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Lactic acid bacteria and yeasts in kefir grains and kefir made from them

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In an investigation of the changes in the microflora along the pathway: kefir grains (A)→kefir made from kefir grains (B)→kefir made from kefir as inoculum (C), the following species of lactic acid bacteria (83–90%) of the microbial count in the grains) were identified: *Lactococcus lactis* subsp. *lactis*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus casei* subsp. *pseudoplantarum* and *Lactobacillus brevis*. Yeasts (10–17%) identified were *Kluyveromyces marxianus* var. *lactis*, *Saccharomyces cerevisiae*, *Candida inconspicua* and *Candida maris*. In the microbial population of kefir grains and kefir made from them the homofermentative lactic streptococci (52–65% and 79–86%, respectively) predominated. Within the group of lactobacilli, the homofermentative thermophilic species *L. delbrueckii* subsp. *bulgaricus* and *L. helveticus* (70–87% of the isolated bacilli) predominated. Along the pathway A→B→C, the streptococcal proportion in the total kefir microflora increased by 26–30% whereas the lactobacilli decreased by 13–23%. *K. marxianus* var. *lactis* was permanently present in kefir grains and kefirs, whereas the dominant lactose-negative yeast in the total yeast flora of the kefir grains dramatically decreased in kefir C.
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Introduction

The wholesome properties of fermented milks (kefir, kumis) and their nutritional value have motivated a considerable interest in such "novel" milk foods [10,20,22–24]. Kefir has been defined as the yogurt of the 21st century [9]. Kefir differs from other fermented milks in its starter, which exists in the form of "grains." The grains have a specified structure and behave as biologically vital organisms. They grow, propagate and pass their properties on to the following generations of new grains. The microflora of kefir grains is remarkably stable, retaining its activity for years if preserved and incubated under appropriate cultural and physiological conditions. Kefir grains have a complex microbiological composition. Lactobacilli, lactic streptococci, yeasts and acetic acid bacteria have been shown to be present in them [23,25,27,28,32,37,38]. The kefir-specific yeasts play a key role in the formation of flavour and aroma [8]. They are represented by the species *Kluyveromyces marxianus*, *Torulaspora delbrueckii*, *Saccharomyces cerevisiae*, *Candida kefir*, *Saccharomyces unisporus*, *Pichia fermentans* and *Yarrowia lypolytica* [1,18,28]. For the formation of a kefir starter culture, which would produce a milk beverage with characteristics similar to those of traditional kefir, more information needs to be acquired about the specific cultures isolated from kefir grains.

The aim of this study was to describe changes in the microbial population in kefir grains while they underwent two subsequent fermentation cycles: kefir grains→kefir→second batch of kefir.

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Materials and methods

Kefir grains and conditions of cultivation

The kefir grains studied were from the collection of the Center of Research, Development and Production (LB Bulgaricum Trading, Bulgaria). The lyophilized kefir grains were activated for 1 month in the following way: Sterilized skim milk (0.1% milk fat, 1-l flasks, 115°C, 15 min) was cooled to 18–20°C, and inoculated using kefir grains (the weight ratio of kefir grains to milk was 1:10) and incubated at 18–20°C for 24 h with three to five stirrings. Kefir grains were separated from the fermented milk by filtration through a sieve and were washed with cooled sterile water before being used in the next culture passage. Activated kefir grains were propagated as follows: From the grown and fresh kefir grains six grains were selected. Each was cut and cultivated separately in sterile skim milk under the conditions given. After daily transfers of the particles of the six grains into sterile milk for a period of 2 weeks, the new kefir grains were cut again. This procedure of transferring and cutting lasted (about 2 months) until a sufficient amount of new kefir grains was obtained from the initial six. The six populations of kefir grains thus obtained were used in the present study. Five grains were taken out of each population; each was cut and the pieces were thoroughly washed with sterile water and suspended in a sterile solution of 0.9% NaCl. By plating the diluted homogenates of every one of the grains on appropriate cultural media, strains of lactic acid bacteria and yeasts were isolated [12–15].

Isolation and identification of lactic acid bacteria from kefir grains

For isolation of the lactobacilli from kefir grains, 0.1-ml portions of the respective dilutions of homogenates were plated on MRS agar

(Fluka RdH, Buchs, Switzerland) and on Rogosa agar with cheese whey (Fluka RdH) [17]. The plates were incubated at 30°C and 37°C for 3–7 days in aerobic and anaerobic (10% CO_2) atmospheres. For isolation of lactic streptococci the diluted homogenates were plated on M-17 medium (Fluka RdH), and azide agar medium (Difco, Detroit, USA) was used aerobically. The plates were incubated at 28°C and 37°C for 4–8 days.

