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COMPARATIVE STUDY OF KEFIR LACTIC MICROFLORA

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Abstract

The microflora of kefir grains of household origin was isolated and identified. At least two lactococci, two lactobacilli, two yeast and one mould were detected. Different methods for kefir grain preservation were studied. Metabolic activity of grains preserved frozen at —20 °C and —80 °C and grains stored at 4 °C was evaluated. Grains stored at -20 °C and -80 °C maintained their microflora and increased their weights at a rate comparable to that found with nonstored grains. Fermented milk obtained with the grains stored at -20 °C and -80 °C showed the same microflora, rheological behaviour, acidity and carbon dioxide content as fermented milk obtained with nonstored grains. Grains stored at 4 °C did not increase their weight and the product obtained did not have the acidity and viscosity of the standard product. Storage at -20 °C is a good method to preserve kefir grains for household manufacture of fermented milk.

Key words: kefir; fermented milk; lactic acid bacteria; yeast; preservation.

INTRODUCTION

Kefir is a fermented beverage originating in the Caucasian mountains and has become popular in many European countries. It is also available in some stores throughout North America.

This beverage 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. They are composed mostly of proteins and polysaccharides in which the complex microflora is enclosed. The chemical composition of kefir grains is 890-900 g/kg water, 2 g/kg lipid, 30 g/kg protein, 60 g/kg sugars and 7 g/kg ash. 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, Leuco-nostocs* and acetic acid bacteria. Bottazzi and Bianchi used scanning electron microscopy to investigate the distribution of microflora within the kefir grain. They suggested that the population of yeasts and lactobacilli were not randomly distributed in the grain. Lactobacilli were at the periphery of the grain, while the yeasts were located inside the grain.

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

proportion, is required. Lyophilized kefir grains are sold by some companies. Some authors recommend storage of kefir grains wet at 4 °C or dry at room temperature for 36-48 h. Dried kefir grains retain activity for 12-18 months, whereas wet grains retain activity for only 8-10 days. Difficulties have been found in maintaining a satisfactory quality in grains to produce a beverage with the appropriate and acceptable viscosity. Characteristics of acidity, viscosity and carbon dioxide content have been described but their variations with storage conditions have not been studied. The purpose of this work was to compare two methods for preservation of kefir grain to be employed as a starter and to evaluate kefir grain metabolic activity when grown in milk after storage.

MATERIALS AND METHODS

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

Chemicals and culture media Yeast extracts and tryptone were obtained from Difco(Detroit, MI, U.S.A.). Agar was obtained from Merck (Darmstadt, Germany). Lactose, sucrose, $CaCO_3$ and K_2HPO_4 were obtained from Merck (Darmstadt, Germany). MRS-agar (11) was also obtained from Merck (Darmstadt, Germany). YGC-agar (yeast extract-glu-cose-chloramphenicol-agar), bromocresol purple (BCP), potassium ferrycyanide, ferric citrate, sodium citrate and malt extract were obtained from Merck (Darmstadt, Germany). Sugars, urea and potassium nitrate were obtained from Merck (Darmstadt, Germany). Lee's medium (12) in g/L was composed of triptone 10, yeast extract 10, lactose 5, sucrose 5, $CaCO_3$ 3, K_2HPO_4 0.5, BCP 0.02, and agar 18, and was pH = 7. Basal medium for sugar fermentation in g/L was composed of yeast extract 6, peptone of casein 15, cysteine chlorhydrate 0.2, Tween 80 1, BCP 0.02, $MgSO_4.7H_2O$ 0.2, and $MnS0_4$ $.4H_2O$ 0.05, and was pH = 6.4. All media were autoclaved at 120 °C for 15 min.

Reconstituted skim-milk was prepared by dissolving 120 g of skim milk in 1 L distilled water and sterilized by three 30-min treatments at 100 °C.

Milk fermentation Two grams of kefir grains, washed with sterile distilled water, were inoculated in 100 mL reconstituted milk. After incubation at 20 °C, the grains were separated from the fermented milk by filtration through a plastic sieve and were washed prior to the next culture passage (subculture).

Isolation of kefir micro flora Kefir grains were broken up in a mortar and resus-pended 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, Gram staining, spore formation, motility, catalase reaction, growth temperature (25, 30 and 37 °C), growth in milk, gas production in basal medium for sugar fermentation with 10 g/L glucose, fermentation in basal medium for sugar fermentation supplemented with different sugars, and citrate fermentation on Kempler and McKay medium. Based on these characteristics genus and species names were determined for each organism following the criteria of Bergey's Manual of Systematic Bacteriology and API system.

