Biostabilization of Kefir with a Nonlactose-Fermenting Yeast

H. S. KWAK,* S. K. PARK,† and D. S. KIM†
*Department of Food Science and Technology, Sejong University, 98 Kunja-dong, Kwangjin-gu, Seoul, 133-747, Korea
†Department of Dairy Science, Kyungpook National University, Sangyuk-dong, Buk-gu, Taegu, 702-701, Korea

ABSTRACT

The biostabilization of kefir was studied using sequential fermentation of milk with a nonlactose-fermenting yeast culture and mixed cultures of lactic acid bacteria. To simulate kefir manufacturing, reconstituted skim milk containing three concentrations of glucose (0.4, 0.5 and 1.0%) was fermented first with the yeast culture, followed by lactic fermentation. The yeast fermentation produced ethanol, but the ethanol production did not continue during lactic fermentation with 0.4 and 0.5% of added glucose. However, some continued ethanol production occurred at 1.0% of added glucose. This pattern appeared to be extended to the refrigerated and room storage. During fermentation, all samples showed similar trends in pH reduction, but higher percentages of added glucose caused faster reduction in pH. The titratable acidity showed trends comparable with the pH changes. With 0.4% added glucose, the yeast culture containing *Saccharomyces cerevisiae* utilized 98% of the added glucose and produced 0.07% ethanol at 31°C during 16 h; the mixed lactic cultures utilized 29% of the lactose at 22°C during the additional fermentation of 16 h. Little change occurred during storage at 5°C for 7 d. The selected strain of *Saccharomyces cerevisiae* appeared to be effective in stabilizing kefir products during storage.

(**Key words**: kefir, biostabilization, yeast, glucose)

INTRODUCTION

Kefir is an ancient fermented milk drink that originated in the Caucasian mountains (3), where it is still manufactured under a variety of names, such as kephir, kiaphur, kefer, knapon, kepi, and kippi (8). Kefir is commonly manufactured by fermenting milk with yeast and lactic acid bacteria (10). Kefir contains a small amount of ethanol and carbon dioxide and has a unique flavor. A traditional Russian product, kefir is now becoming well known in the US, Japan, and European countries. The traditional method of making kefir involves grains that are small, irregularly shaped, yellowish masses resembling individual florets of a cauliflower (10). Kefir grains form the important starter culture for making kefir by harboring a mixture of lactic acid bacteria and yeast.

Kefir grains contain microorganisms belonging to a diverse spectrum of species and genera including lactobacilli, lactococci, and leuconostoc species (1, 9, 10). Lactobacilli species in kefir grain include *Lactobacillus caucasicus*, *Lactobacillus brevis*, *Lactobacillus kefir*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus kefiranofaciens*, *Lactobacillus cellobiosus*, *Lactobacillus bulgaricus*, *Lactobacillus helveticus* ssp. *jugurti*, and *Lactobacillus lactis* ssp. *lactis*. Lactococci include *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis*, *Lactococcus lactis* ssp. *cremoris*, *Streptococcus thermophilus*, *Lactococcus filant*, and *Streptococcus durans*. Common leuconostocs include *Leuconostoc dextranicum*, *Leuconostoc mesenteroides*, and *Leuconostoc kefir* (1, 9).

Yeast is important in kefir fermentation because of the production of ethanol and carbon dioxide. Kefir grains usually contain lactose-fermenting yeasts such as *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Kluyveromyces fragilis*, *Torula kefir*, and *Saccharomyces kefir* (1, 8), as well as nonlactose-fermenting yeasts such as *Saccharomyces cerevisiae* and *Saccharomyces calsbergensis*.

During kefir manufacture with the grains, the lactic acid fermentation practically ceases as the pH declines, but the alcohol fermentation may still proceed. In addition, some yeasts that are capable of fermenting lactose and other sugars still may continue fermentation even after the product is packaged in containers, provided that temperature requirements are met. A drawback for marketing of traditionally produced kefir is that secondary alcohol fermentation tends to occur at the distribution stage, resulting not only in substantial changes in flavor and taste because of the continued formation of ethanol and carbon dioxide gas but also in bulging

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containers and leakage of contents because of the internal pressure created by carbon dioxide produced.

