogenic strains separately and then fermented at $28 \pm 1^\circ\text{C}$ for 20–24 h until the pH is attained to 4.5 ± 1
Traditional yogurt	Traditional kefir
mixed with 100 ml salty water (3% salt)	mixed with 100 ml salty water (3% salt)
Ayran (A)	Modified kefir (MK)
heated at 85°C for two min, cooled to $5\text{--}7^\circ\text{C}$	heated at 85°C for two min, cooled to $5\text{--}7^\circ\text{C}$
Pasteurised Ayran (PA)	Pasteurised modified kefir (PMK)
inoculated with test strains and stored at $5\text{--}7^\circ\text{C}$ for 21 days	inoculated with test strains and stored at $5\text{--}7^\circ\text{C}$ for 21 days

plating media were incubated at 42°C for 24 h for *E. coli* O157:H7, 35°C for 24 h for *L. monocytogenes* 4b and 25°C for 24 h for *Y. enterocolitica* O3. When growth did not appear on selective plating media, the incubation was prolonged for further 24 or 48 h. The colony count method was terminated at the end of 10th day of cold storage, and enrichment procedures were applied to samples from 10th day to 21st day.

Chemical analysis

Determination of titratable acidity was performed by the titration method using NaOH (1/10 N) in

presence of phenolphthalein and the pH was measured with a pH meter equipped with an Orion-gel filled combination electrode (Fisher model 825MP) during the course. The crude fat content of bulk milk samples was determined by the Babcock method at the beginning of experiments (Aurand *et al.*, 1987).

Statistical analysis

Experiments were repeated three times and differences in bacterial counts, acidity and pH were tested for significance by analysis of variance (ANOVA) with $P < 0.05$ and $P < 0.01$.

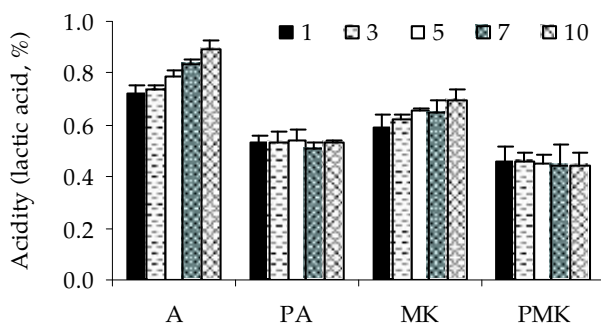


Figure 1. Acidity (lactic acid, %) of samples. A = ayran, PA = pasteurised ayran, MK = modified kefir, PMK = pasteurised modified kefir. The numbers (1, 3, 5, 7, 10) represents analysis days during cold storage

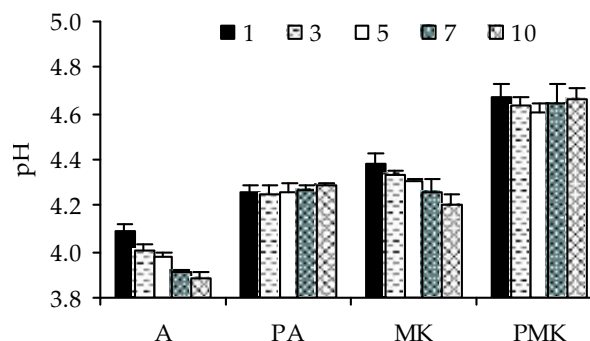


Figure 2. The pH of samples. A = ayran, PA = pasteurised ayran, MK = modified kefir, PMK = pasteurised modified kefir. The numbers (1, 3, 5, 7, 10) represents analysis days during cold storage

RESULTS

Average total crude fat, the titratable acidity and pH levels in bulk milk used for the experiments were 3.3% (± 0.1), 0.13% (± 0.02) and 6.8 (± 2), respectively. Acid and pH levels were not influenced by test strains added to samples as pre- or post fermentation when compared to control samples (data not shown). The corresponding results are represented in Figures 1 and 2.

The cell counts of *E. coli* O157:H7, *L. monocytogenes* 4b and *Y. enterocolitica* O3 increased from 3.75 ± 0.8 to 4.54 ± 1.2 , 4.42 ± 0.7 to 5.18 ± 0.9 , and 3.68 ± 0.9 to 4.23 ± 1.1 logarithmic (log) units CFU/ml at the end of fermentation of yogurt samples, respectively.

At the end of fermentation of kefir, *E. coli* O157:H7, *L. monocytogenes* 4b and *Y. enterocolitica* O3 increased from 4.68 ± 0.9 to 7.7 ± 0.6 , 5.32 ± 1.1 to 6.24 ± 1.0 , and 4.32 ± 0.8 to 7.03 ± 1.1 , respectively.

The populations *Y. enterocolitica* O3 in pasteurised ayran, *L. monocytogenes* 4b pasteurised modified

kefir (Figure 3d) and the both in ayran (Figure 3a) decreased to below the detectable limit at the end of 10th day of cold storage. While *E. coli* O157:H7 and *L. monocytogenes* 4b survived for up to 21 days in all samples, *Y. enterocolitica* O3 survived only for 14 days in modified kefir samples (data not shown).