Yeast identification Yeast and mould characterization was by macroscopic and microscopic orphology, growth in malt extract, type of reproduction, growth at 37 °C, growth in vitamin-free medium, hydrolysis of urea, sugar fermentation, carbon compounds and nitrate assimilation. Yeasts were identified according to criteria described by Kreger-Van Rij.

Enumeration of viable microorganisms The concentrations of viable bacteria and yeasts in kefir cultures and suspensions of kefir grains were determined by plating serial dilutions in 1 g/L of triptone on Lee's medium, MRS-agar and YGC-agar plates. 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 + 0.1 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 Mettler LP16 balance to constant weight. The standard deviation (SD) for the weight determination was 3.65 for 10 independent experiments.

Determination of the CO_2 content in kefir cultures Gaseous samples of 380 xL were analysed in a Shimadzu GC-GA gas chromatograph using a silica gel column at 100°C and a thermal conductivity detector. The experiment was carried out at 125 °C using hydrogen as carrier at a flow rate of 20 mL/min.

Determination of viscosity A rotational viscometer (Haake Rotovisco RV2, Germany) with a thermostatic system, and a sensor NV (Nieder Viskositat) of concentric cylinders was used. Rheological properties were measured at 30 °C. Flow behaviour was analysed through shear stress (T) *vs.* shear rate (D) curves. The following programme was performed: an increasing sequence from 0 to 2769.9 s^{-1} in a period of 3 min, followed by 1 min at the maximum value and a corresponding decreasing sequence in 3 min. Apparent viscosity (n_{ap}) was calculated at 629.5 s^{-1} and expressed as mPa.s.

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

resuspended in milk and frozen at -20 °C and -80 °C. A third batch was centrifuged twice for 5 min at 14,000 rpm, dried with tissue paper and kept at 4 °C in a Petri dish sealed with Parafilm. The fourth fraction was maintained as a control by successive subculturing each 48 h. Each fraction was kept for 120 d 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 homogenate of kefir grains. Bacteria were characterized as Lactic Acid Bacteria (LAB) **(Table 1),** 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 **(Table 2).** 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, lactoba-cilli and yeasts with respect to the total of microorganisms are shown in **Table 3.** 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 heterogeneous distribution of the microflora on the surface and the difficulty in separating the microorganisms from the polysaccharide matrix. However, the coefficients of standard deviations are not very high for lactobacilli and yeasts, being 8.3% and 9.6%, respectively. The standard deviation is 91.45% for the lactococci. The lactobacilli/lactococci ratio is 0.01 and that of bacteria/ yeasts is 3.8.

A ctivity of kefir grains stored under different conditions The microbiological characteristics of the kefir grains stored for 120 d 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 4** 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 -80 °C.

Table 1 Characteristics of LAB strains from Kefir

Strain	CIDCA 821	CIDCA 822
Colony morphology*	Yellowish, glossy, with smooth	White, with smooth border
Cellular morphology	Ovoid, in pairs or short chains,	Ovoid, in pairs or short chains,
Spore forming	-	-
Motility	-	-
Catalase	-	-
Milk coagulation Growth	+	+
	Cel, Mai, Lac, Sac, Tre	Lac, Sac, Tre, DTur
Gas from glucose	-	-
Citrate fermentation	+	-
Genus, species and subsp.	*Lactococcus lactis* subsp.	*Lactococcus lactis* subsp. *Lactis*
Strain	CIDCA 831	CIDCA 832
Colony morphology*	Brown and small, with smooth	Very small, plain and rough
Cellular morphology	Short rods, singly or in chains,	Rods with rounded ends, singly or in
Spore forming	-	-
Motility	-	-
Catalase	-	-
Milk coagulation Growth	d	+
	Lac, Mel, Sac, Tre, Mlz, Gnt	Tre, Mlz, Gnt
Gas from glucose	-	+
Citrate fermentation	n.d.	n.d.
Genus, species and subsp.	*Lactobacillus kefiranofaciens*	*L actobacillus kefir, Lactobacillus*

*On Lee medium.+=positive reaction, —=negative reaction, d = different reactions, n.d. = not determined.Amy = amygdalin, LAra = L-arabinose, Arb = arbutin, Cel = cellobiose, DFru = D-fructose, Gal = galactose, DGlu = D-glucose,Gnt = gluconic acid, Lac = lactose, Mal = maltose, Man = mannitol, Mel=melibiose, Mlz = melezitose, DMne = D-mannose,Rha=rhamnose, Rib = ribose, Sac = sucrose, Sal = salicin, Tre = trehalose, DTur = D-turanose.All assays were carried out at 30°C unless indicated.