The purpose of this study was to investigate biostabilization of kefir by controlling the production of ethanol and carbon dioxide using a nonlactose-fermenting yeast species.

MATERIALS AND METHODS

Microorganisms for Starter Culture

To achieve alcohol fermentation in kefir, a kefir grain containing *S. cerevisiae* was obtained from Wiesby Co. (Postfach, Germany); this grain contained a nonlactose-fermenting yeast. The yeast had an optimal temperature of 31°C and could produce alcohol during 16 h in GYP (glucose, yeast extract, and peptone) broth containing 0.4% glucose (5). For lactic acid fermentation, lyophilized lactic acid cultures were used. This culture, containing *L. casei*, *Lc. lactis* ssp. *lactis*, *Lc. lactis* ssp. *lactis* biovar *diacetylactis*, *Lc. lactis* ssp. *cremoris*, and *Leuconostoc cremoris*, was obtained from Rosell Institute (Montreal, QC, Canada), and *Propionibacterium shermanii* was obtained from Chr. Hansen Laboratorium (Copenhagen, Denmark).

Starter Culture Preparation

The lactic starter cultures were inoculated into reconstituted NDM (11% TS; pH 6.7) at 3% (vol/vol) and propagated twice at 30°C before use in experiments. The *S. cerevisiae* culture was inoculated at 3% into malt extract broth containing 0.5% glucose, prepared for further use by inoculation into GYP broth, and then propagated twice. The *P. shermanii* culture was prepared by inoculation into reconstituted NDM containing 0.5% peptone and incubating at 30°C; the inoculation was duplicated for making the mother culture.

Fermentation for Ethanol and Lactic Acid Production

The yeast starter cultures were inoculated (3%) into the reconstituted NDM containing glucose at three concentrations (0.4, 0.5, and 1.0%) and incubated at 31°C for 16 h for alcohol production as the first stage of the fermentation. All lactic acid bacteria starter cultures were mixed in the same ratio. Then, the mixed cultures were inoculated (3%) into the reconstituted NDM in glass screw-cap tubes (2.7 × 11.0 cm) with teflon sealing; cultures were incubated at 22°C for an additional 32 h as the second stage of the fermentation, the lactic acid production stage. During the fermentation, samples were taken every 4 h. The kefir was also stored at 25°C for 7 d and at 5°C for 14 d.

Measurement of pH and Titratable Acidity

During the lactic fermentation stage, samples were taken every 4 h and analyzed for pH and titratable acidity (15).

Determination of Ethanol

Ethanol was determined by a modified method of Marsili et al. (13). First, 10 ml of sample and 10 ml of acetonitrile were mixed, sealed, and kept at 4°C for 1 h. The mixture was centrifuged at 8000 × *g* (4°C) for 20 min. After centrifugation, the supernatant was filtered through a 0.2-μm membrane filter (Millipore Corp., Bedford, MA). The filtrate was analyzed by gas chromatography (Philips Pye Unicam, Cambridge, England), which was equipped with a flame ionization detector and a packed column (150 × 0.4 cm i.d.) coated with Porapack-Q (120-mesh; Supelco Inc., Bellenfonte, PA). The column used N_2 at a flow rate of 30 ml/min; H_2 flow rate was 33 ml/min, and air flow rate was 330 ml/min. Temperature of the injection port was 230°C. Integration was used to determine peak area with a chart speed of 0.25 cm/min. For quantification, standard ethanol (E. Merck Co., Darmstadt, Germany) was added to each batch of reconstituted NDM at 0.001, 0.005, 0.01, 0.05, 0.1, 0.2, and 0.5% and treated the same as the samples. All samples were analyzed in triplicate.

Determination of Carbohydrates

To determine lactose and glucose, 10 ml of acetonitrile were added to 10 ml of the sample, mixed well, and then centrifuged at 8000 × *g* (4°C) for 20 min. The supernatant layer was filtered through a 0.2-μm membrane filter and analyzed by HPLC with a refractive index detector (Waters Associates, Milford, MA). Samples were analyzed on an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad Laboratories, Richmond, CA), which was operated at 65°C; the mobile phase was 0.009*N* H_2SO_4, flow rate was 0.6 ml/min, and chart speed was 0.5 cm/min (2). A standard lactose solution was used for quantification of lactose in samples. All samples were analyzed in triplicate.

