

Survival of Major *Listeria monocytogenes* Serotypes in Kefir as Pre-fermentation Contaminant

Bahar ONARAN^{1*}, FatmaSeda ORMANCI¹, Muammer GÖNCÜOĞLU¹, and NaimDeniz AYAZ²

¹Ankara University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Ankara, Turkey

²Kirikkale University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Kirikkale, Turkey

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*Corresponding author: Bahar ONARAN, Ankara University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, 06110, Diskapi, Ankara, Turkey, E-mail: dr.baharonaran@gmail.com

Abstract

Kefir is an acidic and mildly alcoholic fermented milk product which is originated from the Caucasus and is commercially produced in Europe, America and Asia. The fermentation is initiated by the addition of kefir grains to fresh milk. In kefir grains, there is a symbiotic cooperation between microorganisms. Many health benefits have been attributed to kefir, including its antimicrobial activity against to a range of Gram-positive, Gram-negative bacteria, and fungi. In many studies that kefir was contaminated with various microorganisms, such as *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Clostridium tyrobutyricum* and *Listeria monocytogenes*, it was observed that the growth of pathogens in the microflora of kefir grains were inhibited. The objective of this study was to determine the behavior of three major serotypes of *L. monocytogenes* which are frequently isolated from foods, after being added to kefir. Kefir grains (2%) were added to pasteurized milk and then incubated at 25°C depending on appropriate growth temperature of their starter microflora. Kefir samples were contaminated with 6.1×10^4 and 6.1×10^6 cfu/ml by inoculation with major *L. monocytogenes* serotypes (1/2a, 1/2b and 4b) and bacteriostatic effect of kefir microflora over *L. monocytogenes* serotypes was demonstrated. During the fermentation process, a gradual increase in acidity was observed from 0.16% LA to 0.37% LA and the pH decreased from 6.84 to 5.80. Kefir microflora had a suppressive effect on these three different *L. monocytogenes* serotypes. The highest reduction was detected in *L. monocytogenes* 4b with a value of 2.37 log cfu/ml in the second hour of 10^4 cfu/ml contaminated kefir.

Keywords: Bacteriostatic effect; Kefir; *Listeria monocytogenes*

Introduction

The function of dairy products as a medium for the transmission of the variety of diseases has been documented. Contaminated milk and dairy products may culture harbour a diverse variants of microorganisms which are responsible for many foodborne outbreaks [1,2].

Kefir is a viscous, acidic, and mildly alcoholic dairy product that is produced by the fermentation of milk using kefir grains as starting culture. The distinct groups of microorganism identified in this beverage perform three different kinds of fermentations, including lactic, alcoholic and acetic fermentations. The increase in lactic bacteria population causes an increase in the lactic acid concentration in the beverage, whereas the increase in yeast population supports the ethanol formation. Alcohol fermentation is the result of the addition of yeasts in the form of kefir grains. Because of the multiple fermentation process, the resulting product possesses flavor that is characterized by a balance of lactic acid, diacetyl, acetaldehyde, acetoin, ethanol and CO₂. Moreover during the fermentation, vitamin B₁, B₁₂, calcium, amino acids, folic acid and vitamin K increase in the kefir [3-6].

The microorganisms in the kefir grains produce bacteriocidal components, which inhibit the development of degrading and

pathogenic microorganisms in the kefir milk. In general, the antimicrobial activity of kefir is attributed to lactic acid, volatile acids, hydrogen peroxide, carbondioxide, diacetyl, acetaldehyde, and/or bacteriocins produced by lactic acid bacteria. Since properly fermented kefir inhibits many pathogens, kefir is generally considered to be safe due to its antimicrobial activity [7-9].

Kefir is claimed to have suppressive effects against the pathogen microorganisms such as *Helicobacter*, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, *S. enteritidis*, *Shigella flexneri*, *Enterococcus faecalis*, *Yersinia enterocolitica*, and *Clostridium tyrobutyricum*. Among these microorganisms, *Listeria monocytogenes* is one of the most important foodborne bacterial pathogens cause encephalitis, meningitis and septicemia especially in immunocompromised individuals [2,8,10-12]. *L. monocytogenes* has been involved in many outbreaks and sporadic cases of disease primarily associated with the consumption of pasteurized milk, cheeses made from unpasteurized milk and other dairy products that serve as favorable medium for the growth and survival of many pathogenic microorganisms [13-15].

The objective of this study was to analyze the behavior of three selected serotypes of *L. monocytogenes* after being added to kefir, besides the effect of kefir grains against *L. monocytogenes* strains.