DISCUSSION

The fermentation process and natural flora are the same for yogurt and ayran. But ayran, a salted (1%) and diluted (20–40% excess water) version of yogurt which has different flavor than yogurt, is consumed as rehydration drink during hot seasons. A diluted and salted version of kefir can also be preferred an alternative to other cold beverages by Turkish people. Pasteurised ayran (Ayran, long-life) is also produced industrially in Turkey. But this procedure may be rejected for kefir due to degrata-

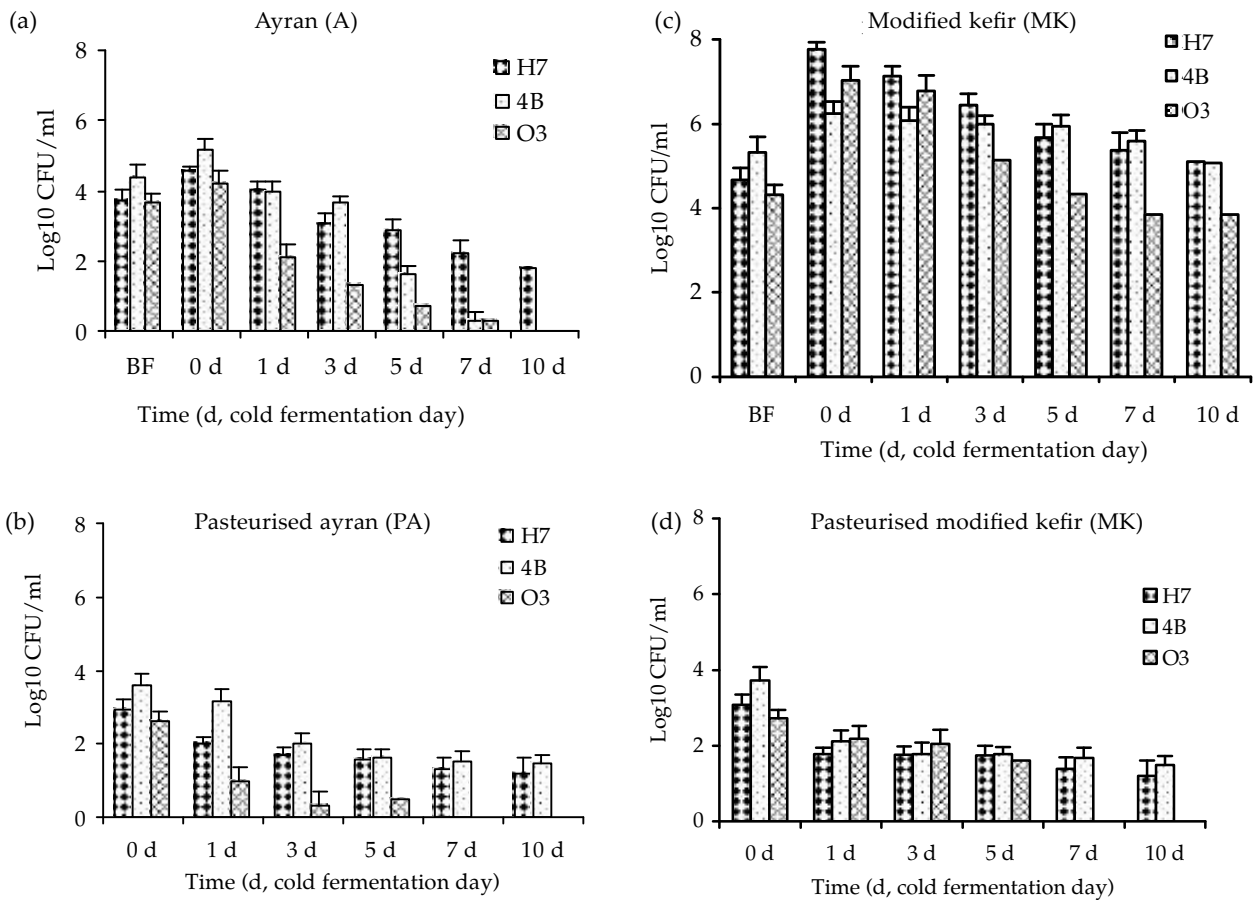


Figure 3. Populations of *E. coli* O157:H7 (H7), *L. monocytogenes* 4b (4B) and *Y. enterocolitica* O3 (O3) in ayran (a), pasteurised ayran (b), modified kefir (c) and pasteurised modified kefir (d) during fermentation and 10 days of cold storage. BF; contamination levels of test strains in samples before fermentation, O; start time of cold storage (each value represents the mean of three replica)

tion of beneficial flora during pasteurisation. Thus, we aimed to ensure uniformity among samples by applying the same procedures that were carried out for ayran to kefir samples. The principal purpose of this study was to determine the antibacterial differences during yogurt and kefir fermentation, also between pasteurised and non pasteurised (natural flora) ayran and kefir samples.

