ISSN 0026-2617, Microbiology, 2015, Vol. 84, No. 4, pp. 561–569. © Pleiades Publishing, Ltd., 2015.
Original Russian Text © A.A. Khokhlacheva, M.A. Egorova, A.N. Kalinina, N.B. Gradova, 2015, published in Mikrobiologiya, 2015, Vol. 84, No. 4, pp. 466–475.

EXPERIMENTAL ARTICLES

Trophic Patterns of Functioning and Microbial Profile of the Evolutionally Established Associated Kefir Grains Culture

A. A. Khokhlacheva[a, 1], M. A. Egorova[b], A. N. Kalinina[c], and N. B. Gradova[a]

[a] *Mendeleyev University of Chemical Technology of Russia, Moscow, Russia*
[b] *Lomonosov Moscow State University, Moscow, Russia*
[c] *State Research Institute of Genetics and Selection of Industrial Microorganisms, Moscow, Russia*

Received December 15, 2014

Abstract—The associated culture of kefir grains was analyzed by molecular methods for determination of the functional activity of microbial isolates and molecular genetic techniques for their identification. A combination of 16S rRNA analysis and denaturing gradient gel electrophoresis was used to determine the microbial profile of kefir grains and to reveal lactic acid bacteria of two physiological groups, differing in their ability to use lactose for lactic acid fermentation. The role of inducible β-galactosidase of lactic acid bacteria for the functional stability of the microbial community was shown in the study of the functional activity and microbial profile of the kefir grains after long-time cultivation (over 4 years) on lactose-free milk. The results obtained improve our understanding of the possible trophic interactions in such microbial communities and may be used to develop the algorithm for experimental production of a stably associated culture of kefir grains.

Keywords: kefir grains, evolutionally established microbial community, trophic relationships, microbial profile

DOI: 10.1134/S0026261715040104

Formation and functioning of morphologically structured, evolutionarily established microbial communities which are considered to be the dominant, the most stable, and most metabolically active form of existence of microorganisms in natural and some man-made ecosystems is of great scientific interest nowadays. In those ecosystems, such communities occupy particular ecological niches. Such communities attract attention not only as an object to study mechanisms of organization of natural systems, but also as a basis for developing new ways to construct novel functionally-targeted microbial communities of practical importance.

In recent years, the role of subtle mechanisms based on chemical communication of microorganisms in the formation of microbial communities was shown [1, 2]. However, trophic relationships play the crucial role in the transformation of such microbial communities into a system acting as a single defined entity [3–5].

Kefir grains are one type of the stably functioning microbial communities which have been used for a long time in the food industry to produce kefir, a fermented dairy product. Years of investigation of their microbial composition and functional properties resulted in identifying the major trophic flows and their participants: lactose-utilizing homo- and heterofermentative lactic acid bacteria that form lactic acid; yeasts using lactic acid in ethanol fermentation; and acetic acid bacteria utilizing ethanol [6, 7]. The composition of microbial communities was found to vary in kefir grains from different man-made systems. Over 20 various species of lactic acid bacteria and about 20 species of yeasts, either lactose-utilizing or non-utilizing, were detected in kefir grains [8].

According to these data, kefir grains might represent a system with variable microbial composition, which, however, perform similar functions, preserving the pathways of substrate transformation. However, this assumption can not be proved by the published data. Many questions concerning the trophic links between microbes in kefir grains remain unsolved. In particular, producer microorganisms in this system are not identified, the relationships between different species and genera of lactic acid bacteria are not clarified, etc. We are still lacking the data required for the development of a functional model of a mature kefir grain consortium, which is essential for developing the ways of controlling this system and constructing stable consortia experimentally.

The goal of the present work was to study the microbial profile of kefir grains used in the manufacturing of different dairy products and to study the trophic relationships between microbial components of these consortia as a basis for construction of a functional model of such system.

[1] Corresponding author; e-mail: sasha-88888@yandex.ru

MATERIALS AND METHODS

Subjects of study. We studied kefir grains used in kefir production at dairy factories in Stavropol (KGS) and Gagarin (KGG) and lyophilized kefir grains used at Moscow factories (KGM). The primary studies were carried out with kefir grains used for kefir production at the dairy plant in Stavropol.

Kefir grains were cultured under microaerophilic conditions on native milk (Parmalat), 0.5% fat (lac+) and on lactose-free milk (Valio Zero Lactose), 1.5% fat (lac–). The total amount of carbohydrates was nearly the same in all studied samples of milk.

Functional properties of kefir grains were determined as the effect of cultures obtained after kefir grains were cultivated on milk and their subsequent separation (in the food industry called leaven) on milk. The cultures were used as inocula in amount of one mL per 10 mL of milk. Standard indicators used as criteria of the functional activity of kefir grains and isolated microorganisms were clot formation and its appearance, changes in pH of the medium, and titrated acidity of milk (°T), which was used to calculate the amount of formed lactic acid and the amount of fermented lactose [9].

