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SHORT COMMUNICATIONS

Analysis of Kefir Grains from Different Regions of the Planet Using High-Throughput Sequencing

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Abstract—The taxonomic composition and spatial localization of yeast and bacteria in kefir grains (KG) obtained for study from different regions of the planet were investigated. The diversity of their microbiome has been demonstrated by high-throughput sequencing of bacterial 16S rRNA genes and the ITS1 region of the 18S-ITS1-5.8S-ITS2-28S complex of yeast rRNA. It has been established that the main representatives of the complex community of KG from different regions are lactic acid bacteria (LAB; lactobacilli, lactococci, and *Leuconostoc* spp. in different ratios) and different types of yeast of the genus *Kazachstania* (family *Saccharomycetaceae*). Acetic acid bacteria and a small percentage of yeast *Kluyveromyces marxianus* were detected in the KG from Tibet, and yeast *Pichia kluyveri* was detected in the KG from Ossetia.

Keywords: yeasts, kefir grains, lactobacilli, microbiome, high-performance sequencing
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INTRODUCTION

In recent years, much attention has been paid to the creation and introduction of useful products with probiotic microorganisms into the human diet for the organization of functional nutrition. One of the most popular dairy products, with undeniable benefits from consumption, is kefir, which can be prepared from various types of milk, such as cow, goat, buffalo, sheep, or camel, by microbial fermentation (milk fermentation with kefir grains, KG) [1, 2]. Kefir is a traditional drink considered a "functional food" due to its nutritional and health benefits. It is produced by the fermentation of milk lactose by microbial species present in KG [1]. Kefir grains contain probiotic microorganisms that exist in a complex matrix of proteins and polysaccharides [3, 4]. There is a symbiotic relationship between the microorganisms present in KG, in which bacteria and yeast survive and share their bioproducts as energy sources, and microbial growth factors. This consortium of microorganisms is responsible for homo- and heterofermentative lactic acid fermentation and alcoholic fermentation [5, 6]. Lactic acid bacteria (LAB) are the main population in KG with a small content of yeast immersed in polysaccharide layers of kefiran, which is a heteropolysaccharide consisting of equal proportions of glucose and galactose, formed by *Lactobacillus kefiranofaciens* [4, 7]. More than 23 different types of yeast have been isolated from KG and from drinks of various origins obtained with their help, while different types of yeast, being probiotic cultures, were the predominant species [6, 8, 9]. Fermentation of milk by KG produces many functional compounds, such as bioactive peptides (e.g., with antihypertensive, antioxidant, antiallergic, antitumor, anti-inflammatory, and cholesterol-lowering activities) [10], antimicrobial compounds (e.g., organic acids, alcohols, carbon dioxide, and bacteriocins) and heteropolysaccharides (e.g., kefiran) with potential probiotic activity [11]. Studies show that probiotic bacteria are more abundant in the guts of regular kefir consumers, which correlates with improved health [1–3, 12]. In this regard, there is a growing interest in the use of kefir as an important component of functional nutrition.

Owing to the continuous development of modern molecular technologies, such as high-throughput sequencing, a deeper analysis of the complex microbial community of KG is becoming possible. Many laboratories around the world are constantly conducting research to further study of the properties of KG and kefiran to develop new important functional products, dietary supplements, and medicines. It is necessary to create stable probiotic microbial communities from the isolated and studied cultures of KG and

Table 1. KG samples taken for research

Region of KG sample	Culture cipher
Moscow	N5
North Ossetia	OS
Tibet	T2-3

to identify and eliminate possible antagonism between the selected cultures.

The aim of this work was to study the microbiome of KG isolated from different territorial zones in order to create sustainable microbial communities and effective probiotic products that are beneficial to health.

MATERIALS AND METHODS

For research purposes, KG were obtained from private farms in different regions of its traditional use, including the Caucasus (North Ossetia), the Tibet region of China, and Moscow oblast (Table 1). All grains were stored in a freeze-dried state in the collection of cultures of the Department of Microbiology, Faculty of Biology, Moscow State University.

Growing of KG. Freeze-dried KG were activated in sterile 1.5% milk with regular transfers (two times a week) at room temperature (21–22°C). During reseeding, KG were washed on sterile sieves with sterile saline to remove accumulated polysaccharides. The grains in milk were stored at 4°C.

