# Survival of pathogenic microorganisms in *kefir*

# Sobrevivência de micro-organismos patogênicos em *kefir*

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**ABSTRACT**

Kefir is a homemade fermented milk produced by adding kefir grains. The domestic handling and the use of raw materials from different standards and sources, and the lack of inspection by qualified professionals, all this classify kefir as a food which might represent potential risks to human health. This study aimed at evaluating the pathogens survival during the kefir fermentation process. Kefir grains were added into portions of UHT skimmed milk which were experimentally contaminated with *Escherichia coli* O157:H7, *Salmonella* Typhimurium and Enteritidis, *Staphylococcus aureus* and *Listeria monocytogenes*. Analyses of the microorganism isolation in these milk samples were carried out at 0, 6, 12, 48 and 72 hours of fermentation process. *Salmonella* Typhimurium and Enteritidis survived for a 24-hour period in fermenting kefir. *Escherichia coli* O157:H7, *Staphylococcus aureus* and *Listeria monocytogenes* were recovered in less than 72 hours after the fermentation process was initiated. Under the conditions and the microorganisms concentrations established in the present study, the analyzed pathogenic bacteria survived for a period longer than those used for homemade kefir fermentation, and this one might be a potential hazard for human consumption.

**Keywords.** kefir, fermented milk, inhibition, pathogens.

**RESUMO**

Kefir é leite fermentado produzido de forma artesanal pela adição de grãos de kefir ao leite. A manipulação doméstica e o uso de matéria-prima de diferentes padrões e origens, aliados à falta de inspeção por profissional competente, fazem do kefir um alimento capaz de apresentar perigos potenciais para a saúde humana. No presente trabalho, foi avaliada a capacidade de sobrevivência de micro-organismos patogênicos durante a fermentação do kefir. Os grãos de kefir foram adicionados a porções de leite UHT desnatado, as quais foram experimentalmente contaminadas com *Escherichia coli* O157:H7, *Salmonella* Typhimurium e Enteritidis, *Staphylococcus aureus* e *Listeria monocytogenes.* As amostras preparadas foram analisadas quanto à presença dos micro-organismos após 0, 6, 12, 24, 48 e 72 horas de fermentação. *Salmonella* Typhimurium e Enteritidis sobreviveram por 24 horas no kefir em fermentação. *E. coli* O157:H7, *S. aureus* e *L. monocytogenes* foram recuperados até 72 horas após o início da fermentação. As bactérias patogênicas estudadas, nas concentrações e condições do presente trabalho, sobreviveram por tempo superior àquele normalmente utilizado para a fermentação do kefir preparado artesanalmente, o qual representa perigo potencial para o consumo humano.

**Palavras-chaves.** *kefir*, leite fermentado, inibição, agentes patogênicos.

## INTRODUCTION

Kefir is a kind of fermented milk, usually homemade, originated in the Caucasus, produced by the addition of kefir grains to milk. These grains consist of jellylike lumps that contain both bacteria and yeasts immersed in a protein-and-polysaccharide-matrix. The most commonly isolated microorganisms in kefir grains are the genera *Lactobacillus* (*L. brevis*, *L. casei*, *L. kefiri, L. acidophilus*, *L. plantarum*, *L. kefiranofaciens* subsp. *kefiranofaciens*, *L. kefiranofaciens* subsp. *kefirgranum*, *L. parakefir*), *Lactococcus* (*L. lactis* subsp. *lactis*), *Leuconostoc* (*L. mesenteroides*), *Acetobacter*, *Kluyveromyces* (*K. marxianus*) and *Saccharomyces*[1,2,3,4].