Measurement of Viscosity

Viscosity was measured in triplicate at 5°C in a 200-ml sample by a viscometer (model BH; Tokyo Keiki Seizosho Co., Tokyo, Japan) with rotor number 3 at 10 rpm for 30 s.

RESULTS AND DISCUSSION

Ethanol

Figure 1 shows the production of ethanol during two-stage fermentation of reconstituted NDM containing 0.4, 0.5, and 1.0% of glucose. A nonlactose-fermenting yeast species, *S. cerevisiae*, was used at 31°C for 16 h, followed by lactic fermentation at 22°C for 32 h with mixed lactic cultures. In samples containing 0.4% glucose, the concentration of ethanol increased steadily until 16 h of fermentation reaching 0.07%, but no further increase in ethanol was observed thereafter. The samples containing 0.5% glucose showed a trend similar to that of 0.4% glucose, but ethanol concentration increased to 0.08% at 20 h. However, samples containing 1.0% glucose showed a considerably different trend. It showed a sharp increase in ethanol from 4 to 36 h, reaching 0.24% ethanol, and then leveled off. This concentration was about three times higher than that of samples containing 0.5% glucose. The yeast in the samples containing 1.0% glucose fermented it continuously up to 36 h because of its high availability.

To test the effect of storage, samples were stored in containers at 25°C for 7 d and 5°C for 14 d after completion of the fermentation (48 h). According to the data (not shown), ethanol was not produced further in samples with 0.4% added glucose at either temperature but developed in samples with 0.5 and 1.0% added glucose. This observation suggests that addition of 0.4% glucose could make the product more biostable because of no further ethanol production during storage. In addition, the samples with 0.4% added glucose showed no strong yeasty flavor according to our experience with the products.

Although our study showed that 0.07% alcohol provided a suitable ethanol content in kefir, ethanol contents in kefir ranged widely in the literature. For example, Duitschaever et al. (3) reported that 0.12 to 0.18% ethanol was produced in sequential fermentation of kefir. Marshall and Cole (12) reported that 0.17% ethanol made a good kefir, and Libudzisz and

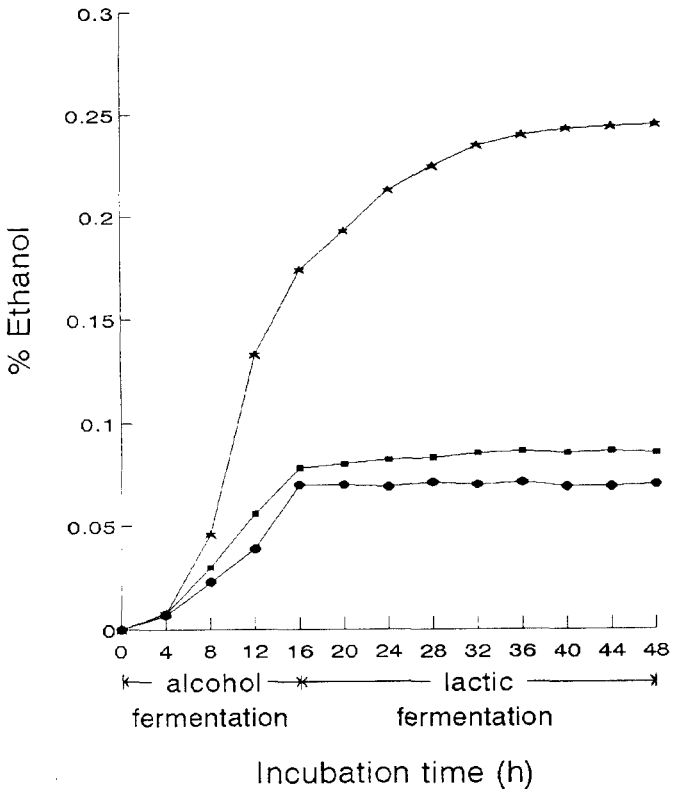

Figure 1. Production of ethanol during alcohol fermentation using a nonlactose-fermenting yeast in reconstituted skim milk containing 0.4 (●), 0.5 (■), and 1.0% (★) of glucose at 31°C for 16 h, followed by lactic fermentation with mixed lactic acid cultures at 22°C for 32 h.