Microscopy of KG. To establish the spatial arrangement of microorganisms in the KG, scanning electron microscopy of grain preparations isolated from different zones, closer to the surface and in the center, was performed [6]. KG particles washed with sterile water were fixed for 30 min in 2.5% glutaraldehyde in phosphate buffer (1.8 mM KH_2PO_4, 10 mM $Na_2HPO_4 \cdot 12H_2O$, 137 mM NaCl, 2.7 mM KCl) and then dehydrated in a series of alcohols of increasing concentration (30, 50, 70, 80, 90, 100%) and then in mixtures of absolute alcohol and acetone (3 : 1, 1 : 3, 1 : 3). The samples were then placed in absolute acetone and the hydrated material was dried by the "critical point" method (CCP) in an HCP-2 Critical Point Dryer (Hitachi, Japan). Dried preparations were attached to special tables with double-sided adhesive tape and sprayed with an Au–Pd mixture in an ion-spray setup (Eiko IB-3 Ion Coater, Hitachi, Japan).

The samples were studied using a JSM-6380LA scanning electron microscope (JEOL, Japan) at the Center for the Collective Use Electron Microscopy in Life Sciences of Moscow State University (a unique scientific installation "Three-Dimensional Electron Microscopy and Spectroscopy"), and using an SU-8010 scanning electron microscope (Hitachi, Japan) at Shenzhen MSU-BIT University.

High-throughput sequencing of the KG microbiome. DNA was isolated from the samples using the Fast DNA Spin Kit for Soil (MP Biomedicals, United States) according to the manufacturer's instructions. Amplicon collections of 16S rRNA gene fragments were obtained by polymerase chain reaction with universal primers to the V4 region according to the previously described method [13]. The following primers were used: 515F: 5' GTGBCAGCMGCCGCGGTAA-3' [14]; Pro-mod-805R: 5'-GACTACNVGGGTMTCTAATCC-3' [15]. Sequencing was performed on a MiSeq System (Illumina, United States) using a MiSeq Reagent Micro Kit v2 (MS-103-1002, Illumina, United States) that read 150 nucleotides from each end. Demultiplexing, subsequent processing, and analysis of the obtained sequences were carried out using the corresponding scripts QIIME 2 software ver. 2019.1 [16]. The table of operational taxonomic units (OTU) was compiled in the SILVAngs program (https://ngs.arb-silva.de/silvangs/). The ITS1 region of the 18S-ITS1-5.8S-ITS2-28S rRNA complex was amplified from KG DNA using the BITS (5'-ACCTGCGGARGGATCA-3') and B58S3 (5'-GAGATCCRTTGYTRAAAGTT-3') polymerase chain reaction primer set for the region ITS1 [17]. After amplification, the obtained regions were purified with AMPure XP magnetic beads (Beckman Coulter, Inc., United States) and prepared for sequencing using the Nextera XT DNA kit according to the manufacturer's instructions (Illumina, Inc., United States). Sequences were collected, filtered, and dereplicated using the UPARSE system [18]. OTUs were pooled into groups with ≥97% sequence identity, from which chimeras were removed using the SILVAngs pipeline (https://ngs.arb-silva.de/silvangs/) for the V4 region of the 16S rRNA gene and knomics/biota pipeline (https://biota.knomics.ru) for the ITS1 region of yeast DNA.

Taxonomic identity was assigned using BLASTn and the Fittings v. 1-2 [19]. The taxonomy and operational taxonomic units were converted into a table using the BIOM file format version V1.3.1 [20].

Statistical processing of results. All experiments were carried out in three independent biological replicates. Statistical data processing was performed using Excel (Microsoft Office, United States). The significance of differences was assessed by Student's *t*-test.

RESULTS AND DISCUSSION

KG can be described as gelatinous white or slightly yellowish masses of elastic consistency, ranging in size from 0.3 to 3.5 cm in diameter, similar to small heads (heads) of cauliflower immersed in a matrix of exopolysaccharides (Figs. 1a, 1b).

Spatial localization of yeast and bacteria in KG. The studied KG demonstrated a close spatial relationship between bacteria and yeasts on the grain surface