Lactobacilli viable concentration decreases between 0.46 and 0.6 times when stored at -80 °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 (Fig. 1). Grains stored at 4 °C showed a negligible increase in weight. In contrast, the grains stored at -20 °C and -80 °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 -80 °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 (Fig. la) and keeping the concentration of inoculum at 20 g/L (Fig lb). 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

Characteristics of the fermented milk. Milk fermented with kefir grains contains the same types of microorganisms as isolated from the kefir grains. However, *Lactobacillus kefir* CIDCA 832 was not detected, probably because it was at a very low concentration. It can be observed in Fig. 2 that

immediately after the addition of grains to the milk the lactococci reach a concentration of 10^3 cfu/mL and the yeasts and lactobacilli reach 10^4 cfu/mL. This indicates that part of the microflora contained in the kefir grain is transferred to milk.

Table 2 Characteristics of yeasts from Kefir

Strain	CIDCA 811		CIDCA 812		CIDCA 813	
Colony	White to cream, dull,		White to cream, waxy,		White, firm, fimbriate,	
Colony	Unicellular, globose to		Unicellular, globose to		Pluricellular , lateral	
Reproduction	Multilateral budding		Multilateral budding		Fission budding	
Growth in malt	Turbidity and sediment		Turbidity, sediment and		Turbidity and superior ring	
Mycelium						
pseudo	*-1-*		-/+		+/+	
Growth at	-		-		-	
Fermentation						
Lactose	—		—		—	
Glucose	+				w	
Sucrose	—		—		—	
Maltose	-		-		-	
Galactose	+		-		-	
Assimilation of						
compounds	Glu +	Gal	Glu +	Gal	Glu +	Gal +
	LSor -	Sue	LSor -	Sue	LSor +	Sue
	Mai	Cel	Mai	Cel	Mai -	Cel
	Tre	Lac	Tre	Lac	Tre	Lac -
	Mel	Raf	Mel	Raf	Mel -	Raf
	Mlz	DXyl	Mlz	DXyl	Mlz -	DXyl +
	LAra -	DRib	LAra -	DRib	LAra -	DRib -
	DMan -	Ino	DMan +	Ino	DMan +	Ino -
	St +	LRha	St	LRha	St	LRha -
	Ery -	Cit	Ery +	Cit	Ery -	Cit +
	Dul		Dul		Dul -	
Assimilation of	-		-		-	
Growth in						
medium	-		-		+	
Hydrolysis of	-		+		-	
Genus and	*Saccharomyces*		*Saccharomyces*		*Geotrichu*	*candidum*

*On YGC medium, 3 d at 30°C. + = positive reaction, -=negative reaction, w=weak reaction. LAra = L-arabinose, Cel = cellobiose, Cit = citric acid, Dul = dulcitol, Ery=erythritol, Gal = galactose, DGlu =D-glucose,Ino = inositol, Lac = lactose, Man = mannitol, Mel = melibiose, Mlz = melezitose, DMne = D-mannose, Raf=raffinose,Rha = rhamnose, Rib = ribose, St = oluble starch, LSor = L-sorbose, Suc = sucrose, Tre = trehalose, DTur = D-turanose.All assays were carried out at 30°C unless indicated.

Table 3 Microflora of kefir grain

	cfu/g±SD *n**	Percent of $10^{\sim 7}$ microorganism*
Lactococci	1.64±1.50	0.9
Lactobacilli	159.00± 13.20	78.3
Yeasts	42.30±4.05	20.8

