Kefir grain tolerance to Escherichia coli contamination–industrial advantages

Kolakowski, Ozimkiewicz

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NOTE

Kefir grain tolerance to *Escherichia coli* contamination—industrial advantages

Piotr Kolakowski · Magdalena Ozimkiewicz

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Abstract Kefir grains are used in dairy plants for mother or bulk starter culture production. The aim of the study was to evaluate the possibility of the re-use of kefir grains grown at 18 °C for 24 h in pasteurized *Escherichia coli* contaminated milk up to 6.1×10^2 cfu.mL^{-1}. The contamination rate was adapted to a realistic situation which may take place in plant. *E. coli* was enumerated in kefir grains, postharvest broth, and in milk incubated without kefir grains. Determination of the replication ability of the affected grains and acidification activity of postharvest broth was performed. The impact of milk contamination on total lactic acid bacteria (LAB), citrate-fermenting and vancomycin-tolerant LAB, and yeasts of the kefir grains and postharvest broth was evaluated. The grains separated from fermented milk and washed with water were not *E. coli*-contaminated. In the postharvest broth (starter culture), *E. coli* was found at inoculation rate, whereas in contaminated milks incubated without grains, *E. coli* population increased at least by 2 log. Separated from contaminated and uncontaminated milk, grains displayed similar growth dynamics in five subsequent transfers into freshly prepared milk. The postharvest broth obtained from last transfer was not contaminated and showed standard acidification milk profile. *E. coli* contamination of milk did not influence adversely the ratio of citrate-fermenting, vancomycin-tolerant LAB, yeasts, and total LAB counts in kefir grains and postharvest broth. These findings would have economical value for kefir producers, but it should be considered that only one strain with low contamination rate was tested.

开菲尔粒对大肠杆菌污染的耐受性

P. Kolakowski (✉) · M. Ozimkiewicz
Danisco Biolacta, Innovation, 1A Tuwima Str, 10-747 Olsztyn, Olsztyn, Poland
e-mail: piotr.kolakowski@danisco.com

M. Ozimkiewicz
e-mail: moz@wp.pl

摘要：开菲尔粒在乳品工厂中常被用做发酵剂。本文评价了在18 °C条件下，开菲尔粒在污染了大肠杆菌（6.1 × 10^2 cfu.mL^{-1}）的巴氏杀菌奶中重复使用性。感染速率符合工厂的实际生产情况。采用菌落计数法测定了开菲尔粒、发酵剂和未接种开菲尔粒牛奶中的大肠杆菌。测定了污染的开菲尔粒的复制能力和发酵培养液酸化能力。评价污染奶对总乳酸菌（LAB）、发酵柠檬酸盐乳酸菌以及耐万古霉素乳酸菌的影响。从发酵乳中分离的开菲尔粒经水洗后没有检测到大肠杆菌的污染。在发酵剂中，大肠杆菌与菌种的生长速率相同，但是在未添加开菲尔粒的污染奶中，大肠杆菌以2倍对数值的速率生长。从污染牛乳和未污染的乳中分离出的开菲尔粒，通过连续5次接种到新鲜调制乳中，大肠杆菌表现出相似的生长动力学。最后一次接种中获得的发酵剂没有被大肠杆菌污染，而且表现出标准的酸乳特点。被大肠杆菌污染的牛乳并不能影响发酵柠檬酸盐乳酸菌、耐万古霉素乳酸菌、酵母进和总乳酸菌在开菲尔粒和发酵剂中总数。本研究结果发对开菲尔生产者来说有很重要的经济价值，但是应该考虑本研究只是针对一株菌，并且污染率较低的情况下得出的结论。

Keywords Kefir grains · Kefir · *Escherichia coli* · Lactic acid bacteria · Yeasts