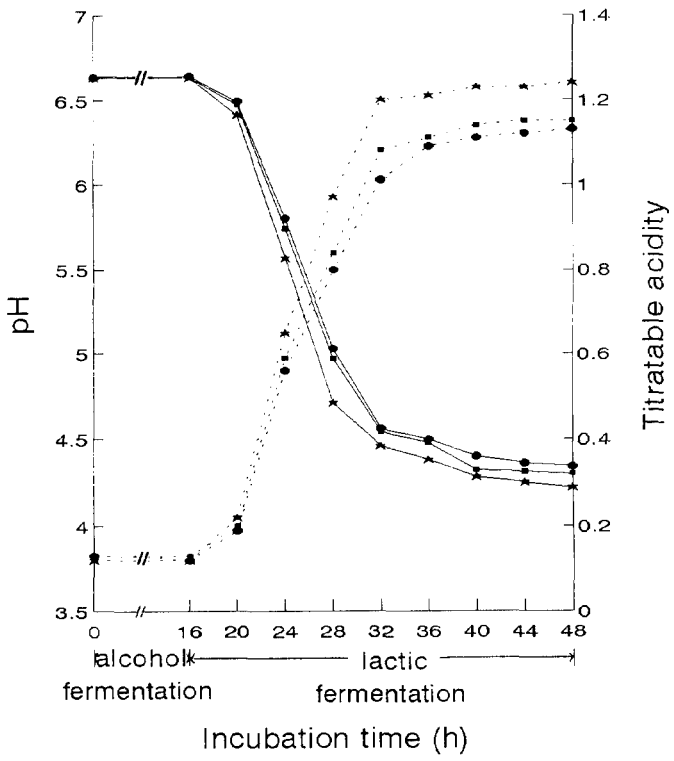

Figure 2. Changes of pH (———) and titratable acidity (– – –) during alcohol fermentation by using a nonlactose-fermenting yeast in reconstituted skim milk containing 0.4 (●), 0.5 (■), and 1.0% (★) of glucose at 31°C for 16 h, followed by lactic fermentation with mixed lactic acid cultures at 22°C for 32 h.

Piatkiewicz (11) reported the appropriate range of ethanol in Polish kefir was 0.035 to 2.0%. Koreleva (8) reported, however, that more than 2.0% ethanol was produced in kefirs made in a Russian farm, but only 0.01% to 0.05 ethanol was found in another kefir made from combined starter cultures. Koreleva (8) indicated that the percentage of ethanol in kefir depended on the starter culture, time and temperature of fermentation, and type of containers.

pH and Titratable Acidity

Figure 2 shows changes of pH and titratable acidity during the two-stage fermentation of reconstituted skim milk with 3 concentrations of glucose (0.4, 0.5, and 1.0%). The pH of all samples did not change up to 16 h. During the second stage, the pH decreased steadily up to 32 h and decreased little thereafter. The pH decreased more rapidly as added glucose increased (Figure 2). Titratable acidity also did not change up to 16 h, but increased sharply from 20 to 32 h of the fermentation and leveled off thereafter.

Similar results were observed by Klupsch (6), who reported that, during fermentation for kefir, pH was about 4.4 after 18 h at 25 to 28°C, and titratable acidity was 1.01% with 0.4% glucose addition, 1.08% with 0.5% addition, and 1.20% with 1.0% addition. Duitschaever et al. (3, 4) reported that pH was about 4.0 after 6 h at 28 to 32°C and after 7 d of storage at 5°C in sequential fermentation of kefir. The authors (3, 4) concluded that the lactic culture utilized more glucose and produced more acid when higher percentages of glucose were available. However, in this study, 0.4% glucose addition was adequate for the development of suitable acidity.