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

Table 4 Microflora of kefir grains stored for 120 d under different conditions

Storage condition	10^{-7} • cfu/g+SD			Lc/Lc.s	Lb/Lb.s	Y/Y.s
	Lactococci	Lactobacilli	Yeasts			
Subculture	11.7+10.7	270.3+22.4	14.90+1.4	1.00	1.00	1.000
-80 °C -20	24.7+22.6	123.3+10.2	1.00+0.1	2.10	0.46	0.070
°C	n.d.	162.9+13.5	0.08+0.0	n.d.	0.60	0.005
4°C	18.1+16.5	558.4+46.3	0.05+0.0	1.50	2.06	0.003

cfu/g = colony forming units per gram of dry kefir grain. Lc = Lactococci, Lb=Lactobacilli, Y = Yeasts, s=subculture.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 -80 °C and -20 °C was 292 and 333 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 kinetics of growth of the yeasts shows that after 30 h the stationary phase is reached. The number of cfu/mL at that stage is 10^6-10^7. After 48 h, both lactococci and lactobacilli reached the stationary phase with a final concentration of 10^9-10^{10} cfu/mL. The kinetics of acidification of the milk inoculated with kefir grain and the increase of CO_2 in the gaseous phase of the kefir culture during the incubation are shown in the Fig. 3. pH values between 4.3 and 4.9 units are reached after 48 h of incubation and between 3.7 and 4.2 after 96 h. The CO_2 content increases during incubation. However, no difference in gas production is observed between 48 and 96 h. Final concentration of CO_2 in both cases was 15.84 mL/g of kefir grain inoculated into the milk.

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 96 h of incubation and the ratio between the number of each microorganism with respect to the corresponding control are shown in **Table 5.**

Table 5 Microflora of fermented milk obtained with kefir grains stored or 120 d under different conditions

Storage condition	10^{-9} • cfu/mL			Lc/Lc.s	Lb/Lb.s	Y/Y.s
	Lactococci	Lactobacilli	Yeasts			
Subculture	6.00	33.00	0.0030	1.00	1.00	1.00
-80 °C	30.00	30.00	0.0150	5.00	0.91	5.00
-20 °C	35.00	20.40	0.0020	5.80	0.62	0.67
4°C	0.31	0.31	0.0002	0.05	0.01	0.07

cfu/mL = colony forming units per millilitre of fermented milk. Lc = Lactococci, Lb=Lactobacilli, Y = Yeasts, s=subculture.

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 have a considerable influence on the final acidity features, gas content and viscosity of the product.

Table 6 shows the characteristics of the fermented milk obtained with kefir grains stored for 120 d under different conditions. Milk obtained with grains stored at 4 °C is noticeably different from those obtained with control grains. No decrease in pH was observed with the control grains. Production of CO_2 per g of grain decreased by 57.1%, and the viscosity was similar to that of nonfermented milk. Milk fermented with grains stored at -20 °C does not show significant differences from the control. The final pH and viscosities are comparable, although the CO_2 concentration decreased by 30.8%. Milk fermented with grains stored at -80 °C shows practically the same properties as fermented milk obtained with nonstored grains.

Table 6 Characteristics of fermented milk obtained with kefir grains stored for 120 d under different conditions

Storage condition	pH±SD	mL CO_2 g grain±SD	$^{n}a_{P}$ (mPa-s)
Subculture	4.62±0.29	15.27+1.74	9.0+2.1
-80 °C	4.43±0.18	13.94+0.61	10.6
-20 °C	4.48±0.45	10.56±0.88	10.8
4°C	6.18±0.49	6.56±0.03	1.6

Milk was incubated with 20 g/L of kefir grains for 48 h at 20 °C. A control milk, without grains, incubated in the same conditions showed a pH of 6.69+0.01, a concentration of CO_2 of 0.02±0.0006 mL CO_2/mL gaseous phase and a n_{ap} of 1.6. Each value is a mean of two to eight experiments.

CONCLUSIONS

In this work the microflora of kefir grains of household origin and the methods used to conserve them were analyzed. Many authors have isolated and identified the yeasts and LAB of kefir grain; some results are reviewed by Zourari and Anifantakis (2). The microflora of kefir grains is variable, depending on the source of the grain. Among the yeasts isolated include: *Candida kefir,* the imperfect form of *Kluyveromyces lactis* (2, 3, 17, 18); *Kluyveromyces lactis* (3, 18); *Saccharomyces cerevisiae* (3, 17, 19, 20); *Saccharomyces delbrueckii* (18, 19); *Torulopsis holmii* (21), *Candida holmii* and *Saccharomyces unisporus* (3, 18); *Tor-ulaspora delbrueckii* and *Candida friedricchii* (3); *Pichia fermentum* and *Kluyveromyces marxianus* (20). *Geo-trichum candidum* is said to be on the surface of many grains but it does not alter the quality of

the product (2, 7). 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. It is important to note that although differences exist between morphological features of the colonies, these are difficult to detect.