After microscopic examination and on the basis of their morphology, the colonies were divided into groups and transferred in triplicate in sterile skim milk and on plates. Some of the lactobacilli, isolated directly from the kefir grains, appeared to have very limited fermentative capability until further passage through laboratory media or sterile skim milk. By microscopic analysis of each culture following incubation, confirming the purity of the isolates and their activation, the cultures were characterized taxonomically according to the criteria described in Bergey's Manual [16,31]. Identification was carried out also on the basis of biochemical tests with API 20A (for identification of anaerobic bacteria) and with API 50CH (test for lactobacilli and lactococci — API 50CHL Medium, bioMerieux, Marcy l'Etoile, France) using the software of the company API LAB Plus.

Isolation and identification of yeasts from kefir grains

Yeasts were isolated by surface spreading on plates of malt extract agar (Fluka RdH) and YM agar (Fluka RdH) [19]. After incubation at 28°C for 3–6 days, colonies of different morphology were obtained. Initial cultures were prepared from these colonies. Inclined agar tubes were inoculated and after incubation (28°C for 3 days) an initial suspension was prepared from each culture. A solution of yeast extract–peptone–glucose (0.45%, 0.75%, 1.0%) was used as medium, whose pH value was corrected to 6.4 by adding tartaric acid. After sterilization, the glucose was added to the medium as a sterile filtered solution. The medium was inoculated with one loop of the inclined agar culture and the sample was incubated to the appearance of distinct darkening. For identification of the yeasts the methods of Kreger van Rij [19] and Barnett *et al* [2] were applied. The yeasts were also identified on the basis of fermentation tests ID 32C (bioMerieux) using API LAB Plus software.

Preparation of kefir, isolation and identification of lactic acid bacteria and yeasts

Using kefir grains A from the six populations obtained, six samples of traditional kefir (kefir B) were prepared as follows: Milk (3% milk fat), pasteurised at 92°C for 15 min, homogenized at 12.5–17.5 MPa and cooled to 18–20°C, was inoculated with 3% kefir grains. Incubation was at 18–20°C for 18 h (pH=4.5–4.6) followed by coagulum cooling to 8–10°C and kefir ripening for 24 h. After separation of the kefir grains from the six kefir B samples, strains of lactic acid bacteria and yeasts were isolated and identified on the basis of taxonomic characteristics and biochemical tests as already described.

The six kefir B samples were used as starter cultures for preparing samples of kefir C. Kefir C was made in the following way: Milk (3% milk fat) was pasteurised at 92°C for 15 min, homogenized at 12.5–17.5 MPa and cooled to 22–23°C. Each of the six milk samples was inoculated with 3% of the sample of kefir B. Fermentation was carried out at 23°C for 8–12 h to pH=4.5–4.6, followed by cooling to 8–10°C with simultaneous ripening for 10–12 h. The microflora of kefir C samples was then isolated and identified as already described.

Analytical methods

In order to calculate the percentage of each characterized microorganism in kefir grains A, kefir B and kefir C, the number of viable bacteria and yeasts expressed as colony-forming units per ml (CFU ml^{-1}) was estimated. Serial dilutions of each sample were plated in triplicate and plates were incubated at 30°C and 37°C (for lactic acid bacteria), and 28°C (for yeasts) until growth of the colonies. The results were the mean of six counts at three different dilutions.

Table 1 Composition of the microflora of kefir grains from six populations

Microorganisms	Colony count in population no.					
	1	2	3	4	5	6
Lactic acid bacteria	22±2.0 (83)[a]	19±2.6 (88)	15±44 (90)	24±3.6 (84)	16±4.4 (87)	21±2.6 (83)
Streptococci	14±2.6 (53)	13±2.0 (61)	11±1.7 (65)	16±2.0 (55)	10±1.7 (54)	13±3.0 (54)
L. lactis subsp. *lactis*	10	12	9	14	8	10
S. thermophilus	4	1	2	2	2	3
Lactobacilli	8±2.0 (30)	6±2.6 (27)	4±1.0 (24)	8±3.0 (29)	6±3.0 (33)	8±2.6 (29)
L. delbrueckii subsp. *bulgaricus*	5	2	2	4	3	4
L. helveticus	2	2	–	2	2	2
L. casei subsp. *pseudoplantarum*	–	1	1	1	–	–
L. brevis	1	1	1	–	1	2
Yeasts	5±1.7 (17)	3±2.0 (12)	2±1.0 (10)	5±1.7 (16)	2±1.0 (13)	5±2.0 (17)
K. marxianus var. *lactis*	1	1	–	1	–	1
S. cerevisiae	2	–	–	2	–	1
C. inconspicua	2	1	1	1	2	2
C. maris	–	1	–	–	–	1
Unidentified	–	–	1	1	–	–

Data represent mean values for five grains from each population and standard deviation.
[a]Data in parentheses represent percentage of total microflora.