Activity of alcoholic fermentation was determined by the classical method in Einhorn-Smith flasks as a share of space occupied by a gas bubble in the closed node and were expressed in relative units 1, 0.5, 0.3, and 0.15 (as a volume of displaced liquid). Incubation was carried out for 3 days in the medium containing the following: yeast water (7–10 g/L dried yeasts), 2% lactose (glucose, galactose), 0.1% K_2HPO_4, and 0.5% NaCl [10, 11].

β-Galactosidase activity of microorganisms was determined according to their ability to form acids [9] and using the chromogenic substrate X-Gal [12].

Concentration of sugars (glucose and galactose) in the culture liquid were determined in dynamics at batch cultivation of *Leuconostoc gelidum* using thin-layer chromatography. *L. gelidum* was cultured under microaerophilic conditions on liquid medium containing the following (g/L): glucose, 30; yeast extract, 15; $MnSO_4 \cdot 5H_2O$, 0.05; $MgSO_4 \cdot 7H_2O$, 1; K_2HPO_4, 2. Thin-layer chromatography was carried out on 60 F_{254} silica gel plates (Merck, Germany). A mixture of isopropyl alcohol: ethyl acetate: water in a ratio 7 : 1 : 2 was used as a solvent; a 2% alkaline solution of 2,3,5-triphenyltetrazolium chloride in ethanol was used as a developer [13].

Ability to utilize various carbon sources for lactic acid fermentation was determined by changes in pH and titrated acidity (°T) in the course of cultivation in liquid medium that contained the following (g/L): $(NH_4)_2SO_4$, 5; KH_2PO_4, 1; KCl, 0.15; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.05; yeast extract, 5, with addition of glucose, lactose, or galactose (20 g/L) [9].

For comparative evaluation of the composition of exopolysaccharides produced by kefir grains, the grains were thermally treated in boiling water, and polysaccharides were isolated by multiple re-precipitation in alcohol [14]. IR spectra of polysaccharide samples were obtained using a Tensor-27 FTIR spectrometer (Bruker) equipped with ATR Miracle (Pike Technologies). The ATR crystal made of Ge for one reflection was used. IR-spectra in the transmissive mode were also registered with Microfocus (Perkin-Elmer) and thin crystalline silicon wafers. In the case of wafers, the samples were applied to the surface of silicon from water solution and the spectra were registered after water evaporation. IR and CR spectra were processed with OPUS v/6.5 software (Bruker).

Structure of the microbial community of kefir grains was determined with commonly used solid media. The grains were minced with a sterile blade, ground in a mortar until a homogeneous mass, and resuspended in 1% tryptone solution (Serva, Germany). The following media were inoculated: (a) yeast medium, g/L: glucose (lactose), 30; yeast extract, 10; $CaCO_3$, 20; agar, 15; (b) Sabouraud medium (glucose-peptone), g/L: glucose, 40; peptone, 10; yeast autolysate, 30; agar, 15; (c) MRS medium, g/L: casein hydrolysate, 10; meat extract, 10; yeast extract, 5; glucose, 20; sodium acetate, 5; ammonium citrate (dibasic), 2; Tween 80, 1; K_2HPO_4, 2; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 4H_2O$, 0.05; agar, 15. The plates were incubated under static conditions at room temperature (22–25°C).

Isolates were identified to the species level based on analysis of their 16S rRNA-coding genes according to the standard methods. To identify the lactic acid bacteria, we have used an AllRussian Collection of Industrial Microorganisms. The following primers were used for amplification of analyzed sequences: 8f—agagtttgatcctggctcag; 926r—ccgtcaattcctttragttt; 1492r —ggttaccttgttacgactt [15]. Sequencing was carried out in an AE3000 automatic sequencer. The sequences were analyzed using specialized phylogenetic software on the RDB II (Ribosomal Database Project II) website for identification of microbial phylogeny and construction of phylogenetic trees [16]. PCR reactions were repeated three times to provide stable playback of results. Microorganisms were referred to particular species with at least 97% homology at sequence length of at least 500 bp [15].

Denaturing gradient gel electrophoresis (DGGE) was applied to compare the microbial profile of kefir grains [17].

Electron microscopy was carried out at Skryabin Institute of Physiology and Biochemistry of Microorganisms, Pushchino, Russia. Kefir grains were fixed with 1.5% glutaraldehyde solution in 0.05 M cacodylate buffer (pH 7.2) at 4°C for 1 h; washed in the same buffer and additionally fixed with 1% OsO_4 solution in 0.05 M cacodylate buffer (pH 7.2). After dehydration

Table 1. Bacterial profile of kefir grains from various technogenic niches

Kefir grains		
KGS	KGG	KGM
Lactococcus lactis		
Leuconostoc mesenteroides		
Lactobacillus plantarum		
Lactobacillus sakei		
Lactobacillus sp.		
Lactobacillus delbrueckii		
Leuconostoc gelidum		
		Lactobacillus kefiri

in alcohols, the samples were covered with platinum–carbon mixture in a JEE-4X vacuum system (JEOL, Japan). The samples were studied under a JSM 6510LV electron microscope (JEOL, Japan) [18].