The production of kefir at industrial scale is limited due to the difficulty in obtaining starter cultures with the required stable composition for maintaining standard quality. Notwithstanding, the consumption of this kind of homemade fermented milk is widespread, mainly due to its alleged nutraceutical properties[5]. Domestic handling and the use of raw materials of different standards and sources, and also the lack of inspection by qualified professionals, classify kefir as a food which may represent potential hazards to human health. Milk is an excellent culture medium for different microorganisms, and plays a major role in food-transmitted disease epidemiology. Among disease-causing microorganisms eventually carried by dairy products are *Salmonella* genus, *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogens*[6]. In order to guarantee consumer safety, it is essential to be aware of the risks brought up by pathogenic microorganisms eventually found in kefir.

This study aims at evaluating *E. coli* O157:H7, *Salmonella enterica* subsp. *enterica typhimurium* and *enteritidis* serotypes, *S. aureus* and *L. monocytogenes* survival during kefir fermentation process.

## MATERIAL AND METHODS

### Bacterial strains

We used *E. coli* O157:H7, by courtesy of Dr. T. Yano (Unicamp, Campinas, Brazil), *Salmonella* Typhimurium LIPOA 2023, previously isolated from pork sausage[7], *Salmonella* Enteritidis LIPOA 2025, previously isolated from ground chicken meat[8], *S. aureus* ATCC 14458 and *L. monocytogenes* ATCC 7644. The *E. coli* O157:H7 strain was successively cultivated on agar for standard plate count (PCA, Acumedia) containing increasing concentrations of a medium of nalidixic acid, up to 100 μg/mL.

### Kefir

Kefir grains were obtained from those used for homemade preparation of fermented milk. The grains were added to skim UHT milk at a 1:10 ratio and kept at 20 °C. The kefir was strained daily on a sterile strainer, and the grains were once more mixed with the milk, returning to incubation. This process was repeated for a week.

### Experimentally contaminated kefir

The bacterial strains were incubated in brain heart infusion (BHI, Acumedia) at 37 °C until stationary phase. Inocula with approximately $10^5$ CFU. $mL^{-1}$ bacterial concentration were prepared from serial dilutions for each culture. 500 mL UHT skim milk was inoculated with 5 mL so as to obtain a final concentration of about $10^3$ CFU.$mL^{-1}$. Following, 50 g of kefir grains were added. This proceeding was made separately for each microorganism. Similarly prepared kefir without inoculation of the studied microorganisms was used as negative control. For positive control, we used 500 mL skim UHT milk inoculated with each microorganism at the same experimental kefir concentration, but without grain addition. Both the experimentally contaminated kefir and the controls were kept at 20 °C.

### Microorganism survival

Research on the studied microorganisms from a sample of each material after 0, 6 and 12 hours of natural fermentation at 20 °C without shaking was done. Two samples were analyzed at 24 and 48 hours of fermentation, and three at 72 hours. During each analysis, pH was determined by the use of a DMPH-2 Digimed pH meter. The *Salmonella* and *L. monocytogenes* research was performed according to US Food and Drug Administration (FDA)[9] recommendations (Andrews and Hammack[10], Hitchins[11]). For *E. coli* O157:H7 research, 25 mL of experimentally contaminated kefir was added to 225 mL buffered peptone water (BPW, Acumedia) and incubated at 37 °C for a 24-hour period. From this culture, spreads on MacConkey agar (Oxoid) added with nalixidic acid at 100 μg.$mL^{-1}$ medium concentration and incubation at 37 °C for a 24-hour period were done. For *S. aureus* research, 25 mL of experimentally contaminated kefir was incubated at 37 °C for a 24-hour period in 225 mL tryptic soy broth (TSB, Acumedia) added with 1% (w/v)

of sodium pyruvate and 10% (w/v) of sodium chloride. Following, spread on Baird Parker agar (Acumedia) and incubation at 37 °C for 24 hours were done. Suspicious colonies were biochemically confirmed according to FDA[9] recommendations (Bennett and Lancette[12]). When growth on selective agar was not observed within the established incubation period, this period was extended for another 24 or 48 hours.

## RESULTS AND DISCUSSION

The ability of pathogenic bacteria that are eventually carried by milk to survive in homemade produced kefir, similarly to that usually found under normal consumption conditions, was researched. It showed that milk fermentation caused by these microorganisms generates unfavorable conditions for the survival of the tested bacteria.