关键词 开菲尔粒 · 开菲尔 · 大肠杆菌 · 乳酸菌 · 酵母菌

1 Introduction

In dairy plants, kefir grains are traditionally used to ferment either skimmed fresh milk or reconstituted non-fat dry milk in order to produce a characteristic drink called kefir. The growth of kefir grains, which is observed as an increase in biomass production, is a continuous process of a daily transfer of grains into freshly prepared milk at the temperature ranging from 18 °C to 24 °C. The initial starter culture for kefir production is a fresh postharvest broth, often called the mother starter culture, which is obtained after separation of kefir grains from fermented milk. Also, a subsequent transfer of the mother starter culture, called the bulk starter culture, may be used to inoculate milk for the production of kefir (Wszolek et al. 2006). The presence of residual microbiota in pasteurized milk and the biomass production of kefir grains in tanks that relies more heavily on manual rather than automatic operations may enhance the development and growth of undesirable microbiota (Hattowska 1984). Even though the risk of contamination is possible, dairy plants have not recorded frequent cases of microbial contamination in the growth process of kefir grains (Danisco, private communication). This phenomenon may be explained by the antimicrobial properties of kefir grains. Kefir and kefir grains have shown to have antimicrobial properties against a number of Gram-positive and Gram-negative bacteria and some yeasts, including *Salmonella* spp. (Garrote et al. 2000; Rodrigues et al. 2005; Santos et al. 2003; Schneedorf and Anfiteteatro 2004; Silva et al. 2009), *Helicobacter* (Schneedorf and Anfiteteatro 2004), *Shigella* spp. (Garrote et al. 2000; Schneedorf and Anfiteteatro 2004; Silva et al. 2009), *Staphylococcus aureus* (Cevikbas et al. 1994; Deeseenthum and Pejovic 2010; Garrote et al. 2000; Rodrigues et al. 2005; Silva et al. 2009), *Pseudomonas aeruginosa* (Rodrigues et al. 2005), *Pseudomonas fluorescens* (Deeseenthum and Pejovic 2010), *Escherichia coli* (Deeseenthum and Pejovic 2010; Garrote et al. 2000; Gulmez and Gueven 2003; Rodrigues et al. 2005; Schneedorf and Anfiteteatro 2004; Silva et al. 2009), *Enterobacter aerogenes, Proteus vulgaris* (Kwon et al. 2003), *Bacillus subtilis*

(Deeseenthum and Pejovic 2010; Garrote et al. 2000), *Bacillus cereus* (Garrote et al. 2000; Kakisu et al. 2007), *Listeria monocytogenes* (Gulmez and Gueven 2003; Rodrigues et al. 2005), *Streptococcus pyogenes, Streptococcus salivarius* (Rodrigues et al. 2005), *Yersinia enterocolitica* (Gulmez and Gueven 2003), and *Candida albicans (*Cevikbas et al. 1994; Rodrigues et al. 2005; Silva et al. 2009).

Many dairy plants conduct daily examination of postharvest broth (filtrate obtained after the separation of kefir grains from fermented milk) for the presence of coliforms and the ability for milk acidification. In the event of contamination of the postharvest broth, the grain multiplication process and kefir production are halted. The process of restarting the production from new kefir grains from their frozen form depends on their activation time in milk and often lasts even 3–4 weeks. The grains' reactivation process is laborious, time-consuming, and energy-costly. It would thus be useful to investigate the microbial safety and the technological properties of kefir grains separated from *E. coli*-contaminated milk, to evaluate the possibility of re-using them for starter production in dairy plants.

2 Materials and methods

2.1 Kefir grains

Fresh active kefir grains manufactured by Danisco Biolacta Poland were used. Before use, the grains were rinsed with ultra-pure water at 18 °C in 1:5 ratio (*w/v*) and separated by filtration through a plastic sieve. The procedure was repeated four times, and last of all, the grains were dried on tissue paper.

2.2 *E. coli*

The strain of *E. coli* 25922 was obtained from the ATTC culture collection. Before use, the strain was activated in trypticase–soy agar supplemented with 5% of sheep blood and multiplied in 10% sterilized skimmed milk at 37 °C for 24 h (inoculation solution).

2.3 Sample preparation and cultivation conditions

Kefir grains were multiplied in rehydrated and pasteurized non-fat dry milk (10% of dry matter), at 95 °C for 30 min. The 0.1, 2, 4, and 8 mL suspension of *E. coli* (ca 10^5 cfu.mL^{-1}) was added to four 1.5-L flasks containing 1,000 mL pasteurized milk cooled to 18 °C under aseptic conditions. After the addition of *E. coli*, 100 g of kefir grains was added to the next four flasks. For the control sample, 100 g of kefir grains was added to 1,000 mL of inoculated pasteurized milk. All nine samples were incubated at 18 °C for 24 h. After incubation, the grains were separated with a plastic sieve, washed four times with ultra-pure water, and then finally dried on tissue paper. The presence of *E. coli* was determined in the kefir grains, postharvest broth, and in the contaminated milk without kefir grains incubated at the same conditions. The experiment was repeated twice.