Fig. 1 Weight increases of kefir grains stored in different conditions. (■) = subculture; (•) = -80 °C; (A) = -20 °C; (*) = 4 °C. Kefir grains were subcultured by successive passage of the total amount of grains in (a) a constant volume of milk and (b) increasing volumes of milk to achieve a concentration of 20 g/L

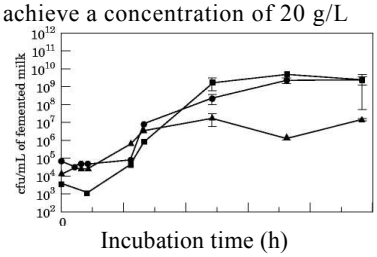

Fig. 2 Kinetics of growth of lactococci (■), lactobacilli (•) and yeasts (A) in milk fermented with kefir grains

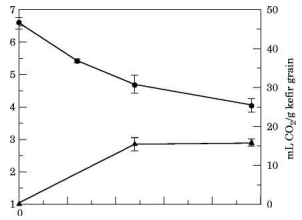

Fig. 3 Kinetics of acidification (•) and CO_2 production (A) in milk fermented with kefir grains

The following are among the lactic acid bacteria described in kefir grains of different sources: *Lactoba-cillus brevis* (3, 22-24), *Lactobacillus viridescens, Lacto-bacillus gasseri, Lactobacillus fermentum* and *Lactobacillus casei* (3), *Lactobacillus kefir* (3, 6), *Lactobacillus acidophilus* (3, 17, 23), *Lactococcus lactis* subsp. *lactis* (3, 17, 23), *Leuconostoc* (3, 17, 24), *Lactobacillus kefiranofaciens* (25, 26). Two novel species were recently described. They are *Lactobacillus kefirgranum* and *Lactobacillus parakefir* (27).

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. It is difficult to differentiate these genera by pattern of sugar fermentation.

The distribution of microorganisms in the grain and kefir culture was different. In the grains there were 10^7 cfu/g lactococci, 10^9 cfu/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 10^6-10^7 cfu/mL yeast. Our results agree with those previously published by Marshall (17). Marshall found that there are 10^9 cfu/g lactobacilli and 10^8 cfu/g yeast in the grain and 10^9 cfu/mL lactococci, 10^8 cfu/mL lactobacilli and 10^6 cfu/mL yeast in the fermented milk.

When milk is inoculated with kefir grains, some microorganisms are dispersed into the milk phase. As **Fig.** 2 shows, the numbers of lactobacilli and yeasts displaced into the milk are 100-fold and 10-fold higher respectively, than lactococci. This can be explained by the distribution of the microflora within the grain, as published previously. Lactobacilli and yeasts seem to be located on the surface of the grain (28, 29). Results dealing with this subject are controversial: Bottazzi and Bianchi (5) found that lactobacilli are at the periphery of the grain and yeasts within the grain. Marshall *et al.* (6) showed that some regions of grains are composed of lactobacilli and others of yeast.

Kefir quality depends on properties such as chemical composition, microflora, rheology and organoleptic features (2). Storage at 4 °C, wet or dried, has been proposed as an alternative method for preserving kefir grains (7, 8). However, no quantitative comparison between them is available. Reports about the parameters evaluated to decide the best method to preserve kefir grains are available. 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, rheological properties, acidity and carbon dioxide content.

The data in **Table 4** show that preservation at -80 °C alters the microbiological composition of kefir grains to a lesser extent than when done at -20 °C or 4 °C. In addition, milk fermented with grains frozen at -80 °C have the microflora, level of acidity, viscosity and carbon dioxide content similar to fermented milk obtained with control grains **(Tables 5** and **6).** Grains stored at 4 °C showed a great change in the microflora **(Table 4)** and gave an atypical product with unpleasant characteristics **(Tables 5** and **6).**

Subculturing allows good-quality products to be obtained. However, it is susceptible to contamination. A good product can be obtained with grains stored at -80 °C avoiding subculture.

Results obtained in this study show that freezing is better than other methods described earlier to preserve kefir grain. This storage condition maintains grain activity needed to ferment the milk. Grains stored at -20 °C

and -80 °C showed a greater increase in grain weight by successive subculturing (Fig. 1). The growth rate of kefir grain (wet weight increase/g of wet grain per d) means that the microflora present in the grain is active (30). 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 -80 °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. Taking into account that the products obtained with kefir grains stored at -20 °C and -80 °C are similar, storage in a freezer at -20 °C can be used to maintain the grains for household kefir production.

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