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Investigation of the Yeast Flora in Dairy Products: A Case Study of Kefyr

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Summary

The traditional starter for manufacture of kefyr is kefyr grain which consists of a gelatinous matrix in which lactic acid bacteria, yeasts and mostly also acetic acid bacteria are embedded. The typical microflora of kefyr is, however, not exactly defined. The aim of this project was to isolate and characterise yeasts from kefyr grains and kefyr in order to learn about the diversity of the yeast flora. The isolated yeasts were characterised and grouped by electrophoretic karyotyping in the contour-clamped homogeneous electric field and by traditional methods.

In the five investigated kefyr grains and kefyr obtained with them, the following yeast species could be identified: Candida kefyr, Kluyveromyces marxianus, Candida colliculosa, Torulaspora delbrueckii, Saccharomyces unisporus, Brettanomyces anomalus and a still unknown yeast U. The composition of the yeast flora was strongly dependent on the production procedure. In grains with at least two different species yeast U always dominated; only when absent, Sacch. unisporus was predominant. But during fermentation, the lactose fermenting yeasts K. marxianus and C. kefyr could catch up with yeast U. When kefyr was taken as inoculum, the composition of yeast flora changed completely during fermentation. Where K. marxianus or C. kefyr were present, they outweighed all other species; if absent, however, Sacch. unisporus dominated, yeast U disappeared and the total yeast count decreased.

It can be concluded that the composition of yeast flora in traditional kefyr is quite homogeneous and depends strongly on the inoculum, i.e. whether kefyr grain or kefyr is used. Three out of five isolated yeast species were non lactose fermenting. Karyotypes for yeasts other than K. marxianus or C. kefyr were species specific.

Keywords: kefyr, yeast flora composition, electrophoretic karyotypes, identification

Introduction

Kefyr is an acidic and mildly alcoholic fermented milk originating from the Caucasian mountains. It is prepared with gelatinous granules, the kefyr grains, which consist mainly of the polysaccharide kefiran and of a protein matrix *(1,2)*. The grains represent a natural symbiosis of different microorganisms which include: yeasts, lactic acid bacteria and mostly also acetic acid bacteria *(3,4)*. The typical microflora of kefyr is, however, not exactly defined.

Some authors claim that only lactose-fermenting yeasts should be considered as specific for the kefyr flora because of their leading role in the alcoholic fermentation *(3,5)*. The endproducts of alcoholic fermentation, ethanol and carbon dioxide, are very important for the flavour and refreshing taste of kefyr. Nevertheless, a high percentage of the yeasts found in kefyr are lactose negative (3). Investigations have shown that in the grain, the lactose negative yeasts such as *Candida holmii, Saccharomyces cerevisiae, Saccharomyces exiguus, Saccharomyces unisporus* and *Candida colliculosa* are predominant, whereas in the fermented milk, the lactose positive *Candida kefyr* and *Kluyveromyces marxianus (4, 6–10)* are present.

The aim of this project was to isolate and characterise yeasts from a number of kefyr grains and kefyr in order to learn about the diversity of the yeast flora.

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Material and Methods

Kefyr grains

For this study, five kefyr grains of different origin were used (HB, Laboratory of Dairy Science, Switzerland; KP, Biolacta-Texel, Poland; WT, Toni AG, Switzerland; KK, a private source, Croatia; HM, a private source, Germany)

Yeast count and culturing methods

To 1 g of grain or 2 g of fermented milk, a nine-fold quantity of a peptone saline solution *(11)* was added. The suspension was then mixed for 1 min by a Polytron homogeniser (Kinematica GmbH, Switzerland) and a Stomacher blender (Müller + Krempel AG, Switzerland), respectively. After preparing a decimal dilution series, the total yeast count was determined at 25 °C (3 d) using PYA medium (Phytone Yeast Extract Agar, BBL, Becton Dickinson, Cockeysville, USA). The same medium was also used for propagation and maintenance of the isolated yeast colonies.

Experimental procedure

Table 1 shows the fermentation procedure applied in this work and the stages at which the total yeast count was determined and yeasts isolated for identification purposes. To calculate the proportion of a certain yeast species, the count and share of each colony type was determined separately and also considered for the isolation of the 10 colonies.