RESULTS AND DISCUSSION

When determining producers in kefir grain communities, we started from the postulate that, in the trophic chain of a microbial community, the producer is considered to be the most active microorganism under studied conditions, one which metabolizes the major food resource that enters the system [3–5].

Our previous studies [19] and reports of other authors [20] revealed lack of objectivity of the results obtained using pure culture isolation methods due to morphological uniformity, small colony size of lactic acid bacteria, and close symbiotic relations between yeasts and bacteria.

In the current work, we therefore applied a different technique. Colonies developing on solid media that exhibited even slight differences in morphology were isolated and their physiological properties were determined to characterize their functional activity.

Over 80 isolates were obtained in the course of comparison of microbial profiles of kefir grains used at three dairy factories during cultivation on native milk (lac+). Strains with the highest level of functional activity were selected, taking into account morphology of their cells and colonies. Obtained isolates were identified by 16S rRNA gene sequencing. All three kefir grain samples demonstrated almost identical structure of lactic acid bacteria community (Table 1).

We studied the ability of 33 isolates obtained from kefir grain KGS grown on native milk (lac+) to utilize various sources of carbon for lactic acid fermentation on synthetic medium. It was shown that 46% of isolates possessed β-galactosidase activity and performed lactic acid fermentation not only on lactose but also on glucose and galactose as well (Table 2). At the same time, 54% of isolates did not possess β-galactosidase activity but used glucose and galactose (36%) or only glucose (18%) for lactic acid fermentation. According to the obtained results, kefir grains consortia include lactic acid bacteria of various physiological groups, able to use both lactose and carbohydrates formed in the course of its hydrolysis for lactic acid fermentation. It should be noted that microorganisms of the first physiological group were not homogeneous in lactic acid fermentation activity. Only 15% of the isolates demonstrated a high level of lactic acid fermentation activity with titrated acidity of the medium up to 56°T on lactose. At the same time, other isolates of this physiological group demonstrated lower levels of lactic acid fermentation activity with titrated acidity about 25°T.

The theoretical possibility of glucose and galactose release into the medium at lactose metabolizing by lactic acid bacteria was shown by Molotov et al. [21] and confirmed in our experiments. It was demonstrated on bacterium *L. gelidum* isolated from kefir grain and possessing β-galactosidase activity during its cultivation on lactose-containing medium. As bacterial cells consumed lactose, the level of glucose and galactose in the medium increased (Fig. 1). The latter could be used as a substrate for lactic acid fermentation by microorganisms of the second physiological group.

Table 2. Capacity for lactic acid fermentation in bacterial components of kefir grains at cultivation on synthetic medium with various carbon sources

Total amount of isolates 33			Titrated acidity, °T			β-Galactosidase activity
			lactose	glucose	galactose	
Of them, %	46	15	48	57	43	+
		31	25	51	28	+
	54	36	18	47	27	–
		18	18	40	18	–

"+" and "–" designate the isolates possessing β-galactosidase activity, i.e. able to synthesize the enzyme β-galactosidase, and the isolates devoid of β-galactosidase activity, i.e. not able to synthesize the enzyme β-galactosidase, respectively.

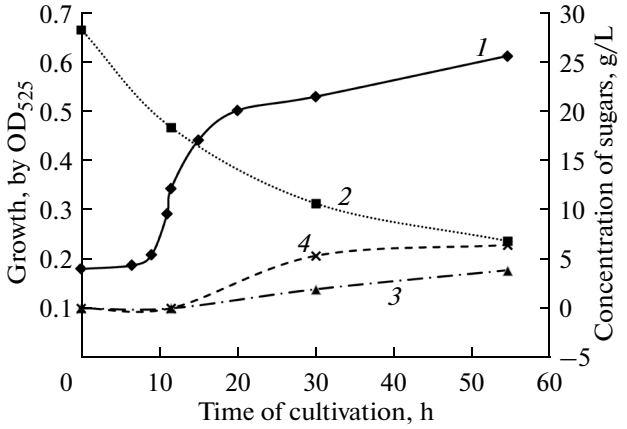

Fig. 1. Concentration of glucose and galactose in the medium at batch cultivation of *L. gelidum* using lactose as carbon source: culture growth, OD_{525} nm (*1*); concentration of lactose, g/L (*2*), concentration of glucose, g/L (*3*); and concentration of galactose, g/L (*4*).

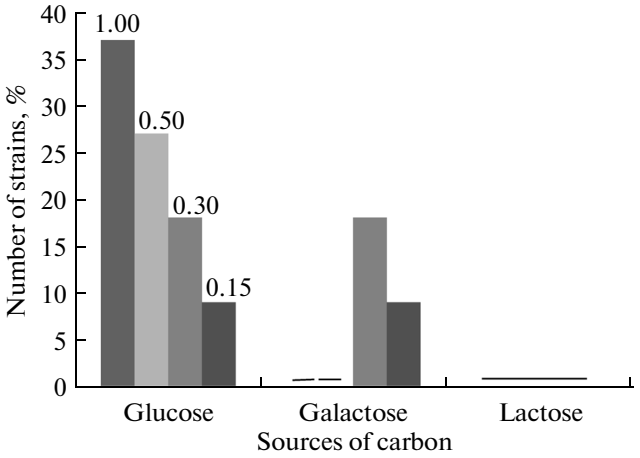

Fig. 2. Activity of alcoholic fermentation of yeasts on various carbohydrates (number of strains, %). The values 1, 0.5, 0.3, and 0.15 indicate activity of ethanol fermentation in relative units.