Table 2 Composition of the microflora of kefir samples (kefir B) prepared with kefir grains from six populations

Microorganisms	Colony count in population no.					
	1	2	3	4	5	6
Lactic acid bacteria	24±3.6 (94)[a]	24±5.2 (94)	20±6.2 (92)	24±3.6 (92)	20±2.6 (96)	20±2.6 (95)
Streptococci	19±2.6 (74)	20±2.6 (78)	16±2.0 (76)	20±2.6 (77)	16±4.3 (78)	16±4.3 (76)
L. lactis subsp. *lactis*	15	18	13	17	12	13
S. thermophilus	4	2	3	3	4	3
Lactobacilli	5±1.7 (20)	4±1.0 (16)	4±2.0 (16)	4±1.7 (15)	4±1.0 (18)	4±1.7 (19)
L. delbrueckii subsp. *bulgaricus*	2	2	2	3	2	2
L. helveticus	2	–	–	1	1	2
L. casei subsp. *pseudoplantarum*	–	1	4	–	–	–
L. brevis	1	1	1	–	1	–
Yeasts	1±0 (6)	2±1.0 (6)	2±1.7 (8)	2±1.0 (8)	1±0 (4)	1±0 (5)
K. marxianus var. *lactis*	1	1	–	1	–	1
S. cerevisiae	–	–	–	1	–	–
C. inconspicua	–	–	1	–	1	–
C. maris	–	1	–	–	–	–
Unidentified	–	–	1	–	–	–

Data represent the mean values from three kefir samples from each population and standard deviation.
[a]Data in parentheses represent percentage of total microflora.

Viable streptococci were estimated on plates of M-17 agar, viable lactobacilli count on plates of MRS agar and the total yeast count was determined using YM agar.

Lactic acid and alcohol were determined by enzymatic methods as described by Boehringer Mannheim [4]. CO_2 content (as dissolved gas) was analysed by the enzymatic method for dissolved CO_2 [3,6]. Viscosity (cst) was measured on an Oswald (Sibata, Japan) cinematic viscosimeter.

Results

The distribution of the species of lactic acid bacteria and yeasts in the kefir grains A and in the samples of kefir B and kefir C is given in Tables 1–3. Two species of homofermentative lactic streptococci were identified: the mesophilic species *Lactococcus lactis* subsp. *lactis* (*Streptococcus lactis*) and the thermophilic species *Streptococcus thermophilus*. These two species were the predominating microflora in all kefir grains (53–65% of the total microflora) and samples of kefir (74–78%; 79–86%). *S. lactis* prevailed over *S. thermophilus* (up to 12-fold in kefir grains and up to 14-fold in kefir samples). During fermentation the proportion of streptococci increased and reached 79–86% of the total microflora in kefir C. The following homofermentative lactobacilli were isolated: *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus* and *Lactobacillus casei* subsp. *pseudoplantarum*. The heterofermentative lactobacillus *Lactobacillus brevis* was also isolated. Lactobacilli constituted about 24–33% of the total flora in the kefir grains. A twofold decrease in the quantity of lactobacilli in the pathway "A→B→C" was noted. Of the isolated lactobacilli in all the

Table 3 Composition of the microflora of kefir samples (kefir C) prepared with B as inoculum

Microorganisms	Colony count in population no.					
	1	2	3	4	5	6
Lactic acid bacteria	17±4.0 (94)[a]	17±2.6 (96)	18±4.4 (96)	19±2.6 (95)	22±2.6 (96)	16±2.0 (95)
Streptococci	14±2.6 (79)	15±4.4 (84)	16±2.0 (85)	17±2.6 (86)	20±2.6 (86)	13±2.6 (79)
L. lactis subsp. *lactis*	11	14	14	13	15	10
S. thermophilus	3	1	2	4	5	3
Lactobacilli	3±1.0 (15)	2±1.7 (12)	2±1.0 (11)	2±1.7 (9)	2±1.0 (10)	3±1.7 (16)
L. delbrueckii subsp. *bulgaricus*	1	1	1	1	1	1
L. helveticus	1	–	–	1	1	1
L. casei subsp. *pseudoplantarum*	–	1	1	–	–	–
L. brevis	1	–	–	–	–	1±0
Yeasts	1±0 (6)	1±0 (4)	1±0(4)	1±0 (5)	1±0 (4)	1±0 (5)
K. marxianus var. *lactis*	1	1	–	1	–	1
S. cerevisiae	–	–	–	–	1	–
C. inconspicua	–	–	1	–	–	–
C. maris	–	–	–	–	–	–
Unidentified	–	–	–	–	–	–

Data represent the mean values from three kefir samples (kefir C) prepared with kefir B as inoculum and standard deviation.
[a]Data in parentheses represent percentage of total microflora.