Methods

Bacterial Strains and Media

L. monocytogenes serotypes 1/2a (ATCC 19111), 1/2b (N 7155) and 4b (RSKK 475) was provided by Kirikkale University, Faculty of Veterinary Medicine, Kirikkale, Turkey. Kefir grains were provided from Ankara University, Faculty of Veterinary Medicine, Ankara, Turkey. Each strain was maintained on Tryptone Soy Agar (Oxoid, CM0131) with 0.6% yeast extract (Oxoid, LP0021) at $4 \pm 1^\circ\text{C}$ with monthly transfer. Before use, each strain was activated by inoculation of Brain Heart Infusion broth (Oxoid, CM1135) and incubated for 24 h at 30°C.

Sample Preparations

To prepare experimental samples, two liters of raw milk was heated to 85°C for 30 min, and immediately cooled to inoculation temperatures in an ice bath. Afterwards, two liters of pasteurised milk was divided into 7 groups, each one including 250 ml. Kefir grains (2%) were added to milk samples and then incubated at 25°C depending on appropriate growth temperature of their starting microflora.

Microbiological Analysis

Kefir samples were serially (10-fold) diluted in sterile phosphate

buffer (pH 7.0). Subsequently, 50 µl aliquots of each sample were plated onto selective agar media in duplicates. Samples were contaminated with different *L. monocytogenes* serotypes 1/2a, 1/2b and 4b. The samples were contaminated with 10^4 and 10^6 cfu/ml from each strain with six groups as 10^4 and 10^6 serotype 1/2a (A- 10^4 , A- 10^6), 10^4 and 10^6 4b (R- 10^4 , R- 10^6), 10^4 and 10^6 1/2b (N- 10^4 , N- 10^6), additionally the seventh group, not contaminated with *L. monocytogenes* serotypes, was used for control.

Bacteriostatic effect of kefir microflora for *L. monocytogenes* serotypes were detected in 0, 0.5, 2, 4, 8, 12, 24 hours. During the fermentation process, acidity and pH were also analyzed. Determination of acidity was performed by the titration method using NaOH (0,1 mol l⁻¹) in presence of phenolphthalein and the pH was measured with a pH meter.

To enumerate the bacterial colonies, Tryptone Soy Agar (TSA) was used as a nonselective medium, and Modified Oxford Medium (MOX) agar was used as a selective medium for *L. monocytogenes*. Kefir samples were plated on TSA and MOX agar, then incubated for 24 to 48 h at 35°C [2].

Results

During the fermentation process, a gradual increase in acidity was observed from 0.16% LA to 0.37% LA and pH decreased from 6.84 to 5.80.

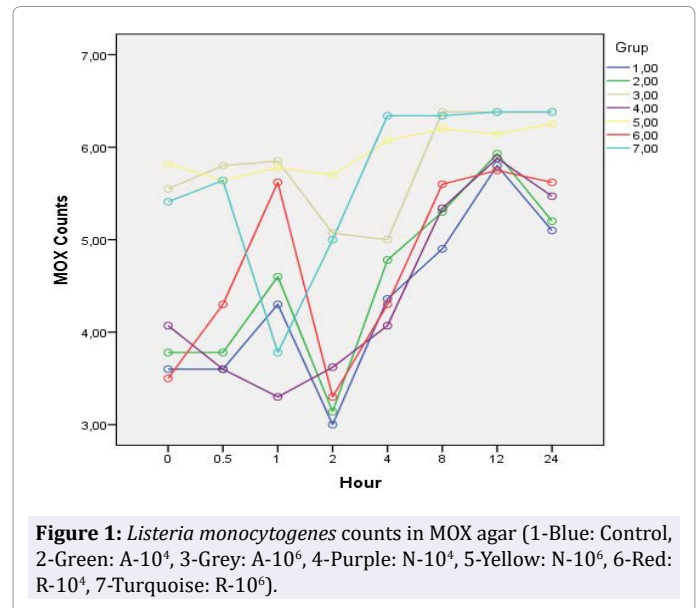
Before performing the statistical analysis, data were examined for normality as the parametric test assumptions. Descriptive statistics for each variable were calculated and were presented as "Mean ± Standard Deviation". To test the differences in TSA and MOX between time sampling in bacteria groups, General Linear Models with repeated measures design were used. When a significant difference was revealed, all significant terms were compared by Simple effect analysis with Bonferroni adjustment. The significant level for all analyses was appointed as $p < 0.05$. SPSS® for windows 14.1 (Licence No:9869264) was used in analysis of the data.

According to the statistical analysis, when the time effect was ignored, there was a statistically significant difference in bacterial groups ($p < 0.001$). When the bacterial groups effect was ignored, there was a statistically significant difference in time sampling effect in both TSA and MOX agars ($p < 0.001$). While there was a statistically significant TSA and MOX (time)*bacteria group interaction, TSA and MOX agar counts in time were significantly differed in bacteria groups ($p < 0.001$).