Investigation of 11 isolates obtained from all colonies morphologically typical for yeasts revealed that yeasts present in the microbial community did not possess β-galactosidase activity and utilized glucose and galactose, but not lactose, as substrates for alcoholic fermentation (Fig. 2).

Obtained results make it possible to conclude that microorganisms of the first physiological group, most active in utilizing lactose for lactic acid fermentation, can act as the major producers in kefir grain systems. Microorganisms of the second and third groups use the products of lactose metabolism and may be in relations of either passive antagonism or cooperation with each other.

Producer detection in the studied system was based on the elective culture approach suggested by Winogradsky, comparison of microbial profiles and functional activity of kefir grains cultured on native milk (lac+) and milk containing hydrolyzed lactose (lac–) but with the same total content of carbohydrates and constant composition of other organic components of the medium. It made possible to eliminate from the system of microorganisms that actively use lactose for lactic acid fermentation.

Cultivation of kefir grains on lactose-free milk (lac–) was carried out for more than four years. Their functional activity was preserved during the whole period: a fine, dense clot was formed on milk and pH decreased to 4.0 ± 0.3. No changes in functional activity were revealed when KG lac– kefir grains were transferred to native milk. Comparative analysis of leaven effect in native milk showed that the absence of lactose in milk did not influence the functional activity of KG lac– kefir grains, and such functional characteristics as titrated acidity, rate and features of clot formation, or the amount of produced lactic acid did not change either (Fig. 3). Obtained results demonstrate rapid adaptation to changed substrate of kefir grains KG lac–, when transferred to native milk, with induction of β-galactosidase synthesis in the cultures able to utilize lactose in lactic acid fermentation.

Comparative assessment of probiotic properties of kefir obtained using lac+ and lac– KG did not reveal differences in such parameters as sensitivity of microorganisms to bile, phenol, NaCl, alkaline environment, antibiotics, or to their antagonistic relations to pathogens [22].

IR spectra of exopolysaccharides isolated from kefir grains KG lac+ and KG lac– (Fig. 4) did not demonstrate any differences. Position and intensity of absorption bands in the 4000–650 cm^{-1} region proved the identity of the composition of these exopolysaccharides. During prolonged cultivation on milk in the absence of lactose, no significant changes appeared in the polysaccharide envelope that plays a structural role in kefir grains. Scanning electron microscopy did not reveal any morphological differences between studied kefir grains KG lac+ and KG lac– (Fig. 5).

To compare the microbial profiles of kefir grains cultured for a long time on lactose-free and native milk 80 and 73 isolates were obtained respectively. According to the functional activity studies of obtained isolates, comparable numbers of isolates with different functional activity were present in kefir grains (Table 3). Thus, 33 and 30% of isolates from KG lac– and KG lac+ kefir grains, respectively, demonstrated β-galactosidase activity and high levels of functional activity when developed on native milk, with titrated acidity as high as 100°T and pH values of 4.3–5.6. The second group of isolates (35 and 22% of the isolates) differed from the first group by their functional activity when developed on native milk; in this case titrated acidity reached 38°T and pH decreased slightly (pH values from 5.8 to 6.8). Isolates of the third group (32

