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COMPARATIVE STUDY REGARDING LACTIC MICROFLORA STORAGE

TIMAR A. V.

University of Oradea – Faculty of Environmental Protection University of Oradea – Faculty of Environmental Protection atimar@uoradea.ro

Abstract

We try to find differences between methods for kefir grain storage. The microflora of kefir grains was isolated and identified. We find lactococci, lactobacilli, yeasts and moulds. Grains stored at different low temperatures maintained their microflora and increased their weights. Fermented milk with the stored grains showed the same microflora like fresh strains. Grains stored at 4°C did not increase their weight. Storage at -20 °C is a good method to preserve kefir grains for fermented milk but the best results we obtained with grains stored at -60°C .

Key words: lactic microflora; milk; lactic bacteria; yeast; microflora storage.

INTRODUCTION

Kefir is a fermented product originating in the Caucasian mountains and has become popular in many European countries.

This diary product differs from other milk products because it is not the result of the metabolic activity of a single species. The milk is fermented with a mixed microflora confined to a matrix of discrete 'kefir grains', which are recovered after fermentation. Kefir grains have a structure similar to tiny florets of cauliflower which vary in size from 0.3 to 3.5 cm diameter.

The activity of the grain depends on the viability of the microflora. Yeast and lactic acid bacteria (LAB) coexist in a symbiotic association and are responsible for an acid-alcoholic fermentation. The microbial composition of grains depends on the origin. Various yeasts and LAB have been described. Among them the genera most frequently reported are homofermentative and heterofermentative *Lactobacillus, Lactococcus, Leuconostocs* and acetic acid bacteria.

For propagation of the starter culture, presence of all the microorganisms composing the kefir grain, in their desirable and adequate proportion, is required.

The purpose of this work was to compare preservation of kefir grain as a starter and to evaluate kefir quality when grown in milk after storage.

MATERIALS AND METHODS

Starter culture Kefir grains were obtained from a household in Romania, Bihor county.

We use fresh UHT pasteurized milk.

Milk fermentation Two grams of kefir grains, washed with sterile distilled water, were inoculated in 100 ml milk. After incubation at 20°C, the grains were separated from the fermented milk by filtration through a metalic sieve.

Kefir grains were broken up in a mortar and in triptone 1 g/l. The microflora was isolated by surface-spreading on plates and incubation at 30°C for 48 h in an aerobic atmosphere. For isolation of lactic acid bacteria (LAB) MRS-agar and Lee's medium were used. YGC-agar was used for yeast isolation.

LAB identification.LAB were classified by cellular morphology, identified according to criteria described by Apostu 2004.

Yeast identification.Yeast were identified according to criteria described by Apostu 2004.

Enumeration of viable microorganisms.The results were expressed as colony forming units per milliliter (cfu/ml) of fermented milk or colony forming units per gram of dry kefir grain (cfu/g).

Determination of wet weight of kefir grains. Kefir grains were washed with sterile water, dried between tissue paper, and weighed with a Sartorius analytical balance (with a precision of + 0.01 mg). Further drying by the same procedure did not significantly affect the wet weight of the grains.

Determination of dry weight of kefir grains.Dry weight was determined at 100 °C in a Sartorius analytical balance to constant weight.

Storage of kefir grains. Kefir grains were washed with sterile distilled water, dried in tissue paper and divided into four batches. Two of them were introduced in milk and frozen at -20°C and -60°C. A third batch was centrifuged twice for 5 min at 15,000 rpm, dried with tissue paper and kept at 4°C in a Petri dish sealed. The fourth fraction was maintained as a control by successive subculturing each 48 h. Each fraction was kept for 100 day in each condition.