2.4 Determination of the replication ability of kefir grains in milk

One hundred grams of kefir grains, inoculated in milk contaminated with highest level of *E. coli* (6.1×10^2cfu.mL^{-1}), and 100 g of non-treated grains were added to freshly prepared milk (1,000 mL) and incubated at 18 °C. After 24 h incubation, the kefir grains were separated by filtration with a plastic sieve containing a double layer of gauze and dried on tissue paper. Then, the grains were weighed and added to freshly pasteurized milk. This process was repeated until the kefir grains doubled their mass. The number of culturing days and the increase in the biomass of grains expressed in grams per liter were recorded. *E. coli*, total lactic acid bacteria (LAB) count, citrate-fermenting LAB, and vancomycin-tolerant LAB and yeasts were enumerated in kefir grains and postharvest broth after the biomass was doubled. The experiment was repeated twice.

2.5 Acidification activity of post-harvest broth

Three percent of postharvest broth was added to 500 mL of 10% skimmed milk at 23 °C (reconstituted from powder and pasteurized milk at 95 °C for 30 min). Changes of pH value were recorded at 1-min intervals with the use of CINAC—a computerized system for the determination of lactic starters' acidifying activity (Ysebaert Dairy Division, Fre'pillon, France).

2.6 Microbiota analyses

A 10-g sample of kefir grains was placed in sterile stomacher bag and diluted with 90 mL of sterile saline solution (9 g.L^{-1}). Samples were homogenized for 3 min in a Stomacher (BigMixer, Interscience, France) at its maximum speed. The postharvest broth was used without any pre-treatment. For enumeration of viable microbiota, the samples were serially diluted in 0.1% peptone water and plated onto the following media: modified Nickels–Leesment (N-L) medium for total count of LAB and citrate-fermenting LAB (Nickels and Leesment 1964); Nickels–Leesment medium supplemented with 200 μg.mL^{-1} filter-sterilized vancomycin (Sigma-Aldrich, St. Louis, USA) for vancomycin-tolerant LAB enumeration (Kołakowski et al. 2004). Plates were incubated aerobically at 25 °C for 48 h. Colonies surrounded by a clear zone on N-L medium were considered to be citrate-fermenting-positive and belonging to *Leuconostoc* and heterofermentative *Lactobacillus* genera and *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* spp. (IDF Standard 149:2010). *Leuconostoc* and mesophilic, mainly heteofermentative, *Lactobacillus* of kefir grains demonstrate high tolerance to vancomycin, and media for total LAB count enumeration supplemented with the antibiotic are often used for their determination or isolation from dairy products (Benkerroum et al. 1993; Cogan et al 2007; Kołakowski et al. 2004). Yeast counts were determined by plating the samples on yeast extract chloramphenicol agar and aerobic incubation at 25 °C for 120 h as described by Rea et al. (1996). *E. coli* were enumerated on VRBL using aerobic incubation at 30 °C for 48 h (Merck, Darmstadt, Germany).

The average count of duplicate plates from each sample was used to determine total viable cell counts. Counts were recorded as colony-forming units per gram.

3 Results and discussion

3.1 Kefir grain tolerance against *E. coli*

In dairy plants, kefir grains are multiplied in skimmed pasteurized milk in a continuous process of a daily transfer of kefir grains into freshly prepared milk (Kołakowski and Ozimkiewicz 2012). During fermentation, microbiota of kefir grains produced lactic acid and many other metabolites including antimicrobial substances (Wszolek et al. 2006). The influence of 24 h fermentation process of milk at 18 °C on the development of *E. coli* in kefir grains and postharvest broth, depending on initial contamination rate, is presented in Table 1. Milk contamination rate with *E. coli* at ranges from 2.0×10^{1} to 6.1×10^{2} cfu.mL^{-1} was adapted to a realistic situation which may take place in a dairy plant. Kefir grains separated from the fermented milk were not contaminated. *E. coli* was not detected in any of the kefir grain samples independently at their initial cell count added to milk before fermentation. In postharvest broth, as opposed to kefir grains, *E. coli* was detected, but their cell count, in comparison to the initial count in milk, did not increase during 24 h of fermentation. For example, when milk before fermentation was contaminated with 6.1×10^{2} cells per mL in postharvest broth, 6.3×10^{2} cells per mL was detected. Even at very low rate contamination of milk (20 cfu.mL^{-1}), *E. coli* was detected in postharvest broth after 24 h of fermentation (Table 1), and because of the reason, the postharvest broth cannot be used as a starter culture for kefir production.