Utilization of Carbohydrates

The utilization of glucose and production of ethanol during alcohol fermentation at 31°C for 16 h of yeast fermentation, the depletion of lactose, and the change of pH during lactic acid fermentation at 22°C for 32 h are shown in Figures 3 and 4. In the samples with 0.4% added glucose, about 98% glucose was used at

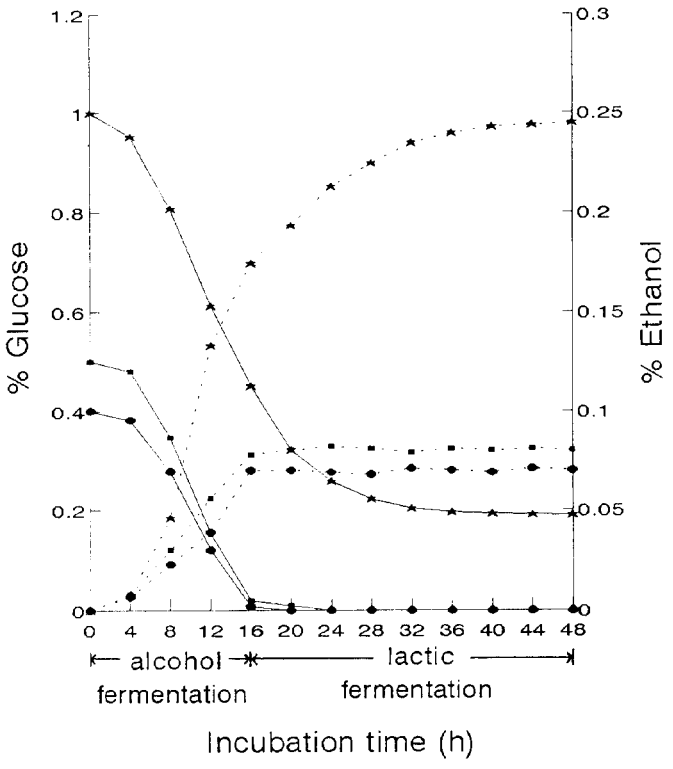

Figure 3. Ethanol production (- - -) and use of glucose (———) during alcohol fermentation by a nonlactose-fermenting yeast in reconstituted skim milk containing 0.4 (●), 0.5 (■), and 1.0% (★) of glucose at 31°C for 16 h, followed by lactic fermentation with mixed lactic acid cultures at 22°C for 32 h.

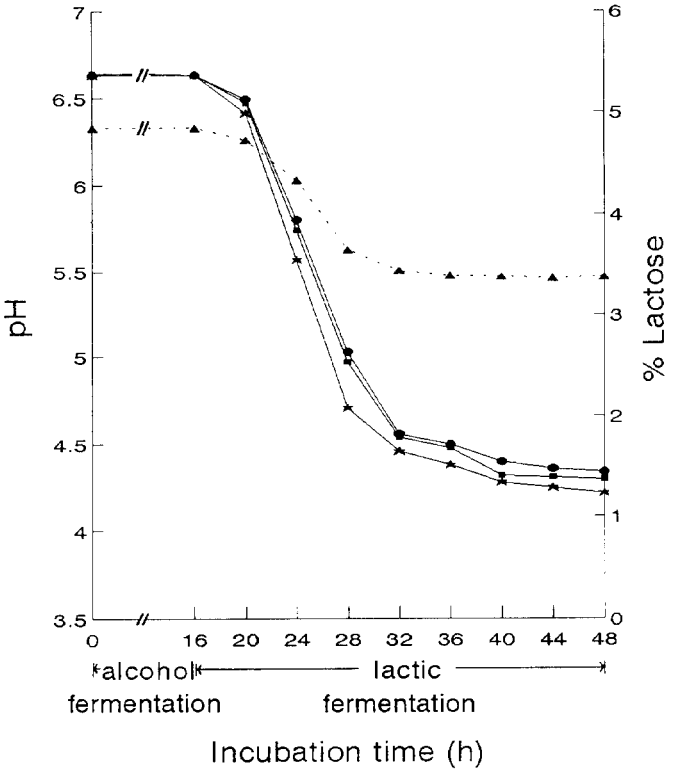

Figure 4. Changes of pH (———) and use of lactose (- - -) during alcohol fermentation by a nonlactose-fermenting yeast in reconstituted skim milk containing 0.4 (●), 0.5 (■), and 1.0% (★) of glucose at 31°C for 16 h, followed by lactic fermentation with mixed lactic acid cultures at 22°C for 32 h.