Table 4 Characteristics of kefir B and kefir C after 24 h storage at 4°C

Component	Kefir B[a]	Kefir C[b]
pH	4.5±0.26	4.35±0,20
Lactic acid ($g\ l^{-1}$)	8.18±0.61	8.20±0,57
Alcohol (%)	0.25±0.04	0.09±0,04
Carbon dioxide ($g\ l^{-1}$)	0.85±0.15	0.15±0,06
Mesophilic streptococci ($CFU\ ml^{-1}$)	$8(\pm4.58).10^9$	$6(\pm2,64).10^9$
Thermophilic streptococci ($CFU\ ml^{-1}$)	$3(\pm2,64).10^6$	$4(\pm2,00).10^6$
Homofermentative lactobacilli ($CFU\ ml^{-1}$)	$8(\pm2,64).10^6$	$2(\pm1,00).10^6$
Yeasts ($CFU\ ml^{-1}$)	$2(\pm2,64).10^5$	$1(\pm2,00).10^4$
Viscosity (cst)	1.075±0,21	1.043±0,28
Aroma	strong, typically yeasty	very light yeasty
Flavour	kefir-specific, refreshingly pungent	predominating lactic acid
Texture	homogeneous, creamy, abundantly effervescent	slightly viscous, very slight effervescence

[a]Kefir B was prepared with kefir grains No. 4 and received the best organoleptic evaluation of kefirs B, prepared with the six populations.
[b]Kefir C was prepared with kefir B[a] as inoculum.
Data represent the mean values from three kefir B and kefir C, and their standard deviations.

samples *L. bulgaricus* prevailed, followed by *L. helveticus*. During the two consecutive fermentations *L. helveticus* decreased. The heterofermentative species *L. brevis*, present in five out of the six populations of kefir grains, dramatically decreased, and in kefir C it was found in two out of six samples. *L. casei* subsp. *pseudoplantarum* was found in three out of six samples of kefir grains in kefir B and kefir C.

The lactose-fermenting yeast *K. marxianus* var. *lactis* was found in kefir grains A, kefir B and kefir C together with non-lactose-fermenting yeasts (*S. cerevisiae*, *Candida inconspicua*, *Candida maris* and two unidentified yeasts). The yeast microflora of kefir grains was marked by the prevailing presence of lactose-negative yeasts (60–100% of the total quantity of yeasts). Among then the species *C. inconspicua* predominated, followed by *S. cerevisiae*.

In the preliminary organoleptic evaluation of kefirs B prepared with kefir grains from the six populations, kefir B prepared with kefir grains from population No. 4 ranked first with the best characteristics. The data comparing the physicochemical and microbiological composition and organoleptic properties of kefir B with population No. 4 and those of kefir C, prepared with kefir B, are shown in Table 4.

Discussion

Lactic acid bacteria represented 83–90% of the microbial count of the grains. In the microbial populations of the kefir grains, the homofermentative species of lactic acid streptococcus *S. lactis* prevailed in kefir B and kefir C (40–55% and 60–80%, respectively), which determined it as the main fermenting organism. Our results differ from those studies that found a very scarce presence of streptococci (a single diplococcal cell) in the grain-associated population [5,26]. However, a number of authors consider *S. lactis* as an integral part of the microflora of kefir grains irrespective of its low numbers [11,30].

Within the lactobacilli, the thermophilic homofermentative species *L. bulgaricus* and *L. helveticus* (70–87% of the isolated lactobacilli) predominated. There are reports on the presence of *L. helveticus* as a prevailing member of the lactic acid microflora in kefir grains [35]. The mesophilic homofermentative species *L. casei* subsp. *pseudoplantarum* was found in the populations from three kefir grains and in two samples of kefir B and kefir C. The presence of this species in kefir grains has been noted in other studies [7]. According to some authors, the heterofermentative lactobacilli predominate over the homofermentative ones [1], and according to others, they are the only representatives of the lactobacilli [38]. The isolated species *L. brevis* decreased along the pathway A→B→C. These results are very similar to those of Takizawa *et al* [33], who found that 90% of the bacterial species in kefir grains were homofermentative lactobacilli. In other studies, however, the lactobacilli represented 65–80% of the microbial count of the grain, with streptococci and various yeasts as the remainder [1,23].

