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An *in vitro* study on the antibacterial effect of kefir against some food-borne pathogens

Kefirin gıda kaynaklı patojenlere karşı antimikrobiyal etkisi üzerine bir in-vitro çalışma

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SUMMARY

Kefir which is acidifying and alcoholic fermented milk is accepted as a good example of a probiotic mixture of bacteria and yeast. In this study; we planned to investigate the antimicrobial effect of 24 and 48 hours fermented kefir against Staphylococcus aureus (ATCC 29213), Bacillus cereus (ATCC 11778), Salmonella enteritidis (ATCC 13076), Listeria monocytogenes (ATCC 7644) and Escherichia coli (ATCC 8739) by using disk diffusion method. The best antimicrobial effect of 24 and 48 hours kefir was seen against Staphylococcus aureus with the diameter zones of 21.4 and 21.1 mm respectively at the end of fermentation period. Similar results were obtained from 24 and 48 hours fermented kefir on the 1st, 4th and 7th days of storage at + 4 ºC. In all cases, although the antimicrobial activity decreased or did not change during the storage; it increased only against Salmonella enteretidis.

Keywords: *Kefir; antimicrobial activity, disk diffusion method, food-borne pathogens, probiotic*

ÖZET

Kefir asidik ve alkolik fermentasyonla oluşmuş bir süt içeceği olmakla birlikte, probiotik bakteri ve maya karışımı için iyi bir örnek oluşturmaktadır. Bu çalışma disk difüzyon metodu kullanarak, 24 ve 48 saat fermentasyona bırakılmış deneysel kefir örneklerinin Staphylococcus aureus (ATCC 29213), Bacillus cereus (ATCC 11778), Salmonella enteritidis (ATCC 13076), Listeria monocytogenes (ATCC 7644) ve Escherichia coli'ye (ATCC 8739) karşı antimikrobiyal etkisini incelemek üzere planlanmıştır. 24 ve 48 saatlik kefirin gösterdiği en kuvvetli antimikrobiyal etkinin 21.4 ve 21.1 mm'lik etki alanı yarı çaplarıyla Staphylococcus aureus'a karşı olduğu gözlemlenmiştir. + 4 ºC'de muhafaza süresi boyunca 1., 4., ve 7. günlerde de benzer sonuçlar elde edilmiştir. Bütün durumlarda, antimikrobiyal etki değişmez ya da azalırken sadece Salmonella enteretidis'e karşı artış tespit edilmiştir.

Anahtar kelimeler: *Kefir; disk difüzyon metodu, gıda kaynaklı patojenler, probiotik*

INTRODUCTION

Probiotic foods contain live bacteria and have some beneficial effects against pathogenic microorganisms and one of the mechanisms of antimicrobial activity is the inhibitory effect of acidifying microorganisms in probiotic foods (1). The term "probiotic" dates back to 1965 when it referred to any substance or organism that contributes to intestinal microbial balance (2). Kefir which is an acidifying and mildly alcoholic fermented milk originated from the Caucasian mountains, is accepted as a good example of a probiotic mixture of bacteria and yeast (3). It is described as a symbiotic association between lactic and acetic bacteria and yeast and claimed to act against the pathogenic genera *Salmonella, Helicobacter, Shigella, Staphylococcus* and *E. coli* (4). With the emergence of antiobiotic-resistant bacteria, it is reasonable to explore new sources of natural compounds with antibacterial compounds. This property makes the kefir use of alternative treatment for these pathogenic infections (5). The function of the microorganisms constituting the kefir may include production of lactic acid, antibiotics and bactericides, which inhibit the growth of undesirable and pathogenic microorganisms (6).

Kefir drink contains lactic acid, mainly the L(+) form, of about 0.8-0.9%, as well as formic, succinic and propionic acids, CO_2, ethyl alcohol, different aldehydes (propionic, acetic) and trace amounts of isoamyl alcohol and acetone (7). This probiotic dairy drink is widely used in various parts of the world for the treatment of tuberculosis, cancer and gastrointestinal disorders that caused by pathogenic microorganisms (8). Koroleva (9) indicated that kefir is used in hospitals and sanatoria for a variety of illnesses, including metabolic disorders.

However scientific interest in kefir is growing due to its health benefits, a limited number of *in vitro* experimental studies were performed in order to understand the antimicrobial mechanism of kefir's microbial flora (10). It has been reported that kefir causes a bacteriostatic effect against *E. coli* possibly due to competition for nutrients between kefir microbiata and the test strain and/or due to substances that could appear at early stages in the fermentation of milk (1).

Alm (11) and Korneva *et al.* (12) assessed the antibacterial activity of kefir against *Salmonella, Shigella* and *Staphylococcus* species. Cevikbas *et al.* (13) studied the antibacterial and antifungal activities of kefir and kefir grain on *Staphylococcus aureus*, *Staphylococcus epidernidis, Pseudomonas aeruginosa, Proteus vulgaris, Klebsiella pneumonia, Bacillus subtilis* and some *Candida* spp. It is reported that *Lactocoocus lactis* DPC3147 a strain isolated from an Irish kefir grain produces a bacteriocin with a broad spectrum of inhibition (14). Rodrigues *et al.* (15) investigated the antimicrobial and healing activity of kefir and kefiran extraction.