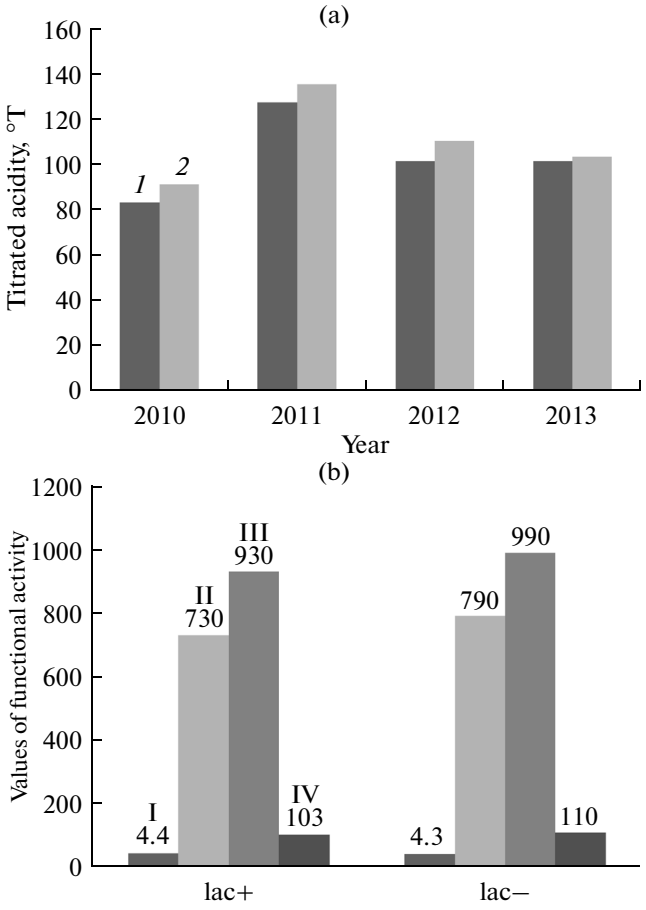

Fig. 3. Functional activity of kefir grains leaven: (a) cultured for a long time on lac+ (*1*) and lac– (*2*) milk, averages of milk titrated acidity by years; (b) average values of functional activity for 4 years of cultivation, where I is pH, II is the amount of fermented lactose, mg, III is the amount of lactic acid, mg, and IV is titrated acidity, °T.

and 48% of the isolates) were characterized by the absence of β-galactosidase activity and were functionally inactive.

The most functionally active strains isolated from each of the physiological groups were identified by sequencing of variable sites of their 16S rRNA genes, primal screening of GenBank and RDP-II databases, and phylogenetic comparison with homologous strains (Table 4). The cultures were identified as belonging to a certain species in the case of at least 97% homology. Classical microbiological methods including pure culture isolation did not reveal any significant differences in the microbial composition of kefir grains cultured on lactose-free milk for a long time compared to initial kefir grains cultured on native milk.

According to obtained results, in the microbial community of kefir grains cultured on lactose-free milk, the role of producers may be played either by lactic acid bacteria of the first physiological group (Table 2), active in metabolizing glucose in the absence of lactose, or by bacteria of the second group, active in utilizing glucose for lactic acid fermentation.

Taking into account the difficulties of studying microbial profiles of kefir grains using pure culture isolation-based methods, comparative study of kefir grains cultured for a long time on lactose-free and native milk was carried out using denaturing gradient gel electrophoresis (DGGE). This method is considered the most informative one for comparison of microbial communities because it gives an opportunity to study microbial profiles without pure culture isolation. Analysis of electrophoregrams of the amplicons demonstrated two kefir grain samples with the same band number, the thickness of bands, and the distance between them to be identical (Fig. 6).

Thus, our study revealed no differences in microbial profiles of kefir grains from different technological niches. It is confirmed by pure culture isolation methods with their subsequent identification by 16S rRNA analysis, as well as by results of DGGE without pure culture isolation. Lactic acid bacteria, components of kefir grains cultured on native milk, were shown to fall into two physiological groups: (1) possessing β-galactosidase activity, utilizing lactose in lactic acid fermentation; and (2) with no β-galactosidase activity, using

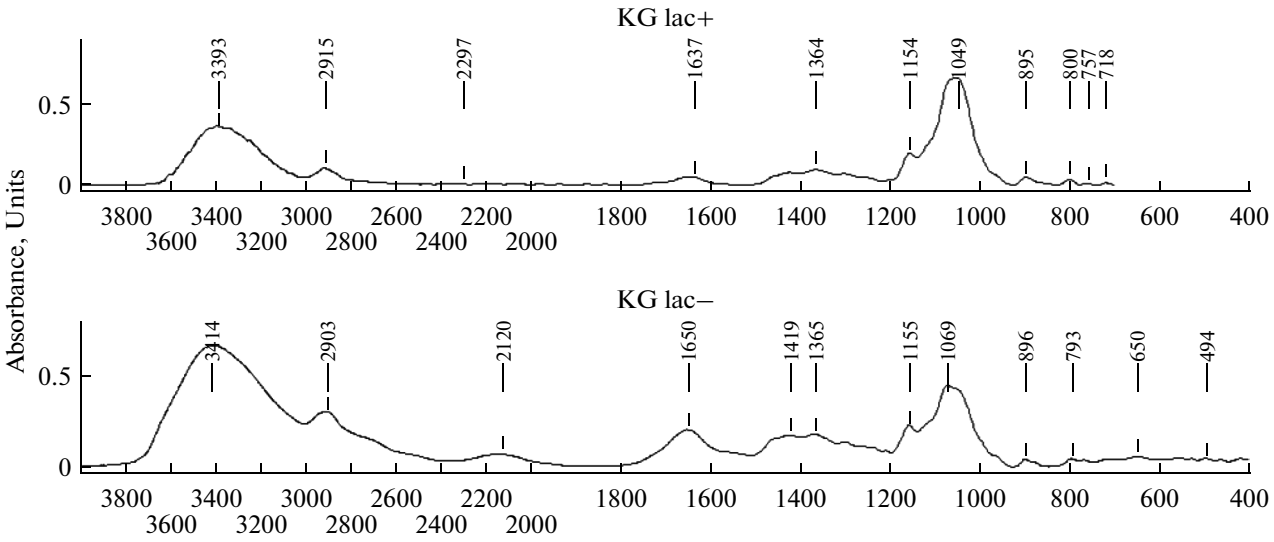

Fig. 4. IR spectra of exopolysaccharide samples isolated from kefir grains KG lac– and KG lac+.

glucose and galactose for lactic acid fermentation. Yeasts isolated from the studied kefir grains did not possess β-galactosidase activity and did not ferment lactose for alcoholic fermentation, but carried out alcoholic fermentation (at high rates on glucose and at low rates on galactose).