In many studies, growth (Ahmed *et al.*, 1986; Schaack and Marth 1988; McIngvale *et al.*, 2000) and/or survival (Aytac and Ozbas, 1994; Leyer *et al.*, 1995; Massa *et al.*, 1997; Guraya *et al.*, 1998; Basaran, 2000) of *E. coli* O157:H7, *L. monocytogenes* and *Y. enterocolitica* in acidified dairy foods including yogurt (pH 4.0 to 4.5) even the 30 to 40 days has been stated. In this study, all the three pathogens grew during fermentation and survived (except for *Y. enterocolitica* O3) during 21 days of cold storage in all samples. Contrary to our findings, *E. coli* O157:H7 has been suggested not to survive during fermentation process of yogurt and presence of this organism in ready to eat yogurt would indicate the postprocessing contamination (Dineen *et al.*, 1998).

In contrast to yogurt, kefir has been stated to cause a bacteriostatic effect on *E. coli* possibly due to competition for nutrients between kefir microbiota and the test strain and/or due to substances that could appear at early stages in the milk fermentation (Garrote *et al.*, 2000). Contrary to the researchers, we did not monitor a bacteriostatic effect in yogurt or in kefir during fermentation (Figures 3a,c). Based upon our findings, it may be speculated that the pathogenic microorganisms used in this study grow easily in the early stage of yogurt and kefir fermentation when the development of acidity and other antimicrobial substances produced by fermentative cultures is limited. Pre-fermentation contamination appeared to cause more health risk than postfermentation contamination due to the growth of pathogens during fermentation period and, hence, its possible adaptation to the matrix. Nonetheless, it should be taken into consideration that the test strains added to the milk samples before fermentation were from the fresh culture, and we tested only one strain from each pathogen strain in this study. Ahmed *et al.* (1986) has also demonstrated that while *Y. enterocolitica* O:3 has grown during fermentation of yogurt, *Y. enterocolitica* O:8 population has not increased. For that reasons, our results will not fully reflect the behaviour of all pathogenic *E. coli*, *L. monocytogenes* and *Y. enterocolitica* strains in modified kefir.

Many factors including the type of culture microorganisms, and fermentation and storage conditions affect the growth and/or survival of the pathogen microorganisms in the fermented dairy foods (Presser *et al.*, 1997; Dineen *et al.*, 1998; Pitt *et al.*, 2000; Ogwaro *et al.*, 2002). Acid levels of ayran and modified kefir were about 15 to 40% higher than that of pasteurised ayran and modified kefir samples maintained at cold storage (Figure 1). High decrease rate of the pathogens in samples with natural flora than in pasteurised samples can be explained by this findings.

The acid tolerance of *E. coli* O157:H7 is a general characteristic shared by many enteric bacteria such as *E. coli* and *Shigella* spp. (Park *et al.*, 1999). Gahan *et al.* (1996) demonstrated that acid adaptation of *L. monocytogenes* can enhance the survival of this organism in acidic dairy foods during fermentation. Cheng and Chou (2001) have stated that acid adaptation has enhanced the survival of *E. coli* O157:H7 in Yakult and low-fat yogurt stored at 7°C. *L. monocytogenes* 4b was the most resistant strain in pasteurised samples (Figures 3b,d), and *E. coli* O157:H7 was the most resistant strain in non pasteurised samples (Figures 3a,c). Behavior of the each strain in natural ayran or in modified kefir samples did not confirm another in the pasteurised version of the samples. Possible competitive inhibition of the natural flora of yogurt and modified kefir neither prevented the growth of the test strains during fermentation nor fully suppressed their survival during cold storage (Figure 3). While heat treatment decreased the titratable acid levels of the samples (Figure 1), relative decrease in the population of the strains in these samples continued during cold storage (Figures 3b,d). Inhibitory power of yogurt on *Salmonella java* and *Shigella sonnei* has been found to be high than that of the other fermented milk samples including kefir by Alm (1983). The researcher has also stated that pasteurisation did not inhibit antibacterial activity of the samples. In this study, ayran (a version of diluted yogurt) appeared to be more safe than modified kefir ($P < 0.05$) and pasteurisation appears not to affect antibacterial properties of the samples as stated by Alm (1983).

Casey and Condon (2002) has stated that *E. coli* can use NaCl to counteract acidification of its cytoplasm by organic acids, and in addition, that combinations of antimicrobial agents can not always be relied upon to additional antimicrobial effects. In this study, samples were contained a 1% NaCl. A possible support of this agent on survival

of tested microorganisms should be taken into consideration.

From this study, despite the high acidity of ayran and modified kefir, it could be potentially hazardous to the public health if contaminated with the pathogens studied herein. Such risk would increase if the product was contaminated before the fermentation period.

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