RESULTS AND DISCUSSION

Influence of storage conditions on the activity of kefir grains

Microbiological features of the kefir grain. At least four types of bacteria, two yeasts and one mould were detected in the kefir grains. Bacteria were characterized as Lactic Acid Bacteria (LAB), being Gram-positive, nonspore-forming and catalase-negative rods and cocci. The two cocci were identified as *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis*

subsp. *diacetylactis*. The homo-fermentative bacillus seemed to be *Lactobacillus kefir-anofaciens* or *Lactobacillus kefirgranum;* the hetero-fermentative bacillus showed a pattern of sugar fermentation similar to *Lactobacillus brevis, Lactobacillus kefir* or *Lactobacillus parakefir*. The lactose-nonfermenting yeast was characterized as *Saccharomyces*. The principal characteristics of this genus are : multilateral budding, pseudomycelium production, no film on liquid medium, fermentation with gas production and negative nitrate assimilation. The mould that frequently was present on the kefir culture surface was characterized as *Geotrichum candidum.*

The number of colony forming units per dry weight of kefir grains and the percentage of lactococci, lactobacilli and yeasts with respect to the total of microorganisms are shown in Table 1. The number of viable microorganisms was expressed by grams of dry weight of kefir grain. The exact number of microorganisms cannot be easily detected, due to the distribution of the microflora on the surface.

Activity of kefir grains stored under different conditions The microbiological characteristics of the kefir grains stored for 100 day under different conditions were studied (Table 4). The results are shown as cfu/g and ratio between the number of each type of viable microorganism in stored kefir grains.

The data in Table 2 show that the viable yeast concentration decreases with all conditions tested. However, the relative decrease is lower with respect to the control when kefir grains are stored at -60°C

Lactobacilli viable concentration decreases between 0.46 and 0.6 times when stored at -60°C and -20°C. However, the concentration decrease is higher with storage at 4 °C. This can be explained either as a consequence of the rupture of the bacterial chains or by the growth of cells during the first days of storage. The concentration of lactococci is not affected by storage conditions within the experimental error of the determinations.

The weight of the kefir grains increases with successive sub culturing. Grains stored at 4°C showed a negligible increase in weight. In contrast, the grains stored at -20°C and -60°C increased their weights at a rate comparable to that found with non stored grains. After eight sub culturings in milk, the increase of the total weight of the grains stored at -20°C and -60°C was 4-8 times higher than those stored at 4°C. This type of assay was carried out by sub culturing the total amount of grains in the same final volume of milk and keeping the concentration of inoculum at 20g/l. In both cases, the same trend was observed. These results confirm that the grains stored at 4°C lose their ability to produce some components for the production of the matrix.

Influence of storage conditions of kefir grain on the fermented milk

Milk fermented with kefir grains contains the same types of micro-organisms as isolated from the kefir grains. However, *Lactobacillus kefir* was not detected, probably because it was at a very low concentration. After the addition of grains to the milk the lactococci reach a concentration of 10^3cfu/ml and the yeasts and lactobacilli reach 10^4cfu/ml. This indicates that part of the microflora contained in the kefir grain is transferred to milk.

Table 1. Microflora of kefir grain

	cfu/g±SD, *n**	Percent of 10^{-7}, microorganism*
Lactococci	1.58±1.47	0.87
Lactobacilli	148.00± 12.60	77.63
Yeasts	40.70±3.55	20.56

cfu/g = colony forming units per gram of dry kefir grain. *Percent with respect to the total of microorganisms. Mean value of 10 independent assays of dry weight was 226.5g/kg with a standard deviation of 36.5g/kg.

Table 2. Microflora of kefir grains stored under different conditions

Storage condition	10^{-7} • cfu/g+SD		
	Lactococci	Lactobacilli	Yeasts
Subculture	10.7+9.7	264.3+21.4	12.70+1.2
-60 °C	21.7+20.6	120.3+9.4	1.00+0.1
-20 °C	n.d.	158.9+12.3	0.07+0.0
4°C	17.6+16.8	532.4+42.1	0.05+0.0

cfu/g = colony forming units per gram of dry kefir grain. n.d. = not determined. To calculate the number of microorganisms per gram of dry kefir grain, the dry extract for each case was considered. Dry extract of the grains stored in milk at -60 °C and -20°C was 278 and 321 g/kg, respectively, when stored at 4°C.

The number of microorganisms in the product varies along the fermentation, probably due to the growth of microorganisms in the milk and to the transfer of cells growing in the grain to the milk. The dynamic of growth of the yeasts shows that after 25 h the stationary phase is reached. The number of cfu/ml at that stage is 10^6-10^7. After 36 h, both lactococci and lactobacilli reached the stationary phase with a final concentration of 10^9-10^{10}cfu/ml.