Results of our study make it possible to characterize the trophic chain and to propose a functional model of the kefir grain microbial community (Fig. 7).

Lactic acid bacteria of the first physiological group are producers in kefir grains microbial community, which possess β-galactosidase activity, actively use lactose for lactic acid fermentation, and rapidly acidify the system, e.g., members of the species *L. lactis*, which can grow at neutral pH as well as at low pH and titrated acidity level up to 200°T. In the case of further pH decrease, lactose can be utilized by members of genus *Lactobacillus*, which can develop in the more acidic medium at titrated acidity up to 300°T.

Glucose and galactose, released to the medium at lactose transportation into the cell and its metabolism [21], are utilized in lactic acid fermentation by bacteria of the second physiological group and in ethanol fermentation by yeasts. In kefir grains consortia, these microorganisms perform a regulatory function, removing glucose from the medium and thus eliminating its inhibiting effect on β-galactosidase synthesis.

No differences in microbial profile and functional activity of kefir grains were discovered in kefir grains cultured on native milk (lac+) and kefir grains cul-

Table 3. Functional activity of the isolates obtained from kefir grains KG lac– and KG lac+ cultured on native milk (lac+)

Kefir grains	KG lac–	KG lac+	Functional activity characteristics		
number of isolates	80	73	β-galactosidase activity	titrated acidity, °T	medium acidification, pH
Of these, %	33	30	+	40–100	4.3–5.6
	35	22	+	≤38	5.8–6.8
	32	48	–	18	6.9

"+" designates the isolates possessing β-galactosidase activity, i.e. able to synthesize the enzyme β-galactosidase; "–" designates the isolates devoid of β-galactosidase activity, i.e. unable to synthesize the enzyme β-galactosidase.

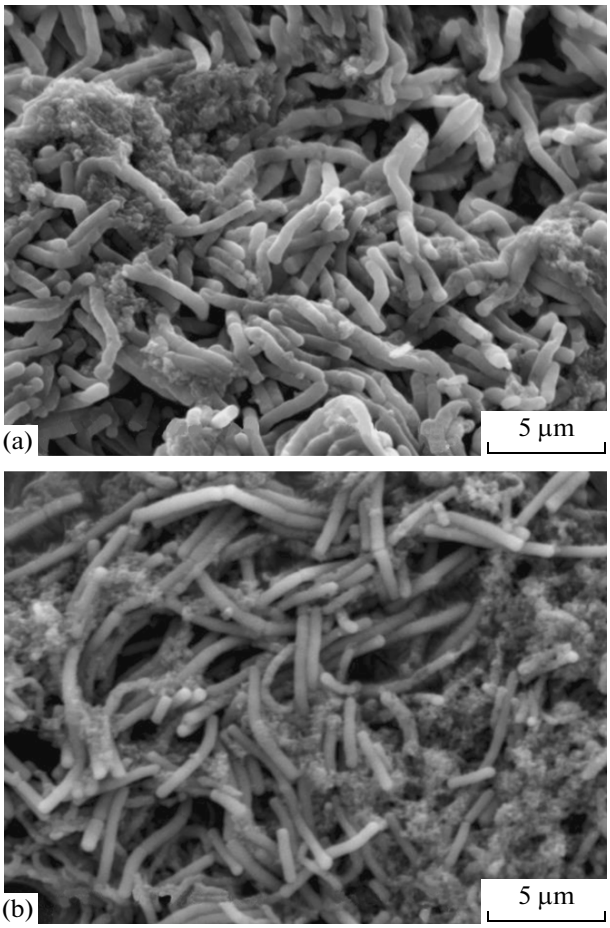

Fig. 5. Scanning electron microscopy of kefir grains KG lac+(a) and KG lac– surface (b).

tured on lactose-free milk for a long time, exceeding 4 years (lac–), nor on kefir grains subsequently transferred to native milk. This fact confirms the ability of components of this consortium to synthesize inducible enzyme β-galactosidase as well as the role of this enzyme in self-regulation of this community.

Table 4. Bacterial profile of kefir grains KG lac– and KG lac+

Basic phenotype parameters	KG lac+	KG lac–
Possess β-gal., 40–100°T, pH 4.3–5.6	*Lactococcus lactis* *Lactobacillus otakiensis* *Lactobacillus sankii*	*Lactococcus lactis* *Lactobacillus otakiensis*
Possess β-gal., ≤38°T, pH 5.8–6.8	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides*
Do not possess β-gal., 18°T, pH 6.9	*Acetobacter* sp. *Lactobacillus plantarum*	*Acetobacter* sp.