All the steps in the experimental procedure were carried out in a sterile 2 L beaker which was covered with tin foil to avoid contamination. In order to maintain the activity of the grains, they were incubated at least every two weeks in 1 L of UHT skimmed milk as described in Table 1. They were stored in the kefyr at 5 °C and then, to prepare them for the next fermentation, washed with running tap water in a sterile sieve. Since all of the grain mass from 1 L of the fermented product was used, the quantity for incubation varied among the grains (HB: 3.7 g, HM: 3.0 g, KP: 4.2 g, KK: 3.4 g, WT: 1.7 g). The pH of the kefyr, however, reached a value in the narrow range of 4.3–4.5. The mixing of the grain with the milk was done with the help of a magnetic stirrer.

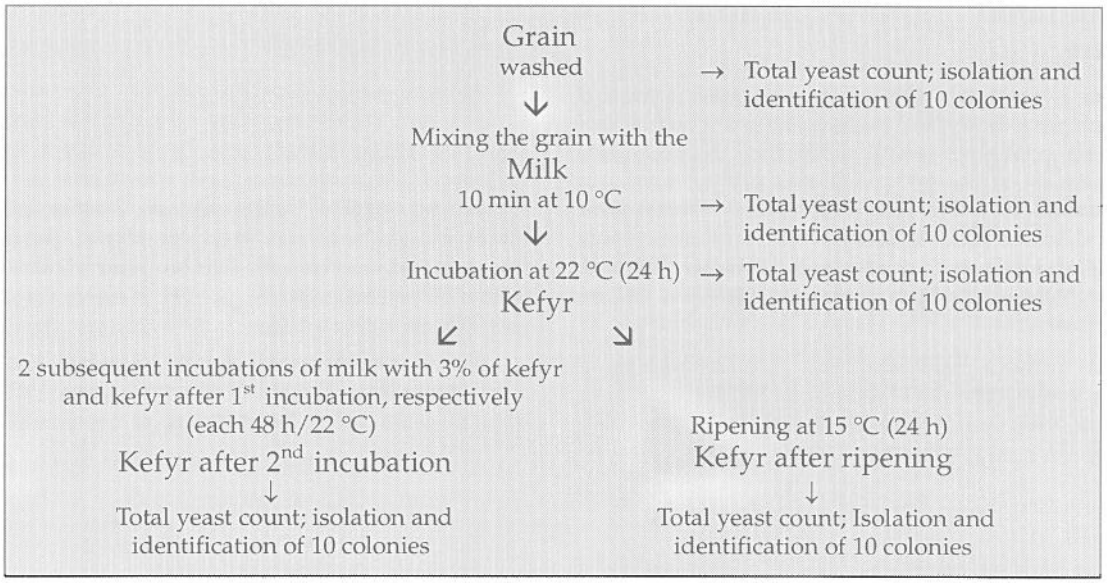
Yeast identification methods

At each production step, 10 yeast colonies were isolated and purified on PYA medium. The isolates were characterised and grouped by electrophoretic karyotyping in the contour-clamped homogeneous electric field (CHEF) according to Schütz and Gafner *(12)* and by a specific polymerase chain reaction (PCR) method which had been described previously *(13)*. Furthermore, representatives of the resulting groups were identified by the CBS Yeast Division in Delft, Holland using traditional methods *(14)*. A comparison with corresponding CBS and DSM (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) strains by the two molecular-biological methods mentioned above followed. The reference strains from CBS and DSM were: *Kluyveromyces marxianus* (E.C. Hansen) van der Walt var. *marxianus* CBS 834 and CBS 2762, *Kluyveromyces marxianus* (E.C. Hansen) van der Walt DSM 70073, DSM 70801 and DSM 70804, *Torulaspora delbrueckii* (Lindner) Lindner CBS 705 and CBS 1146, *Saccharomyces unisporus* Jörgensen CBS 2420, *Saccharomyces pastorianus* Reess ex E.C. Hansen CBS 1513, *Saccharomyces bayanus* Saccardo CBS 425 and *Dekkera anomala* M.T. Smith & van Grinsven CBS 7654.