In pasteurized milk incubated at the same conditions (at 18 °C for 24 h), without kefir grains, *E. coli* counts increased at least 2 log per mL of milk, regardless of the inoculation rate. The higher the contamination rate, the bigger the population of *E. coli* detected in milk after incubation. The maximum concentration of 1.0×10^{6} of cells per mL was observed at the highest contamination rate of milk (6.1×10^{2} cfu.mL^{-1} of milk). The population of *E. coli* exceeded 8 log per mL in the tested milk when the incubation temperature was 37 °C (data not shown). The results demonstrated stronger inhibitory activity of kefir grains against *E. coli* than the

Table 1 *E. coli* multiplication during 24 h of kefir grain incubation in milk

Count of *E. coli* in			
Milk before incubation, cfu.mL^{-1}	Milk after incubation without grains, cfu.mL^{-1}	Kefir grains after incubation, cfu.g^{-1}	Postharvest broth, cfu.mL^{-1}
nd (control)	nd	nd	nd
2.0×10^{1} (±2.0)	5.0×10^{4} ($\pm2.9\times10^{3}$)	nd	1.8×10^{1} (±3.0)
4.1×10^{2} (±64.0)	4.5×10^{4} ($\pm3.0\times10^{3}$)	nd	4.2×10^{2}(±16.0)
5.3×10^{2} (±37.0)	7.3×10^{5} ($\pm5.8\times10^{3}$)	nd	5.1×10^{2} (±27.0)
6.1×10^{2} (±64.0)	1.0×10^{6} ($\pm3.5\times10^{4}$)	nd	6.3×10^{2} (±27.0)

The data represent the mean value of two independent trials; in each trial, two parallel samples were analyzed (in brackets, standard deviation)

nd not detected in 0.1 g

postharvest broth. Growth inhibition of *E. coli* in the presence of kefir or during milk fermentation with kefir grains has been reported in many studies (Deeseenthum and Pejovic 2010; Garrote et al. 2000; Gulmez and Gueven 2003; Rodrigues et al. 2005; Silva et al. 2009). The inhibitory effect of milk fermented with kefir grains was attributed to the competition for nutrition components between kefir microbiota and the tested strain and/or mainly due to organic acids produced during the fermentation process (Garrote et al. 2000). The authors showed that milk supplemented with lactic acid or lactic acid and acetic acid at the concentrations found in kefir also demonstrated inhibitory activity against *E. coli*. In their studies, Gulmez and Gueven (2003) fermented milk containing 4.68 log per mL of *E. coli* O157:H7 with 5% of postharvest broth at 30 °C. The fermentation process failed to inhibit the growth of *E. coli* O157; its population increased by 3 log, but it systematically diminished during cold storage of kefir. In our study, the limited growth of *E. coli* in milk during fermentation with kefir grains may have been partially due to lower inoculation rate, lower final pH of broth (<4.30), and suboptimal temperature of incubation. In contrast, at 18 °C, kefir grains microbiota is able to develop much faster and produce organic acids (lactic acid and acetic acid) at high concentrations and other microbial substances such bacteriocins and hydrogen peroxide. Yuksekgag et al. (2004) reported that 11 out of 21 strains isolated from Turkish kefir produced hydrogen peroxide at levels 0.04 to 0.19 ug.mL^{-1}.