All bacteria were recovered from experimentally contaminated milk used as positive control after up to 72 hours of storage. No bacterium was recovered from the negative controls.

Among the pathogens studied, *E. coli* had the greatest resistance (Table 1). This microorganism is the most frequently found thermotolerant coliform in unprocessed milk and dairy products that have not been submitted to thermal treatment, indicating that this bacterium can adapt to environments rich in milk components. Gulmez and Guven[13] studied the *E. coli* O157:H7 behavior in kefir after a 24- and 48-hour fermentation period and observed a population growth which was kept viable for 21 days in cooled food. In their study, experimental kefir was prepared by inoculating milk with other previously prepared kefir, differently from this study, in which kefir was produced from the direct inoculation of kefir grains. The procedure adopted by Gulmez & Guven[11] resulted in a much milder fermentation, which did not significantly affect the growth of *E. coli* O157:H7. Moreover, the ensuing cool storage maintenance inhibited the fermentation process and consequently the production of inhibitory factors, allowing the pathogen survival for a longer period. Kasimoglu and Akgun[14], though not working with kefir, analyzed the behavior of *E. coli* O157:H7 in traditional and acidophilus yogurt. By experimentally inoculating milk before fermentation at a $10^4$ CFU.mL$^{-1}$ concentration, these authors did not succeed in recovering the microorganism after a 48-hour period. However, at an initial $10^6$ CFU.mL$^{-1}$ concentration, *E. coli* O157:H7 was recovered after a period of up to 72 hours in traditional yogurt.

**Table 1.** Recovery of pathogenic microorganisms from experimentally contaminated kefir in different fermentation times at 20 °C

| Microorganisms | Kefir (repetition) | Fermentation time (h) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 6 | 12 | 24[1] | 48[1] | 72[1] |
| *Escherichia coli* O157:H7 | 1 | + | + | + | ++ | +++ | +-- |
| | 2 | + | + | + | ++ | +++ | +++ |
| | 3 | + | + | + | ++ | +++ | +++ |
| *Salmonella* Typhimurium | 1 | + | + | + | ++ | --- | --- |
| | 2 | + | - | - | -- | --- | --- |
| | 3 | + | - | - | -- | --- | --- |
| *Salmonella* Enteritidis | 1 | + | + | + | +- | --- | --- |
| | 2 | + | + | + | ++ | --- | --- |
| | 3 | + | + | + | -- | --- | --- |
| *Staphylococcus aureus* | 1 | + | + | + | ++ | --- | --- |
| | 2 | + | + | + | ++ | --- | --- |
| | 3 | + | + | + | ++ | +++ | +++ |
| *Listeria monocytogenes* | 1 | + | + | - | -- | --- | --- |
| | 2 | + | + | + | ++ | +++ | --- |
| | 3 | + | + | + | ++ | +++ | --- |

[1] Two analyses were performed at 24-hour fermentation and three at 48 and 72 hours; +: presence in 25 g; -: absence in 25 g

Even though *Salmonella* Typhimurium and Enteritidis proved to be the least resistant bacteria among the studied pathogens, they managed to survive for up to 24 hours of kefir fermentation. It is possible that the strain used in this study was more adapted to the meat products, where they were found, than to the fermented milk. Czamansky[15], on researching kefir antimicrobial action on Gram-negative microorganisms, observed *Salmonella* inactivation after a 60-minute exposition period. This fast inhibitory action was not observed in the present study, probably because of the inoculum concentration – $10^3$ CFU.mL$^{-1}$ – far higher than that used in Czamansky[15], which was of 10 CFU.mL$^{-1}$.