In this study, it is aimed to investigate the antimicrobial effect of 24 and 48 hours fermented kefir against *Staphylococcus aureus* (ATCC 29213), *Bacillus cereus* (ATCC 11778), *Salmonella enteritidis* (ATCC 13076), *Listeria monocytogenes* (ATCC 7644) and *Escherichia coli* (ATCC 8739) *in vitro* conditions. In order to evaluate the antimicrobial effect of kefir, comparison made with ampicillin and gentamycin. pH changes of the kefir were also observed during the storage period.

MATERIAL AND METHODS

Kefir Production. Commercially bottled, homogenized UHT milk was used to produce kefir. 0.01 U freeze dried, mild aromatic kefir culture PROBAT KC3 (Danisco, Denmark) was used as starter culture to ferment 1 L of milk. It was reported that, using defined cultures to produce kefir is in progress toward standardizing the kefir production (16). Because of that we preferred to use standard, freeze dried culture instead of using traditional kefir grain. The composition of starter culture was *Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. cremoris, Lactococcus lactis subsp. diacetylactis, Leuconostoc mesenteroides subsp. Cremoris, Lactobacillus kefyr, Kliyveromyces marxianus var. marxianus,* and *Saccharomyces unisporus.* Fermentation was done in two groups at 24-26 °C for 24 and 48 hours. At the end of fermentation time, kefir was cooled to approximately to +4 °C and stored at this temperature for 7 days. At the end of fermentation and during storage, antibacterial activity was tested in the 1^{st}, 4^{th} and 7^{th} days of storage. The traditional starter culture of kefir takes the form of grains of variable sizes which resembles cauliflower flowers in shape and these grains contain a wide and varying microflora (17). Because of that commercially prepared and freeze-dried kefir culture was preferred in order to optimize the fermentation and initial flora of kefir.

Preperations of bacterial solutions. *Staphylococcus aureus* (ATCC 29213), *Bacillus cereus* (ATCC 11778), *Salmonella enteritidis* (ATCC 13076), *Listeria monocytogenes* (ATCC 7644) and *Escherichia coli* (ATCC 8739) were used as test microorganisms which are the most common food borne pathogenic bacteria. They were activated in nutrient broth by fermentation at 35°C for 24 hours. A loop full of the bacteria that activated and enriched in nutrient broth were transferred to sterile saline water and emulsified to a turbidity of McFarland 0.5 density. The final bacterial cell concentration approximated to 10^8/ml with spectrophotometric method.

Testing antimicrobial activity. Antibiotic activity of kefir was evaluated using the disk diffusion method as described by the National Committee for Clinically Laboratory Standards (18). Ampicillin and gentamycin were used to compare the antimicrobial activity and 10μg/ml of antibiotics was pipetted on to 5 mm diameter paper disk. 24 and 48 hours fermented kefir were pipetted at the amount of 0.1 ml and 1.2 mg/ml as described by Rodrigues *et al*. (15). The paper disks with antibiotics and experimental kefir were applied to the agar surface previously inoculated with 0.1 ml organism suspension. These plates were incubated at 37°C for 24 hours and the inhibition zones were measured at the end of fermentation period. Experiments were performed in triplicates and mean values were used.

pH Measuring. pH of 24 and 48 hours fermented kefir were measured by Hanna HI 9321 Microprocessor pH meter. pH was measured within the same day of antimicrobial activity testing by taking about 35-40 ml of kefir to a separate glass under aseptic conditions.

RESULTS AND DISCUSSION

The antimicrobial activity was first tested at the end of fermentation period, before storage at +4 ºC. No significant difference was obtained between antimicrobial zone diameters of 24 and 48 hours fermented kefir. The best antimicrobial effect of 24 and 48 hours kefir was seen against *Staphylococcus aureus* with the diameter zones of 21.4 and 21.1 mm respectively. Similar results were reported by other researchers. Cevikbas *et al*. (13) reported the greatest activity of kefir against gram-positive coccus, *Staphylococcus aureus* and gram-positive bacillus. Rodrigues *et al*. (15) obtained the best antimicrobial effect against *S. aureus* and *Pseudomonas aeurigonasa* with the diameter zones of 30.0 and 30.2 mm respectively. Also according to this study kefir causes good inhibition zones against *S. pyogenes* and *S. salivarius* with the diameter zones of 27.2 mm and 24.9 mm respectively. Another study that has investigated the antimicrobial activity was performed by Zacconi *et al*. (19). According to their results; kefir has antimicrobial effect against wide variety of gram-positive and gram-negative bacteria.