The absence of *E. coli* in kefir grains, as opposed to postharvest, can be presumed to be the results of indirect contamination of kefir grains, higher concentration of bacteria, and higher concentration of inhibitive substances in their matrix, which may have created unfavorable conditions for *E. coli* cell absorption and growth (Molska 1988; Wszolek et al. 2006). The antimicrobial activity of kefir grains may be related to their polysaccharide matrix properties (Rodrigues et al. 2005). Kefiran, the main polysaccharide of kefir grains, was able to inhibit the growth of many bacteria and yeasts. Rea et al. (1996) reported that *Lactococcus* spp. are located on the surface of kefir grains matrix, while *Lactobacillus* spp. are present in the deeper layers of the grains. Santos et al. (2003) showed that 43 strains out of 58 *Lactobacillus* spp. isolated from kefir grains demonstrated antimicrobial activity against *E. coli*. Moreover, kefir grains' matrix contained less nutritive substances than milk for *E. coli* growth. An interesting aspect would be to check the ability of *E. coli* cells to bind with the grains matrix. If *E. coli* cells are loosely bound to the grains matrix, they can easily be washed off during sample preparation. It is well known that that

Table 2 Growth dynamics of kefir grains contaminated with *E. coli* in five subsequent transfers into milk

Kefir grains	Number of incubation days				
	1	2	3	4	5
	Kefir grains mass (g)				
Uncontaminated	121.9 (±6.9)	142.0 (±5.0)	163.6 (±5.3)	186.2 (±6.1)	210.3 (±7.4)
Contaminated	120.8 (±5.2)	143.2 (±4.6)	162.6 (±7.4)	183.4 (±6.8)	208.9 (±7.0)

The data represent the mean values of two independent trials; in each trial, two parallel samples were analyzed (in brackets, standard deviation)

Table 3 Microbiota of postharvest broth and kefir grains after five subsequent transfers of *E. coli*-contaminated grains into milk

Samples	Microbiota				
	Total count of LAB, cfu.g^{-1} or ·mL^{-1}	Citrate-fermenting LAB, cfu.g^{-1} or ·mL^{-1}	Vancomycin-tolerant LAB, cfu.g^{-1} or ·mL^{-1}	Yeasts, cfu.g^{-1} or ·mL^{-1}	*E. coli,* cfu.g^{-1} or ·mL^{-1}
Uncontaminated grains	1.8×10^{8} ($\pm2.2\times10^{7}$)	6.9×10^{7} ($\pm3.2\times10^{7}$)	2.0×10^{7} ($\pm1.8\times10^{6}$)	1.8×10^{7} ($\pm2.2\times10^{6}$)	nd
Contaminated grains	2.1×10^{8} ($\pm9.0\times10^{6}$)	7.1×10^{7} ($\pm1.4\times10^{7}$)	2.2×10^{7} ($\pm1.2\times10^{6}$)	1.5×10^{7} ($\pm1.7\times10^{6}$)	nd
Postharvest broth from uncontaminated grains	3.8×10^{8} ($\pm1.8\times10^{7}$)	1.4×10^{8} ($\pm1.2\times10^{7}$)	3.4×10^{7} ($\pm2.3\times10^{6}$)	6.1×10^{6} ($\pm2.6\times10^{5}$)	nd
Postharvest broth from contaminated grains	4.1×10^{8} ($\pm2.2\times10^{7}$)	1.7×10^{8} ($\pm1.5\times10^{7}$)	3.9×10^{7} ($\pm2.7\times10^{6}$)	5.5×10^{6} ($\pm4.0\times10^{5}$)	nd

The data represent the mean value of two independent trials; in each trial, two parallel samples were analyzed (in brackets, standard deviation)

nd not detected in 0.1 g or 0.1 mL

Lactococcus spp. can be more easily flushed with the kefir grain matrix than *Lactobacillus* spp. or yeasts (Wszolek et al. 2006). Our findings can shed a new light on some differences between inhibitive properties of kefir grains versus milk fermented with kefir grains microbiota.

3.2 Technological properties of kefir grains incubated in milk contaminated with *E. coli*

The growth dynamics of kefir grains in milk is a good indicator of their vital and technological functions. It was noted that the lower vitality of kefir grains, the lower their growth dynamics in milk (Kołakowski and Ozimkiewicz 2012). Inoculated with *E. coli* and uncontaminated kefir grains displayed similar growth dynamics, and both samples duplicated their biomass after five subsequent transfers into milk (Table 2). Untreated kefir grains increased their mass from 100 to 210.3 g and treated grains to 208.9 g, respectively. Contamination with *E. coli* did not influence the size of the grains, their appearance, or resilience (data not shown). Microbiota of postharvest broth and kefir grains after five subsequent transfers of inoculated grains into milk is presented in Table 3. *E. coli* was neither detected in postharvest broth nor in kefir grains. Postharvest broth was free of contamination already after the first transfer into milk (data not shown). Ratio of total LAB count to citrate-fermenting and vancomycin-tolerant LAB; yeast in kefir grains influence the properties of kefir, and they are the main indicators of kefir grain quality (Molska 1988). Vancomycin-tolerant LAB is characteristic for kefir grains and kefir, and their ratio to total count of LAB is quite constant (Kołakowski et al. 2004; Kołakowski and Ozimkiewicz 2012).