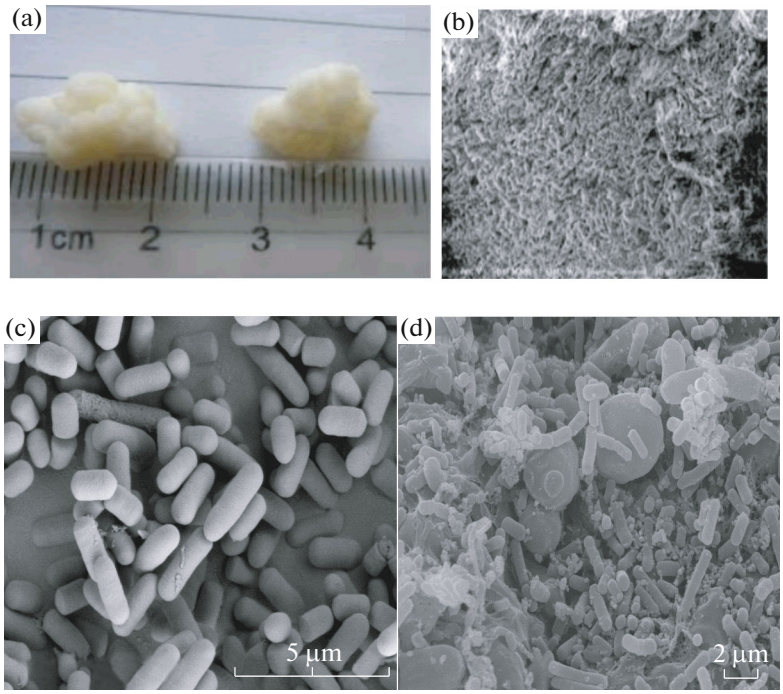

Fig. 1. Micrographs of CG from Tibet (T2-3). (a) Appearance, (b) surface of kefir grain in a scanning electron microscope (Hitachi SU-8010; magnification ×5000), (c) inner part of kefir grain (magnification ×40000), (d) outer surface of kefir grain (magnification ×40000).

(Fig. 1d). Almost complete absence of yeast cells was found in the internal parts of the KG (Fig. 1c); only LAB cells (1–4 μm in length) are visible. Large (7–8 μm) yeast cells with scars from detached buds (Fig. 1d) and closely adjoining LAB cells (1–3 μm in length) are visible on the outer side of the KG.

This reflects the metabolic needs of bacteria and yeast and their relationship to molecular oxygen. Thus, yeasts, being aerobic organisms, adapt their location in the grain closer to the surface (Fig. 1d), while LAB, being aerotolerant anaerobes, are located both on the surface, closely adjacent to yeast cells, and occupy the entire internal volume of the grain. Such a close adherence of bacteria to yeast cells suggests the chemical bonds of these microorganisms and the obligate dependence of LAB on yeast, which supply bacterial cells with biologically active substances, growth components, and vitamins. The close symbiotic relationship between lactic acid bacteria and yeast, which stimulate the growth of LAB, has been noted by many researchers [3–6, 21–24].

Yeast cells, especially those that are not able to metabolize milk lactose, are obligately dependent on LAB, which actively break down milk lactose into glucose and galactose. The complex interactions between yeast and bacteria in KG are not fully understood. However, when the bacteria are separated from the grain, the yeast will not grow so efficiently [2].

Determining the generic composition of bacterial cultures in KG. Research started in the 1980s showed that phylogenetic comparison of microbial species using a conserved region of the genome is more accurate and stable in identifying their similarities or differences. Thus, comparing sequences of genes encoding 16S rRNA has become part of everyday practice in establishing phylogenetic relationships. The 16S rRNA gene consists of many variable and conserved regions; therefore, for the purpose of taxonomic comparison, universal primers were developed for the entire 16S rRNA gene, which includes both conserved and variable regions, and hypervariable regions with the corresponding primers are more often used for taxonomic comparison. High-throughput sequencing of variable regions of genes encoding the synthesis of bacterial 16S rRNA in the microbiome of grains was carried out in order to identify the generic bacterial diversity of samples from Ossetia (OS), sample N5 from Moscow, and T2-3 from Tibet.

When comparing KG from different geographically located zones, it was found that the main bacterial representatives of their composition are *Lactobacillus* and *Lactococcus* (Fig. 2). Based on the results of sequencing, it can be seen that lactobacilli and lactococci are present in our studied KG in almost equal proportions in the sample from Moscow oblast (40 and 41%), with a slight predominance of lactobacilli in the samples from Ossetia (50 to 34%), and more lactococci (46%) than lactobacilli (32%) were detected in KG from Tibet. In all samples, heteroenzymatic LAB *Leuconostoc* spp. were found in small amounts: 11% in the