Results

In Table 2, the yeast species found in five different kefyr grains and kefyr obtained with them are grouped according to their origin. Only the identity of one species, *yeast U*, could not be verified by the methods ap-

Table 1. Fermentation procedure of kefyr and sampling plan



Tab. 2. Yeast species isolated from five kefyr grains of different origin and kefyr obtained with them

Grain HB	Grain HM	Grain KP	Grain KK	Grain WT
C. kefyr (Beijerinck) Meyer & Yarrow [anamorph state of *K. marxianus*]	***K. marxianus*** (Hansen) van der Walt	***Sacch. unisporus*** Jörgensen	***Sacch. unisporus*** Jörgensen	***C. kefyr*** (Beijerinck) Meyer & Yarrow [anamorph state of *K. marxianus*]
Yeast U	***Yeast U***	Yeast U	***T. delbrueckii*** (Lindner) Lindner	
C. colliculosa (Harmann) Meyer & Yarrow [anamorph state of *T. delbrueckii*]		***B. anomalus*** Custers [anamorph state of *D. anomala*]		

B=Brettanomyces, C=Candida, D=Dekkera, K=Kluyveromyces, S=Saccharomyces, T=Torulaspora

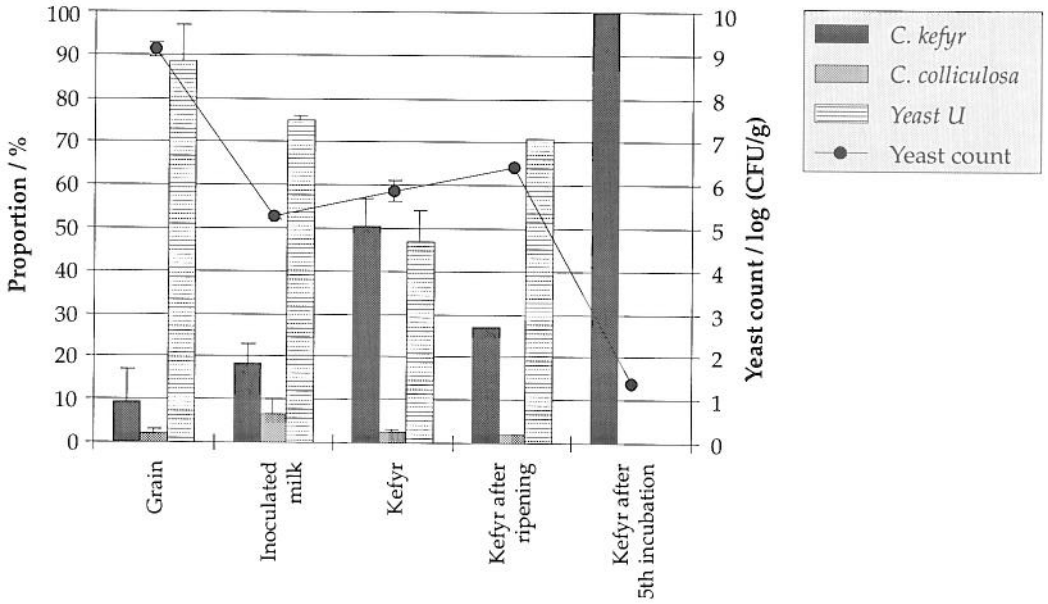


Fig. 1. Kefyr grain HB: Composition of yeast flora and total yeast count

plied in this work *(13)*. Identification by the CBS led to the conclusion that *yeast U* might be a representative of either the species *Sacch. bayanus* Saccardo or *Sacch. pastorianus* Reess ex Hansen.

The investigation according to the diagram in Table 1 was performed twice for the grains HB, HM, KP and once for the grains KK and WT. For grain HB five subsequent incubations were carried out as a preliminary experiment. It became obvious that two subsequent incubations were sufficient to get an understanding of the changes in the yeast flora composition. The results for each grain are discussed below.