*S. aureus* was viable under fermentation in kefir for up to 72 hours. Although it varies according to food conditions and characteristics, an effective dose of this enterotoxin can be produced when the *S. aureus* population exceeds $10^5$ cells.g$^{-1}$ food[9]. The initial concentration used in the experimental contamination of milk added with kefir grains was approximately of $10^3$ CFU.mL$^{-1}$ in the present study, lower than that needed to represent a hazard to human health. The scope of this study was to evaluate the survival ability of this pathogen in kefir, and we did not perform bacterial counts which could allow

**Table 2.**Means of pH values of the experimentally contaminated kefirs and the positive controls in different fermentation times at 20°C.

| Treatments | pH (means ± standard deviation) | | | | | |
|---|---|---|---|---|---|---|
| | 0h | 6h | 12h | 24h | 48h | 72h |
| LIPOA CDT | 6.2±0.1[a] | 5.2 ± 0.1[a] | 4.9 ± 0.4[a] | 4.3 ± 0.4[a] | 4.0 ± 0.2[a] | 3.9 ± 0.[1]a |
| LIPOA ABB | 6.3 ± 0.1[a] | 5.0 ± 0.4[a] | 4.7 ± 0.4[a] | 4.3 ± 0.1[a] | 4.0 ± 0.2[a] | 4.0 ± 0.1[a] |
| LIPOA MH | 6.3 ± 0.1[a] | 5.1 ± 0.5[a] | 5.0 ± 0.2[a] | 4.6 ± 0.3[a] | 4.2 ± 0.1[a] | 4.0 ± 0.0[a] |
| Positive controls(milk with pathogens) | 6.5 ± 0.0[b] | 6.3 ± 0.1[b] | 6.2 ± 0.1[b] | 5.9 ± 0.2[b] | 5.6 ± 0.4[b] | 4.9 ± 0.1[b] |

Means with distinct letters in the columns differ statistically (P<0,001)

a follow-up of the *S. aureus* population behavior during the fermentation process. The likelihood of a quantitative increase of this microorganism during the first hours of fermentation, producing toxin in concentration able to cause human intoxication, however, cannot be discarded based on results obtained. Therefore, the *S. aureus* ability of surviving up to 72 hours in the fermenting product represents a potential hazard to consumers.

*L. monocytogenes* was viable under the conditions generated by the kefir production process for up to 48 hours of fermentation. These results are in agreement with Gulmez and Guven's[13] observations, who, though working with a milder fermentation than that used in the present study, recovered *L. monocytogenes* from kefir after 24 and 48 hours of fermentation.

The fermentation that is caused by microorganisms in the kefir grains triggers a more intense acidification process in kefir than that in milk without the addition of these grains. Thus, the pH values obtained during the fermentation of experimentally contaminated kefir, as well as the negative control values, were lower than those found in positive controls (milk without kefir grains) (Table 2). This fact suggests that the alterations as a result of pH decrease are related to pathogen inhibition, once all inoculated microorganisms in positive controls were recovered after up to 72 hours of contamination. Garrote et al.[2], when studying the kefir supernatant inhibitory action on Gram-positive and Gram-negative microorganisms, observed that the antimicrobial effect is mainly due to the organic acids produced during the fermentation process. However, the possibility that microorganisms on kefir grains produce metabolites with antimicrobial activity cannot be ruled out.

As under normal domestic preparation conditions kefir is generally consumed within 24 hours of fermentation, and rarely after 48 hours, because it loses palatability due to acidification, the risk represented by the likely presence of pathogenic microorganisms is high. The results emphasize the importance of sanitary quality of the milk used as raw material and of hygienic procedures in utensil cleaning and handling during homemade kefir preparation.

The studied pathogenic bacteria, in the concentrations and under the conditions of the present study, survived for a longer period than that normally used for homemade kefir preparation, representing a potential hazard to human consumption. *Salmonella* showed lower survival ability in the environment generated by fermenting kefir as compared to other bacteria in this study. Milk fermentation with kefir grains promoted the conditions that exerted an inhibitory effect on the tested microorganisms. The pH decrease caused by the kefir process seems to be related to the inhibition of pathogenic bacteria.

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