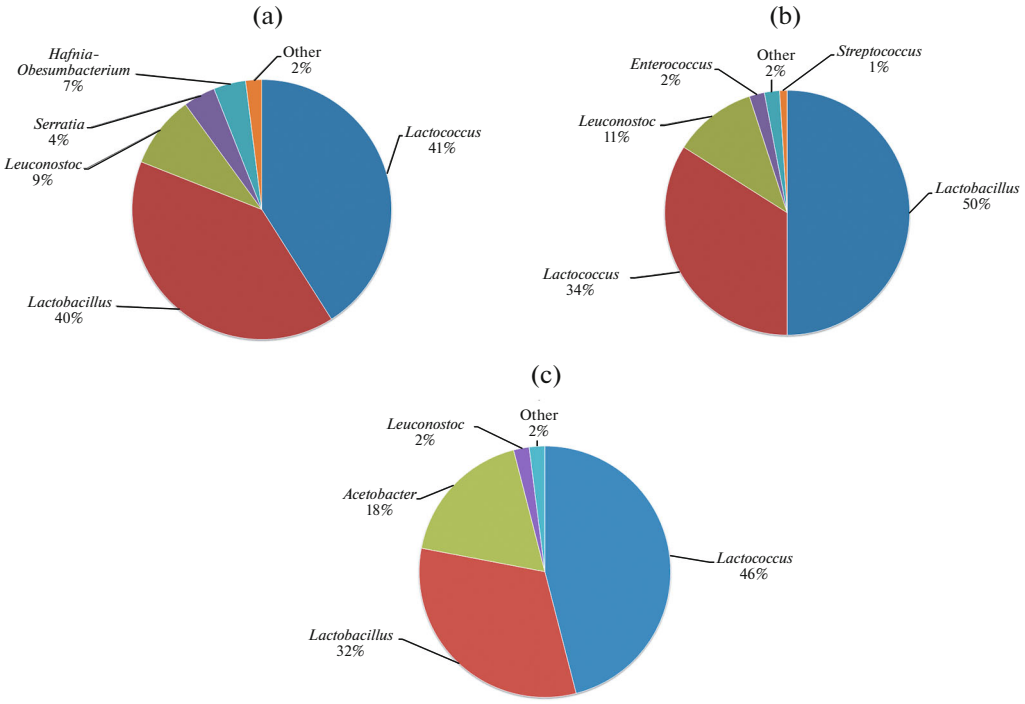

Fig. 2. Bacterial composition of the KG microbiota from different regions according to the results of high throughput sequencing of 16S rRNA genes. Samples: (a) from Moscow (N5), (b) from Ossetia (OS), (c) from Tibet (T2-3).

Ossetian sample, 9% in the sample from Moscow, and only 2% in the Tibetan sample. Leukostoks determine the aroma of kefir, since, being heterofermentative LAB, they form lactic and acetic acid, carbon dioxide, ethyl alcohol, esters, aromatic substances acetoin and diacetyl, and dextran, which promotes the formation of the KG matrix [21, 22].

In the microbiome of KG, other bacteria are also present in small amounts. Representatives of the genus *Acetobacter* (18%) were found in a sample from Tibet, which is consistent with the experimental data of Chinese scientists [21]. Bacteria from the *Acetobacteriaceae* family of the alpha-proteobacteria class oxidize ethanol to acetic acid, while acetate and lactate to CO_2 and H_2O, and create a matrix of bacterial cellulose in KG.

The eukaryotic ribosomal cluster has two internal transcribed spacers, ITS1 and ITS2, which separate the 18S, 5.8S, and 28S RNA genes in ribosomal DNA. These sequences are a combination of nuclear genes arranged in tandem repeats and the ITS1 and ITS2 sequences contain sections of ribosomal genes.

As can be seen from Fig. 3, the microbiota of all KG samples contains the yeast *Kazachstania turicensis*, regardless of the region of their isolation. This is a former species of *Saccharomyces* sp., often isolated from different CGs and mentioned in the works of Chinese scientists [21, 22]. But the yeast *Kluyveromyces marxianus* was also found in KG samples from Tibet and Moscow and was also detected in kefir from Argentina. These yeasts make up the majority or all of the lactose-using yeast population and have probiotic properties [8]. A low percentage (0.12%) presence of yeasts of the *Saccharomycetes* class, *Pichia kluyveri* was found in samples from Ossetia. These yeasts belong to the phylum *Ascomycota*, reproduce by budding, are characterized by rapid growth (they reach maturity in 72 h), and are often found in KG from China or Spain [21, 22, 24]. The coexistence of different types of yeast and bacteria indicates their symbiotic, mutually beneficial relationship.