Kefyr grain HB

As shown in Fig. 1, the three detected yeast species (*C. kefyr, C. colliculosa* and *yeast U*) were always present in the grain, milk, kefyr and the kefyr after ripening, yet not always in the same quantitative distribution. The predominant yeast in the grain was *yeast U* with a share of nearly 90%. After adding the grain and stirring, the milk reflected approximately the same yeast flora diversity as in the grain, except for the yeast count, which was much lower, and the shares of *C. kefyr* and *C. colliculosa*, which increased slightly. Therefore, it could be concluded that *C. kefyr* and *C. colliculosa* are located mostly in the outer layers of the grain, whereas *yeast U* in the inner part. Similar findings had been reported already by other work groups *(15,16)*. In the kefyr, *C. kefyr* could catch up with *yeast U*, most likely taking advantage of its ability to ferment lactose. Its contribution to the typical kefyr flavour (ethanol and carbon dioxide) must be considered as essential. On the other hand, *yeast U* and *C. colliculosa* do not ferment lactose, but both glucose and galactose. A contribution to the typical kefyr flavour compounds must, thus, also be expected. The potential of *yeast U* to assimilate the two possibly available carbon sources mentioned above might be responsible for an increase of this yeast and the total yeast count in the ripened kefyr. After the 5^{th} incubation, a very low total yeast count was found in kefyr, consisting solely of the lactose positive *C. kefyr*.

The total yeast count did not change considerably after fermentation or ripening of the kefyr; it did not even reach 10^7 from an initial count in the milk of 10^5.

Kefyr grain HM

The two species *K. marxianus* and *yeast U* could be detected in all the steps of the fermentation procedure (Fig. 2). Again, *yeast U* dominated in the grain as well

as in the milk after addition of the grain. However, the proportions changed remarkably; the share of *K. marxianus* increased from 10% in the grain to nearly 40% in the milk. This finding refers to the presumption mentioned above that *K. marxianus* is located mainly in the external layers, whereas *yeast U* in the interior. After incubation of the milk with the grain, *K. marxianus*, the lactose fermenting yeast, could catch up with *yeast U*. Ripening of the kefyr did not change the proportions of the two yeast species. Only in the kefyr after the 2nd incubation, a remarkable change in favour of *K. marxianus* occurred. Again, *yeast U* nearly disappeared, as in kefyr HB, probably because of its incapacity to utilise lactose. The development of the total yeast count is comparable to that of grain HB.

Kefyr grain KP

Three different yeasts could be detected in all fermentation stages, *yeast U*, *Sacch. unisporus* and *B. anomalus* (Fig. 3). *Yeast U* made up nearly 90% of the total yeast count and could hold its dominance over the two other species, not only in the grain, but also in the milk, kefyr and the kefyr after ripening. This composition could be attributed to an initial high share and to the absence of the competing lactose fermenting yeasts *K. marxianus* and *C. kefyr*. Only in the kefyr after the 2nd incubation, the share of *yeast U* markedly decreased. *Sacch. unisporus* was the dominant species, probably because it ferments glucose and galactose faster than *yeast U*. The lactose fermenting yeast *B. anomalus* remained at the level of approximately 10% and did not seem to take