According to the results of *Listeria monocytogenes* counts in MOX agar, kefir microflora had a suppressive effect on these three different *L. monocytogenes* serotypes especially in the first and second hours. The reductions occurred in different times of the fermentation and with the different counts as shown in the Figure 1. Especially there was a major reduction in the second hour for all of the three serotypes (A- 10^4 , A- 10^6 , R- 10^4 , N- 10^6) with the counts of 1.46, 0.84, 0.14, 2.37 log cfu/ml, respectively. There was also significant reduction in the first hour for N- 10^4 and R- 10^6 with the counts of 0.85 and 1.88 log cfu/g, respectively. The major *L. monocytogenes* reduction in the study was 2.37 log cfu/ml which was detected in the second hour of 10^4 cfu/ml contaminated kefir experiment with 4b serotype (Figure 1).

Discussion

Santos A, et al. [16] observed the antagonistic behavior of lactobacilli isolated from kefir grains against *E. coli*, *L. monocytogenes*, *Salmonella typhimurium*, *S. Enteritidis*, *Shigella flexneri*, *Y. enterocolitica* and *Listeria monocytogenes*. They detected that *Listeria monocytogenes* CECT 4032 strains were inhibited 50%



in contaminated kefir products. Similarly, in our study, highest reduction counted in *L. monocytogenes* serotype 4b (RSKK 475), were inhibited 42% between at first and second hours of the contamination. Highest reduction counts in second hour can be attributed to pH changes during second hours of fermentation. Sabokbar N, and Khodaiyan F. [17] also indicated in their study that the highest decrease in pH value was observed during the second hours of fermentation. In contrast to our results, Rodrigues KL, et al. [8] demonstrated that there wasn't any significant survival difference for in *L. monocytogenes* ATCC 4957 strains that was added to kefir samples.

Also according to a study, Gulmez M, and Guven A. [18] contaminated kefir samples with *L. monocytogenes* 4b and at the end of the fermentation of kefir, *L. monocytogenes* 4b counts increased from 5.32 to 6.24 log units. They also did not monitor a bacteriostatic effect in kefir during fermentation. In our study, *L. monocytogenes* counts increased from 5.57 to 6.29 log units in 10^6 contaminated samples, while in 10^4 contaminated samples, there were an increase from 3.74 to 5.35 log units.

According to the results, it may be speculated that *L. monocytogenes* grow easily in the early stage of kefir fermentation when the development of acidity and other antimicrobial substances produced by fermentative cultures are 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, and we tested only these strains of the study. For that reasons, our results will not fully reflect the behaviour of all pathogen *L. monocytogenes* strains in the modified kefir.

Many factors including the type of culture microorganisms, fermentation and storage conditions affect the growth and/or survival of the pathogenic microorganisms in the fermented dairy foods [15]. Gahan CG, et al. [19] demonstrated that acid adaptation of *L. monocytogenes* can enhance the survival of this organism in acidic dairy foods during fermentation. Santos A, et al. [16] observed the antagonistic behavior of lactobacilli isolated from kefir grains against *E. coli*, *L. monocytogenes*, *S. typhimurium*, *S. enteritidis*, *Shigella flexneri* and *Y. enterocolitica*. Silva KR, et al. [10] observed the inhibition of

Candida albicans, *Salmonella typhi*, *Shigella sonnei*, *Staphylococcus aureus* and *E. coli* by kefir cultured in brown sugar. On the other hand, Chifiriuc MC, et al. [11] observed that all milk fermented with kefir grains had antimicrobial activity against *Bacillus subtilis*, *S. aureus*, *E. coli*, *E. faecalis* and *S. enteritidis*, but did not inhibit *P. aeruginosa* and *C. albicans*. All these studies indicate that kefir antimicrobial activity is associated with the production of organic acids, peptides (bacteriocins), carbon dioxide, hydrogen peroxide, ethanol and diacetyl. These compounds may have beneficial effects not only in the reduction of food borne pathogens and deteriorating bacteria during beverage production and storage, but also in the treatment and prevention of gastroenteritis and vaginal infections [20,21].

In conclusion, Kefir microflora found to be suppressive on *L. monocytogenes* serotypes studied in this study. Besides, there was significant survival difference between all *L. monocytogenes* serotypes. The major reduction in the study was 2.37 log cfu/ml which was detected in the second hour of 10⁴ cfu/ml contaminated kefir experiment with 4b serotype. Therefore, we can conclude from this study that despite the high acidity of kefir, it could be potentially hazardous to the public health if it is contaminated with the pathogens studied here in. Such risk would increase if the product was contaminated before the fermentation period.

Conflict of Interest

All the authors declared that they have no conflict of interest.

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*Corresponding author: Bahar ONARAN, Ankara University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, 06110, Diskapi, Ankara, Turkey, E-mail: dr.baharonaran@gmail.com.

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