The conducted studies have shown a certain phylogenetic diversity of microorganisms that make up the KG microbiome, selected for research from different regions of their historical origin. Using the method of high-throughput sequencing of the KG microbiome, it was shown that the main representatives of the complex KG community are LAB (lactobacilli and lactococci in different ratios, as well as *Leuconostoc* spp.), and different types of yeast of the genera *Kazachstania* and *Klyveromyces* in KG from different regions. Differences in the composition of their microbiomes were also established, with LAB of the *Lactobacillus* and *Lactococcus* genera accounting for approximately 80% of all readings with bacterial primers, and LAB of the *Leuconostoc* genus from 2 to 11%, which should significantly affect the taste and texture of the final drink. The significant amount of bacteria of the *Acetobacteriaceae* family found in KG from Tibet (18% of all bacterial readings) indicates the inevitable appearance of

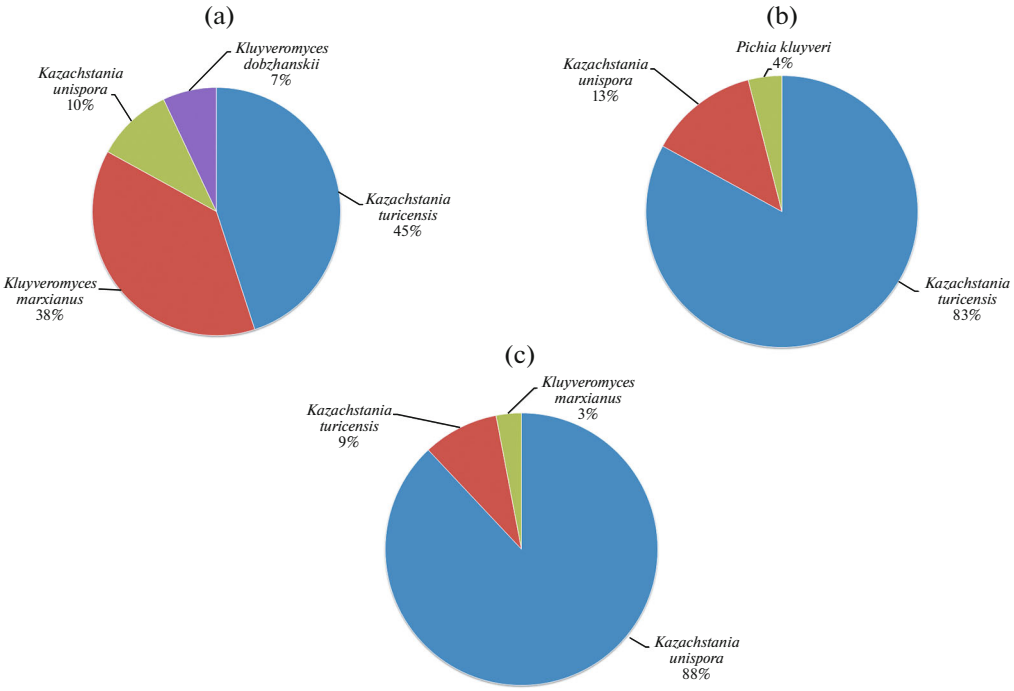

Fig. 3. Diversity of yeasts in KG from different regions according to the results of high-throughput sequencing. Samples: (a) from Moscow (N5), (b) from Ossetia (OS), (c) from Tibet (T2-3).

a vinegar taste and smell in the final product, which in this form may not be liked by the consumer, especially in childhood.

With regard to yeast, the differences are even clearer: for example, in yeast from Tibet and Ossetia, almost complete dominance of representatives of the genus *Kazachstania* was revealed (96 and 94% of all readings, respectively), but the species ratio is exactly the opposite. *K. turicensis* dominates (81% of reads) in KG from Ossetia, while *K. unispora* dominates in Tibetan KG (87% of reads), which clearly indicates the diversity of KG from different regions and the possible influence of yeast and bacterial diversity on the quality of kefirs produced from them. The influence of the established microbial diversity of KG on the consumer properties of kefir drinks has yet to be studied with the involvement of specialists—nutritionists and tasters. The spatial localization of yeasts and bacteria in KG confirms their different relationship to molecular oxygen. At the same time, yeasts tend to the outer layers of the KG, while LAB try to occupy their inner regions, where the oxygen concentration is significantly reduced due to the high respiratory activity of surface yeasts.

The experimental data obtained after the isolation of pure cultures of yeast and bacteria from KG can be used to create sustainable probiotic microbial communities and functional food products needed during the coronavirus pandemic.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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