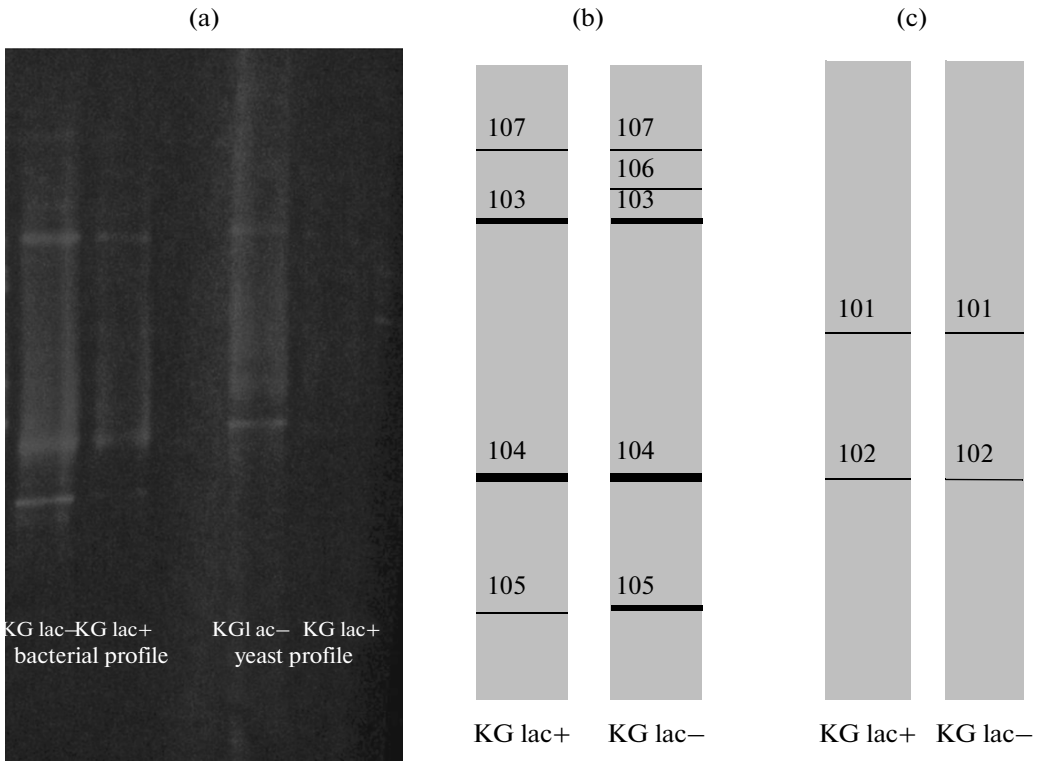

Fig. 6. DGGE of the microbial profile of kefir grains KG lac– and KG lac+ after 4 years of cultivation (research was carried out at the Departament of Microbiology, Moscow State University): DGGE electrophoregram (a); schematic representations of bacterial (b) and yeast (c) DGGE profiles.

Obtained results extend the general knowledge on possible trophic patterns of the studied microbial communities and can be a basis for the development of an algorithm for the experimental formation of a functionally stable kefir grain associative culture.

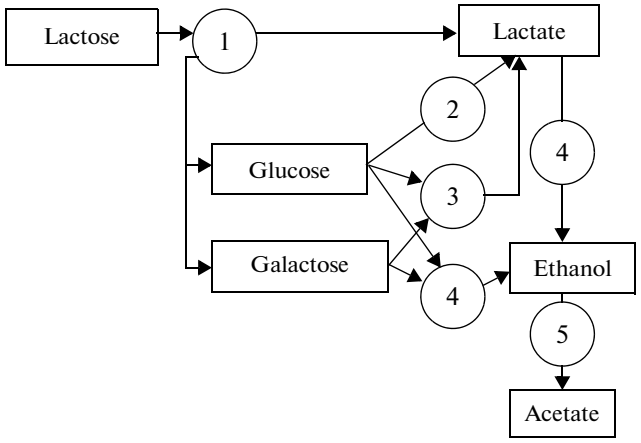

Fig. 7. The general scheme of the trophic chain of KG lac– and KG lac+ associative kefir grains cultures. Designations: 1, lactic acid bacteria synthesizing β-galactosidase; 2, lactic acid bacteria of group 1, in which β-galactosidase synthesis is repressed by glucose; 3, lactic acid bacteria not synthesizing β-galactosidase; 4, yeasts; 5, acetic acid bacteria.

ACKNOWLEDGMENTS

The work was financially supported by Ministry of Education and Science of the Russian Federation: identification number of the project UNU RFMEFI59214X0002 and the project 1/12628 “The study of the functioning and regulation of the composition and activity of practically valuable microbial communities”.