16 h, while producing 0.07% ethanol (Figure 3). The rest of the glucose (0.008%) appeared not to be converted to ethanol. With addition of 0.5% glucose, about 95% glucose was utilized at 16 h with the production of 0.08% ethanol. More ethanol was apparently generated up to 24 h of fermentation. In the samples with 1.0% added glucose, only about 55% glucose was consumed at 16 h. The consumption of glucose continued during 48 h of fermentation with the production of 0.25% ethanol.

As shown in Figure 4, the yeast culture did not use lactose during alcohol fermentation (16 h). However, the lactic culture utilized 29% of the original lactose at 32 h and 30% at 36 h, respectively (Figure 4). However, very little lactose was used thereafter. In addition, the pH change was similar to the utilization of lactose up to 24 h, and showed a rapid decrease during the rest of the fermentation period. Although samples with a higher percentage of glucose yielded lower pH, the patterns of the decreases were similar. Lactose utilization by the mixed lactic culture appeared to have some similarity to other fermented dairy products. For example, Puhan (14) reported that utilization of lactose was generally about 40% by most lactic acid bacteria in lactic acid fermentation in milk. Ko (7) also reported that the utilization of lactose in fermented products was 69% by *Bifidobacterium bifidum*, 40% by *L. acidophilus*, 41% by *S. thermophilus*, and 10% by *L. casei*.

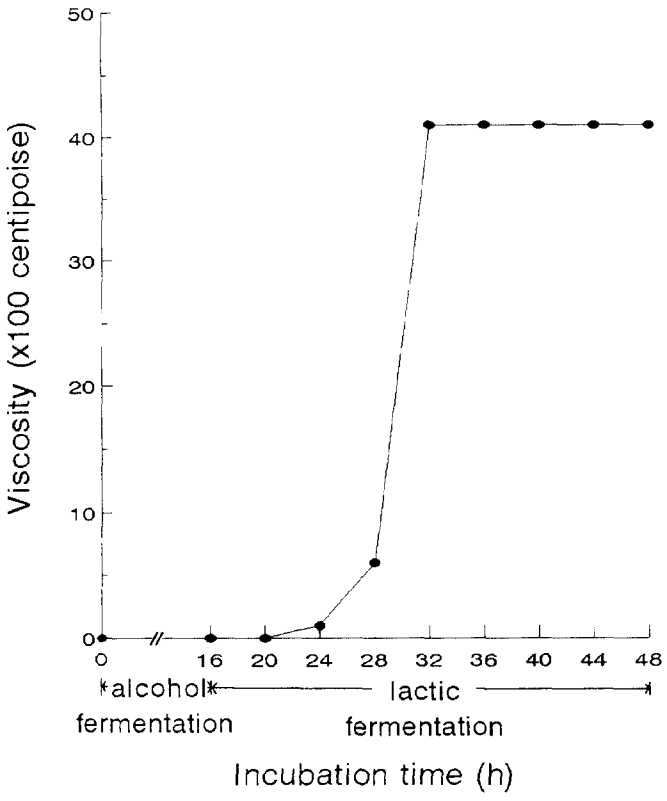

Figure 5. The change of viscosity during alcohol fermentation using nonlactose-fermenting yeast in reconstituted skim milk containing 0.4% glucose at 31°C for 16 h, followed by lactic fermentation at 22°C for 32 h with mixed lactic acid culture.

Viscosity

The viscosity of samples containing 0.4% added glucose was measured during fermentation of alcohol and lactic acid (Figure 5). During alcohol fermentation, viscosity did not change. However, a viscosity increase was apparent at 24 h; viscosity increased rapidly to 4000 cps at 32 h. Curd formation occurred between 28 and 32 h of fermentation. The viscosity changed very little thereafter.

CONCLUSIONS

A two-stage sequential fermentation using a nonlactose-fermenting yeast followed by lactic cultures was advantageous for stabilizing kefir products. The nonlactose-fermenting *S. cerevisiae* strain selected and used in this study appears to be good for kefir manufacture. The addition of 0.4% glucose in reconstituted skim milk was suitable for minimizing fermentation time and producing favorable kefir flavor. At this percentage of glucose addition, concentrations of ethanol and carbon dioxide in the finished products were stable during storage. Therefore, the sequential use of the nonlactose-fermenting yeast and lactic culture with a low amount of glucose is recommended for the development of high quality kefir.

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