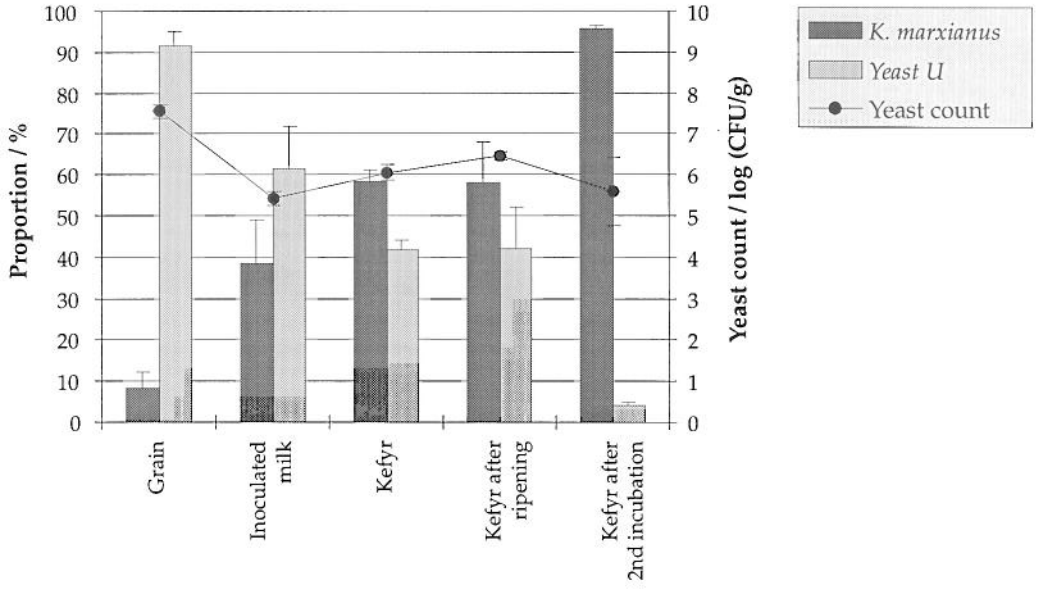


Fig. 2. Kefyr grain HM: Composition of yeast flora and total yeast count

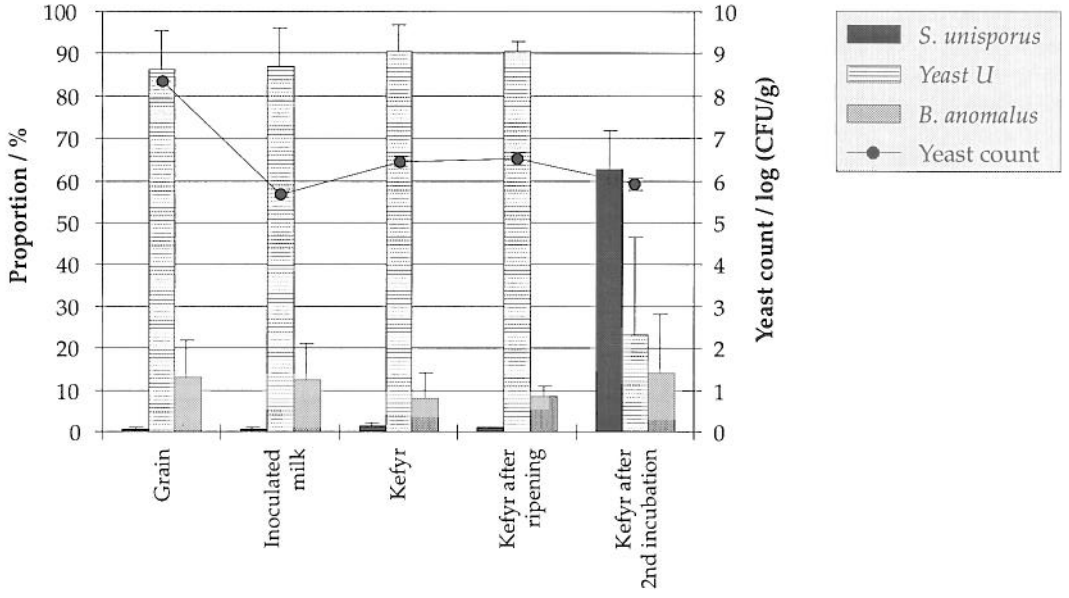


Fig. 3. Kefyr grain KP: Composition of yeast flora and total yeast count

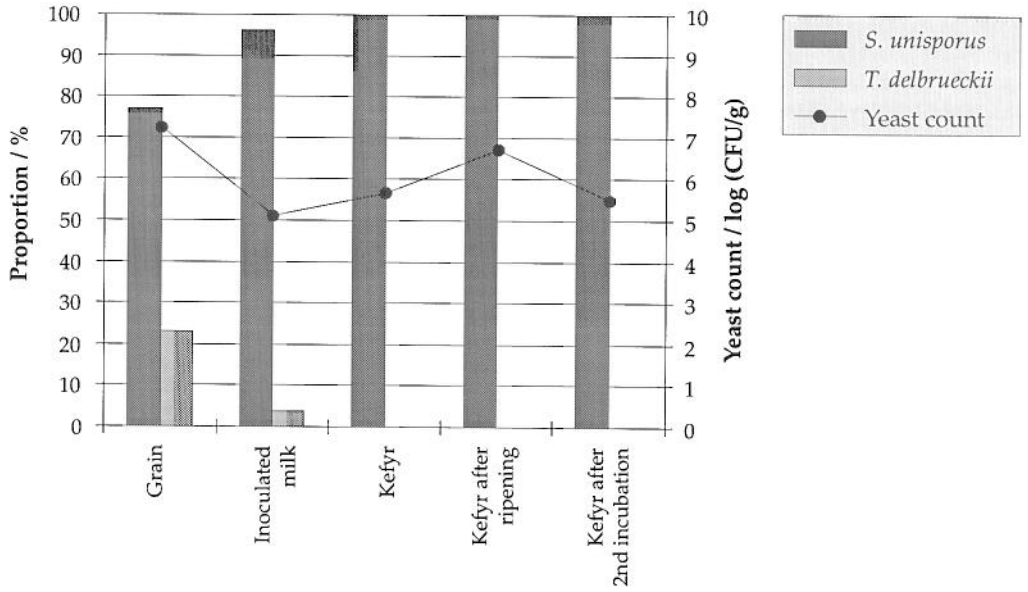


Fig. 4. Kefyr grain KK: Composition of yeast flora and total yeast count

advantage of its ability to utilise lactose for increasing its share. It is, however, known for its slow growth in all media *(17)*. Because of their ability to ferment carbon sources, the contribution of all three species to the typical kefyr flavour is surely of importance.

The development of the total yeast count is comparable to that of grain HB and HM.

Kefyr grain KK

The two yeast species *Sacch. unisporus* and *T. delbrueckii* were dominant in grain KK. After fermentation of the milk, however, the latter even dropped under the detection limit (Fig. 4). Therefore, *Sacch. unisporus* was the only species left in kefyr, kefyr after ripening and kefyr after the 2nd incubation. Again, the total yeast count was similar to that of the grains described previously, despite of the absence of a lactose fermenting yeast species. The typical kefyr flavour is most probably achieved by the ability of *Sacch. unisporus* to ferment glucose and galactose.

Kefyr grain WT

C. kefyr appeared to be the only detectable species in this kefyr (Fig. 5). The development of the total yeast count did not deviate much from that of all the other grains.

Discussion

In grains with at least two different species, *yeast U* always was the dominating species if present; otherwise, *Sacch. unisporus* was found to dominate. Both species are lactose negative. The composition of the yeast flora in the milk after addition of the grains led to the presumption that *K. marxianus* and *C. kefyr* cells are located mostly in the external layers of the grains. During fermentation of the milk, *K. marxianus* and *C. kefyr* could catch up with *yeast U*. Their potential to utilise lactose is, therefore, an advantage for growth in milk. Conse-