The contaminated grains and post-harvest broth were characterized by approximately the same numbers and proportions between the determined groups of microorganisms in comparison with the control samples.

Citrate-fermenting LAB (*Lactococcus*, *Leuconostoc*, and *Lactobacillus*) play a key role in the development of the sensory characteristics of kefir (Hattowska 1984; Molska 1988), whereas the ratio of total LAB count to vancomycin tolerance could influence the speed of milk fermentation (Molska 1988). The dominance of vancomycin-tolerant LAB (mainly *Lactobacillus* spp.) in the postharvest broth may

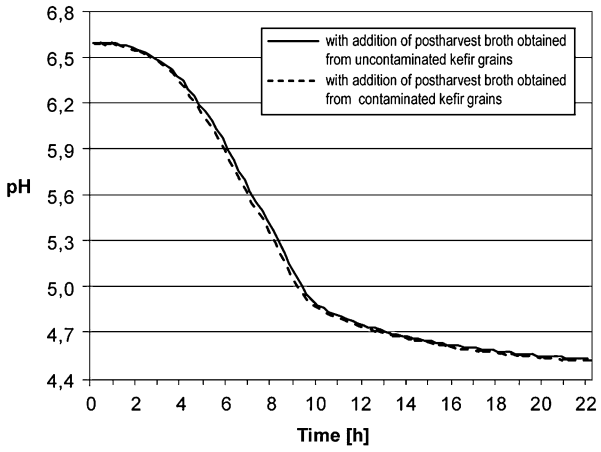

Fig. 1 Fermentation curve of milk incubated at 23 °C with 3% of postharvest broth addition obtained after five subsequent transfers of contaminated kefir grains into milk

significantly extend the time of milk fermentation because they do not grow well in milk (Briggiler-Marco et al. 2007; Cogan et al 2007; Molska 1988). The dominance of *Lactococcus* ssp. in the postharvest broth shortens the time of milk fermentation but can adversely influence the sensory characteristics of kefir (Hattowska 1984; Molska 1988). Incubation of milk with *E. coli* did not have an adverse impact on the microbial indicators in respect of the kefir grains and postharvest broth. Citrate-fermenting LAB consisted of 38% and 37% of total LAB population for uncontaminated samples of kefir grains and postharvest broth and 34% and 41% for contaminated samples, respectively. The ratio of total LAB count, vancomycin-tolerant, and yeasts was 10:1:1 for contaminated and uncontaminated kefir grains and postharvest broth samples (Table 3).

Fermentation time and acidification profile of milk inoculated with postharvest broth, obtained after five subsequent transfers of inoculated kefir grains, was similar to that achieved in respect of uncontaminated kefir grains (Fig. 1). The results confirmed that contamination with *E. coli* did not rather change the counts of and proportions between vancomycin-tolerant and total LAB count in postharvest broth.

4 Conclusions

The kefir grains separated from fermented milk and washed with water were not contaminated when inoculation of milk with *E. coli* before fermentation did not exceed 6.1×10^2 cfu.mL^{-1}. In the postharvest broth, contamination was found at inoculation rate, and in spite of demonstrated inhibitory activities against *E. coli*, it cannot be used as starter culture for kefir production. In contaminated milks incubated at the same conditions without kefir grains, the population of *E. coli* increased by 3 log in average. Kefir grains separated from contaminated milk retained their replication ability in milk and allowed obtaining uncontaminated postharvest broth with similar characteristic to uncontaminated grains in respect of milk acidification profile and ratio of total LAB count to citrate-fermenting and vancomycin-tolerant LAB, and yeasts. The study has demonstrated that kefir grains, after separation from contaminated fermented milk, can be considered for re-use in a dairy plant for starter culture production.

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