REFERENCES

1. Khmel, I.A., Quorum-sensing regulation of gene expression: fundamental and applied aspects and the role in bacterial communication, *Microbiology* (Moscow), 2006, vol. 75, no. 4, pp. 390–397.
2. Zhurina, M.V., Kostrikina, N.A., Strelkova, E.A., Plakunov, V.K., Parshina, E.Y., Yusipovich, A.I., and Maksimov, G.V., Visualization of the extracellular polymeric matrix of *Chromobacterium violaceum* biofilms by microscopic methods, *Microbiology* (Moscow), 2013, vol. 82, no. 4, pp. 517–524.
3. Svirizhev, Yu.A. and Logofet, D.O., *Ustoichivost' biologicheskikh soobshchestv* (Stability of Biological Communities), Moscow: Nauka, 1978.

4. Setrov, M.I., *Organizatsiya Biosistem* (Organization of Biological Systems), Leningrad: Nauka, 1971.
5. Zavarzin, G.A., *Lektsii po prirodovedcheskoi mikrobiologii* (Introduction to Ecological Microbiology), Moscow: Nauka, 2003.
6. Feofilova, E.P., Microflora of kefir grains, *Mikrobiologiya*, 1958, vol. 27, no. 2, pp. 229–234.
7. Fil'chakova, S.A., Microbiological composition of kefir grains and kefir starter, *Pererabotka Moloka*, 2005, no. 7, p. 28.
8. Lopitz-Otsoa, F., Rementeria, A., Elguezabal, N., and Garaizar, J., Kefir: a symbiotic yeasts–bacteria community with alleged healthy capabilities, *Rev. Iberoam Micol.*, 2006, vol. 23, pp. 67–74.
9. Ganina, V.I., Kalinina, L.V., and Bol'shakova, E.V., β-Galactosidase activity of lactic acid bacteria and bifidobacteria, *Molochnaya Promyshlennost'*, 2002, no. 8, pp. 36–37.
10. Omelyanskii, V.L., *Prakticheskoe rukovodstvo po mikrobiologii* (Practical Course in Microbiology), Moscow: Izd. AN SSSR, 1940.
11. Kreger-van Rij, N.J.W., *The Yeasts: A Taxonomic Study*, Amsterdam: Elsevier Sci., 1984.
12. Miller, Dzh., *Eksperimenty v molekulyarnoi genetike* (Experiments in Molecular Genetics), Moscow: Mir, 1967.
13. Kadyrova, R.G., *Tonkosloinaya khromatografiya. Identifikatsiya i razdelenie uglevodov, vitaminov i toksichnykh soedinenii* (Thin-Layer Chromatography. Identification and Separation of Carbohydrates, Vitamins, and Toxic Compounds), Kazan: Kaz. Gos. Energ. Univ., 2010.
14. Piermaria, J.A., de la Canal, M.L., and Abraham, A.G., Gelling properties of kefiran, a food-grade polysaccharide obtained from kefir grain, *Food Hydrocolloids*, 2008, vol. 22, pp. 1520–1527.
15. Eden, P.A., Schmidt, T.M., Blakemore, R.P., and Pace, N.R., Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction-amplified 16S rRNA-specific DNA, *Int J. Syst. Bacteriol.,* 1991, vol. 41, no. 2, pp. 324–325.
16. Ribosomal Database Project II. http://www.cme.msu.edu
17. Jianzhong, Z., Xiaoli, L., Hanhub, J., and Mingsheng, D., Analysis of the microflora in Tibetan kefir grains using denaturing gradient gel electrophoresis, *Food Microbiol.*, 2009, vol. 26, pp. 770–775.
18. Abramov, V., Khlebnikov, V., Kosarev, I., Bairamova, G., Vasilenko, R., Suzina, N., Machulin, A., Sakulin, V., Kulikova, N., Vasilenko, N., Karlyshev, A., Uversky, V., Chikindas, M., and Melnikov, V., Probiotic properties of *Lactobacillus crispatus* 2,029: homeostatic interaction with cervicovaginal epithelial cells and antagonistic activity to genitourinary pathogens, *Probiot. Antimicrob. Proteins.*, 2014, vol. 6, pp. 165–176.
19. Gradova, N.B. and Sarantseva, A.A., Investigation of the microbial profile of kefir, structures associative microbial culture, *Izv. Samar. Nauch. Center, Russ. Acad. Sci.*, 2012, vol. 14, no. 5, pp. 704–710.
20. Elinov, N.P. and Larina, O.G., Microbiota of the "Tibetan rice" natural association, *Probl. Med. Mikol.*, 1999, vol. 1, no. 1, pp. 51–56.
21. Molotov, S.V., Alkhimova, R.A., Pimenova, N.V., and Sukhodolets, V.V., Isolation and properties of lactic streptococci mutants defective for the ability to utilize glucose, *Russ. J. Biotechnol.*, 1994, nos. 11–12, pp. 9–12.
22. Sarantseva, A.A., Gradova, N.B., and Machulin, A.V., Investigation of trophic patterns of the functioning of kefir fungi, an associated microbial culture, *Izv. Gorsk. Gos. Agr. Univ.*, 2013, vol. 50, no. 4, pp. 265–272.

Translated by E. Botchkova