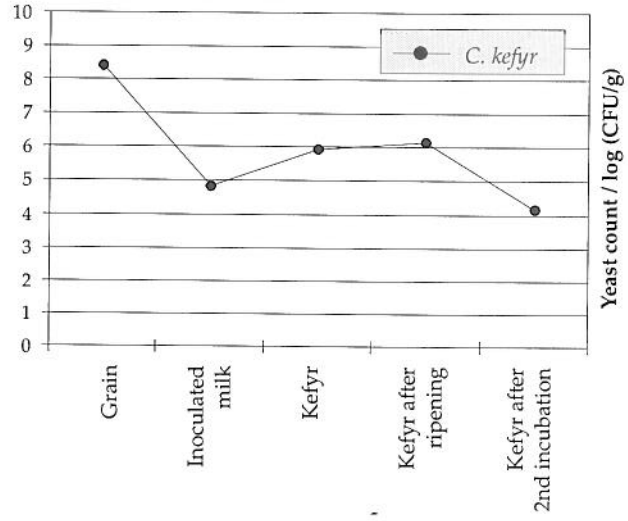


Fig. 5. Kefyr grain WT: Total yeast count

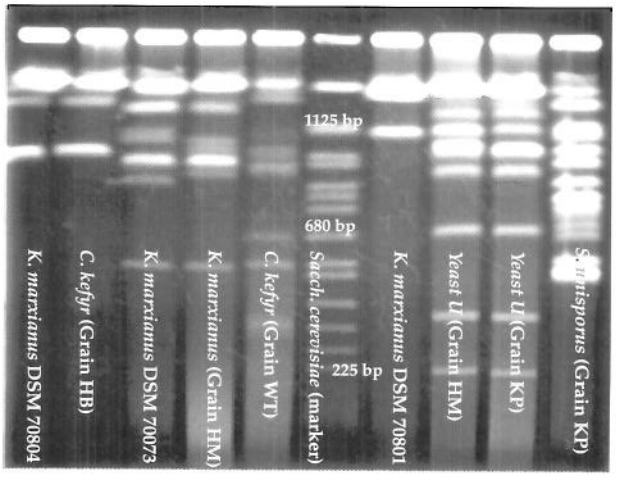


Fig. 6: Karyotypes of yeasts from kefyr and of reference strains

quently, it is not surprising to picture *K. marxianus* and *C. kefyr* mainly in the external regions of the grain, since the grain may still carry the rests of the fermented milk. Only when the competing yeasts *K. marxianus* and *C. kefyr* were absent, *yeast U* dominated, probably due to its high initial share. In cases where kefyr and not grain was used as inoculum, the composition of the yeast flora changed completely. Where either *K. marxianus* or *C. kefyr* was present, it dominated with a share of 80–100%. When both were absent, *Sacch. unisporus* dominated. *Yeast U* disappeared and the total yeast count decreased.

The karyotypes (Fig. 6) of the *K. marxianus* and *C. kefyr* group showed large differences among strains from different grains, but not between strains (data not shown) of the same grain. These findings coincide with the results of Steensma *et al. (18)* who found heterogeneity within the *K. marxianus* species with distinguishable groups, but also between var. *marxianus* strains. This was not the case with all the other species isolated from kefyr: they resulted, within a species, in similar or even identical karyotypes, independently of the grains from which they were isolated (not all data shown in Fig. 6).

The identity of *yeast U* could not be confirmed by the two methods applied in this work (data not shown). Some results had already been published previously (*13*). Since it was a predominant species in three different grains, it would be of high interest to know its identity.

Conclusions

It can be concluded that the composition of the yeast flora in traditional kefyr is quite homogeneous and depends strongly on the inoculum, *i.e.* whether kefyr grain or kefyr is used. Kefyr made without grain as direct culture results in a different product.

Three out of five isolated yeast species were non lactose fermenting. Hence, it can be concluded, that lactose negative yeasts must also be considered as specific for the yeast flora of kefyr. Nevertheless, more grains need to be investigated in order to define a specific yeast flora of kefyr.

The karyotypes of four yeast species were reproducible and species specific. Karyotypes of *K. marxianus* and *C. kefyr* strains of different origin where not comparable.

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Ispitivanje vrste kvasaca u mliječnim proizvodima (kefir)

Sažetak

Uobičajeni starter za proizvodnju kefira jesu zrnca kefira koja se sastoje od želatinoznog matriksa u koji su ugrađene bakterije mliječne kiseline, kvasci, a najčešće i octenokisele bakterije. Međutim, tipična mikroflora kefira nije točno utvrđena. Svrha ovog rada bila je izolacija i karakterizacija kvasaca iz zrnaca kefira i kefira kako bi se proučila raznolikost kvaščeve flore. Izolirani kvasci bili su okarakterizirani i razvrstani prema elektroforetskom kariotipu u homolognom električnom polju i prema tradicionalnim postupcima. U pet istraživanih zrnaca kefira i kefira dobivenog s pomoću njih utvrđene su sljedeće vrste kvasaca: Candida kefyr, Kluyveromyces marxianus, Candida colliculosa, Torulaspora delbrueckii, Saccharomyces unisporus, Brettanomyces anomalus i još nepoznati kvasac U. Sastav kvaščeve flore strogo je ovisio o načinu proizvodnje. U zrncima s barem dvije vrste uvijek je prevladavao kvasac U, a kada ga nije bilo, tada je bio dominantan Sacch. unisporus, dok su tijekom fermentacije kvasci koji fermentiraju laktozu, K. marxianus i C. kefyr, po broju dostizali kvasac U. Kada se kefir koristi kao inokulum, tijekom fermentacije potpuno se izmijeni kvaščeva flora. Ako su u kulturi bili K. marxianus ili C. kefyr, tada su oni prevladavali, a ako nisu, tada je prevladavao Sacch. unisporus, kvasac U nestajao je, a smanjivao se i ukupni broj kvasaca. Može se zaključiti da je kvaščeva flora u tradicionalnom kefiru potpuno homogena i jako ovisi o inokulumu, tj. je li upotrijebljen kefir ili zrnca kefira. Tri od pet izoliranih vrsta kvasaca ne fermentiraju laktozu. Kariotipovi za kvasce, osim K. marxianus i C. kefyr, specifični su za vrstu.