In our study, gentamycin and ampicillin were used to compare the antimicrobial effect and caused similar inhibition diameter zones with the zones caused by kefir. Table 1 shows the diameters of antimicrobial inhibition zones of 0.1 ml of 24 and 48 hours fermented kefir at the end of fermentation and before storage, compared with gentamycin and ampicillin. The results represent the mean zone diameters of triplicate trials in mm.

Table 1. Inhibition zones (diameter in mm) of antimicrobial activity of 24 and 48 hours fermented kefir after fermentation

Strain	Ampicillin	Gentamycin	Kefir (24 h)	Kefir (48 h)
S. aureus	25.3	22.6	21.4	21.1
E. coli	20.2	20.8	19.5	18.6
B. cereus	21.8	21.2	20.6	20.2
S. enteretidis	21.3	19.8	18.4	18.9
L. monocytogenes	20.0	21.5	18.3	18.2

According to our study it was seen that; no significant change in antibacterial activity was occurred in the 1st, 4th and 7th days of storage for 24 hours fermented kefir. The best antibacterial activity was seen against *Staphylococcus aureus* with unimportant changes during the storage days. Table 2 represents the diameters of antimicrobial inhibition zones of 24 hours fermented kefir during the storage at + 4 ºC.

Table 2. Inhibition zones (diameters in mm) of antimicrobial activity of 24 hours fermented kefir during the storage at + 4 ºC.

Strain	Ampicillin	Gentamycin	Kefir (day 1)	Kefir (day 4)	Kefir (day 7)
S. aureus	25.1	22.0	21.2	21.0	21.0
E. coli	20.4	21.4	19.5	19.4	19.4
B. cereus	21.0	21.7	20.5	20.2	20.1
S. enteretidis	21.7	20.2	18.8	20.9	21.2
L. monocytogene	20.4	20.9	18.0	18.0	17.9

Similar results were obtained from the tests on 48 hours fermented kefir. The results of 48 hours fermented kefir's antimicrobial activity testing during the storage at + 4 ºC were given in Table 3.

Inhibition zones of kefir were similar sizes as those found using other probiotics. Matijasik and Rogelsj (20) showed *Lactobacillus* K7 strain produced inhibition zones of 19 and 22 mm against *Clostridium tyrubutyricum* and *C. perfringens*, respectively

Table 3. Inhibition zones (diameters in mm) of antimicrobial activity of 48 hours fermented kefir during the storage at + 4 ºC.

Strain	Ampicillin	Gentamycin	Kefir (day 1)	Kefir (day 4)	Kefir (day 7)
S. aureus	25.5	22.2	21.0	20.8	20.8
E. coli	20.0	21.1	18.4	18.0	18.0
B. cereus	22.1	21.0	20.0	19.8	19.7
S. enteretidis	20.4	19.9	19.0	21.6	22.0
L. monocytogene	19.8	21.2	18.2	18.1	17.9

At the end of fermentation period pH of 24 and 48 hours fermented kefir were 5.52 and 4.89 respectively. During the storage of kefir, pH decreased slightly till the 7th day of storage. Atasever *et al*. (21) observed the pH changes of kefir during 15 days of storage. According to the results of their study; pH was measured as 4.55 at the first day; 4.36 at the 7th day. These results are parallel to the ones that we obtained. In Table 4, pH changes of 24 and 48 hours fermented kefir during the storage at + 4 ºC were given.

In all cases, however the antimicrobial activity slightly decreased or did not change during the storage; it increased only against *Salmonella enteretidis*. As reported previous, kefir plus gastric juice showed inhibition of *Salmonella typhimurium* after one hour (11). This result was thought to be because of the slightly decrease in pH values during storage. pH changes of kefir did not enhance the inhibition of other test bacteria significantly. This result is similar as reported in the literature by Cevikbas *et al*. (13). According to their study, the antibacterial effects of kefir were same at both pH 5.5 and 7.0.

Table 4. pH changes of 24 and 48 hours fermented kefir during the storage at + 4 ºC.

	Storage days under cold chain (+4 ºC)			
	0	1st	4th	7th
24 h kefir	5.52	4.62	4.55	4.52
48 h kefir	4.89	4.47	4.42	4.40

In conclusion; this study demonstrates that kefir possesses antibacterial effect against *Staphylococcus aureus* (ATCC 29213), *Bacillus cereus* (ATCC 11778), *Salmonella enteritidis* (ATCC 13076), *Listeria monocytogenes* (ATCC 7644) and *Escherichia coli* (ATCC 8739) and the present studies mentioned above confirm our conclusion.

It has to be underlined that the inhibitoriest effect was seen against *Staphylococcus aureus*. On the other hand, it was seen that the antibacterial effect decreased or did not change during the storage at + 4 ºC except of *Salmonella enteretidis*. The data presented in this study suggests that kefir may be a good antimicrobial agent in food technology for food safety. More researches have to be performed related to this subject, in order to put kefir's antimicrobial activity in practice for food technology.

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