Characteristics of the product obtained with kefir grains stored in different conditions. Fermented milks obtained using the starter of kefir grains stored under different conditions described above were analysed. It was observed that these milks did not present the organoleptic features and coagulum consistency typical of the product obtained with non-stored kefir grains.

Microflora of the fermented milks after 72 h of incubation and the ratio between the number of each microorganism with respect to the corresponding control are shown in Table 3.

Table 3. Microflora of fermented milk obtained with kefir stored under different conditions

Storage condition	10^{-9} • cfu/ml		
	Lactococci	Lactobacilli	Yeasts
Subculture	6.11	32.46	0.0029
-60 °C	28.88	28.81	0.0142
-20 °C	34.45	20.10	0.0019
4°C	0.28	0.30	0.0002

cfu/ml = colony forming units per millilitre of fermented milk.

The final concentration of yeasts in the milk fermented with grains stored at 4°C was significantly lower than that obtained with frozen and control grains. The concentration of lactobacilli and lactococci in the milk fermented with grains stored at 4°C decreased with respect to those obtained with grains stored in the other conditions. The higher values of lactococci obtained with fermented milk with frozen grains can be attributed to the rupture of cell chains or to a diminution of the retention of these micro-organisms in the matrix after freezing and thawing. These variations in the microflora will have a considerable influence on the final acidity features, gas content and viscosity of the product.

CONCLUSIONS

In this work the microflora of kefir grains of household origin and the methods used to conserve them were analyzed.

Two yeasts and a mould were isolated from kefir grains in this work. The yeast was identified as *Saccharomyces cerevisiae* and the mould as *Geotrichum candidum.* The other yeast, *Saccharomyces lipolytic,* belongs to the group of yeasts that do not ferment lactose and it is only described in the grains studied in this work.

Two cocci and two bacilli were isolated from the grains studied in this work. The cocci were identified as *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *diacetylactis.* In addition, two lactobacilli, one homofermentative and the other heterofermentative, were isolated. The former was identified as *Lactobacillus kefiranofaciens* or *Lactobacillus kefirgranum.* The second could be *Lactobacillus brevis, Lactobacillus kefir* or *Lactobacillus parakefir.* They are heterofermentative lactobacilli with similar metabolic characteristics.

The distribution of microorganisms in the grain and kefir culture was different. In the grains there were 10^7cfu/g lactococci, 10^9cfu/g lactobacilli and 10^8 cfu/g yeast, while in the fermented milk there were 10^9-10^{10}cfu/ml lactococci, 10^9-10^{10}cfu/ml lactobacilli and 10^6-10^7cfu/ml yeast.

When milk is inoculated with kefir grains, some microorganisms are dispersed into the milk phase.

Kefir quality depends on properties such as chemical composition, microflora, rheology and organoleptic features. Storage at 4°C, wet or dried, has been proposed as an alternative method for preserving kefir grains. During storage the characteristics of the grain and the fermented milk must be maintained. The main parameters to consider in the evaluation of the preservation methods are: maintenance of all the microflora species and their relative proportions in the grain.

The data show that preservation at -60°C alters the microbiological composition of kefir grains than when done at -20°C or 4°C. In addition, milk fermented with grains frozen at -60°C have the microflora, similar to fermented milk obtained with control grains**.** Grains stored at 4°C showed a great change in the microflora.

Subculturing allows good-quality products to be obtained, but it is susceptible to contamination. A good product can be obtained with grains stored at -60°C avoiding subculture.

Results obtained in this study show that freezing is better than other methods to preserve kefir grain. This storage condition maintains grain activity needed to ferment the milk.

Lactococci, lactobacilli and yeast can be mutually stimulated to produce the components of the grain matrix. Grains maintained by subculturing or frozen at -20°C and -60 °C increased their weight but those stored at 4°C did not increase their weight after eight transfers into milk.

These results show an alternative procedure to preserve kefir grain and can be used to maintain the grains for household kefir production.

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