Yeasts in kefir grains represented 10–17% of the total isolated microflora with lactose-negative yeast species predominating (66–100%). The inability of these yeasts to ferment lactose makes them dependent on lactic acid bacteria capable of hydrolyzing this disaccharide. The prevalence of lactose-negative yeasts in the grains was consistent with the studies carried out by other authors [29,36]. It is worth noting that the group of lactose-negative yeasts contains the two species *C. inconspicua* and *C. maris*, whose presence in kefir has not been reported previously. *C. inconspicua* predominated in the yeast microflora of the kefir grains and drastically decreased along the pathway A→B→C. *C. maris* was present in the microflora of two kefir grains. These two species did not ferment or assimilate galactose. *C. inconspicua* assimilated DL-lactic acid and was, therefore, completely dependent on the metabolism of lactic acid bacteria. *C. maris* did not assimilate DL-lactic acid. Possibly, its presence in kefir grains was connected not only with the presence of glucose and certain vitamins (thiamine, pyridoxine), but also with the assimilation of some acids produced by lactic acid bacteria by the transformation of pyruvic acid. The lactose-positive species *K. marxianus* var. *lactis* was identified in the isolates from four kefir grains. Its presence in kefir grains has been reported in other studies [1,35,36]. According to some authors, *K. marxianus* var. *lactis* isolated from kefir grains is distinguished by the peculiarity of always occurring with lactose-negative yeasts, which is also confirmed by our results [1].

The composition of the yeast flora in kefir B and kefir C was highly dependent on the starter used. During fermentation and

ripening, alterations occurred only in the group of lactose-negative yeasts. In kefir B samples, the lactose-negative species (five out of nine isolated yeast species) predominated slightly. In all kefir C samples, where *K. marxianus* var. *lactis* was present, other yeast species were not identified. In two of the samples where *K. marxianus* var. *lactis* was not found, the species *C. inconspicua* and *S. cerevisiae* were identified. The unidentified yeast present in kefir B disappeared in kefir C. The quantity of the lactose-fermenting yeast *K. marxianus* var. *lactis* remained the same in the kefir grains, traditional kefir B and kefir C. The total yeast quantity decreased because of the decrease of lactose-negative yeast in the course of the process: kefir grains A→kefir B→kefir C. A number of authors maintain that lactose-fermentative yeast should be defined as one of the main components of the "specific cultures," whereas the lactose-negative yeasts should be designated as recontamination yeast [8,21,34]. Participation of *K. marxianus* var. *lactis* in the microflora of the kefir grains ensures metabolism of lactose through alcohol fermentation and the formation of the typical yeasty flavour and aroma. According to some authors, kefir must contain an approximate minimum of 1×10^5 lactose-fermenting yeasts to obtain the characteristic flavour and aroma [8]. That statement was supported by four samples of kefir C (out of six) and two samples of kefir B (out of six), in which only *K. marxianus* var. *lactis* was present (Tables 2 and 3). On the other hand, special emphasis should be laid upon the role of lactose-negative yeast in the formation of the yeasty flavour of kefir. Although *K. marxianus* var. *lactis* was similar in kefir B and kefir C, the stronger and typical yeasty aroma and the refreshing, pungent taste of traditional kefir B may have been due to the lactose-negative yeast that was present. The remaining samples of kefir B (with populations Nos. 2, 3, 5) and kefir C, which contained lactose-negative yeasts, were noticed to possess the typical yeasty flavour and aroma that were absent in the kefirs with *K. marxianus* var. *lactis* only. Kefir B had the flavour and aroma of traditional kefir, comparatively denser texture and significantly stronger carbonated taste than kefir C (Table 4). There were significant differences in alcohol and CO_2 concentrations (three and six times higher in kefir B), which have an important role in the formation of kefir flavour and aroma.

It can be concluded that the composition of lactic acid microflora in the samples of kefir grains and kefir was quite homogeneous. Along the pathway A→B→C, streptococci increased their proportion in the total kefir microflora by 26–30%, whereas lactobacilli decreased by 13–23%. The composition of the yeast flora undergoes a complete change at the expense of the lactose-negative yeasts. *K. marxianis* var. *lactis* was permanently present in kefir grains and kefirs, whereas the dominant lactose-negative yeasts in the total yeast flora of kefir grains decreased drastically in kefir C. Lactic acid bacteria and yeasts isolated from kefir grains can be used to form kefir starters.

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