Changes in Microbiota During Multiple Fermentation of Kefir in Different Sugar Solutions Revealed by High-Throughput Sequencing

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Abstract

Kefir is a fermented beverage produced through the activity of its grains, which is constituted by lactic acid and acetic acid bacteria and yeasts. We studied the bacterial succession during multiple fermentation of Argentinian kefir in brown sugar, purified molasses or high-test molasses, using 16S high-throughput sequencing. *Firmicutes* was dominant (up to 98% of total population) in grains and beverages made from various sugar substrates, except in high-test molasses beverage, which was dominated by *Proteobacteria* (up to 78% of total population). Major bacterial species in *Firmicutes* were *Liquorilactobacillus nagelii*, *Lentilactobacillus hilgardii/diolivorans* and *Lacticaseibacillus casei/paracasei*, which are active in lactic acid fermentation. *Proteobacteria* comprised *Acetobacter lovaniensis* and *Gluconobacter oxydans/roseus* as major species, which are presumably responsible for the acetic acid formation in sugary kefir beverages. Bacteria differ in abundance depending on the sugar type, as revealed by the competitive dominances between *L. nagelii* and *A. loveniensis*. Purified molasses led to scarce acetic acid bacteria during fermentation, indicating that it is not a suitable substrate for their growth. Our results suggest that acetic acid (and/or ethanol) in sugary kefir modulates the succession and dominance of specific lactic acid bacteria. This study will provide valuable information for designing more sophisticated non-dairy fermented beverages with stable microbial properties.

Introduction

Kefir is a beverage made from fermentation of milk or sugar solutions with kefir grains. Kefir grains are a consortium of lactic and acetic acid bacteria and yeasts. Kefir beverage has multiple health benefits, including antitumor, antibacterial, antioxidant, and cholesterol-reducing properties [1–3].

There is stark contrast between our current knowledge of milk kefir and sugary kefir, in that the former is better known, better studied, and consumed more often [4]. Therefore, it is necessary to fully understand the microbial profile of sugary kefir to achieve a successful product with the best properties. Kefir has been studied for decades using various techniques. In the past, kefir and its microorganisms were studied using culture-dependent methods [5–7]. Classic

microbiological studies suggest that bacteria constitute up to 90% of the microbial population in the grains [7]. Lactic acid bacteria (LAB) such as *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc* and acetic acid bacteria (AAB) such as *Acetobacter* have been described as major species [5, 6]. Although these early studies provided substantial amount of information, their methods may be limited by low sequence coverage in the data. In recent years, culture-independent methods have been increasingly used to provide more accurate and in-depth analysis while avoiding the limitations mentioned above. High-throughput DNA sequencing has led to a great advancement in research on microbial ecosystems such as those in fermented food [8, 9]. Indeed, this technique has been used to study the microbial population of kefir [10, 11]. However, previous studies only focused on the microbial variety between grains with different origins, or between grains and their final products [10–13]. None of them investigated the bacterial succession during multiple cycles of fermentation. In our previous study, we conducted the culture-dependent analysis on bacteria inhabiting sugary kefir, and demonstrated that diverse bacterial succession might be occurring in three different sugar substrates (brown sugar, purified molasses, and high-test molasses) during

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seven cycles of repetitive inoculation [1]. We detected various LAB species including *Liquorilactobacillus nagelii* (formerly *Lactobacillus nagelii*), *Lentilactobacillus hilgardii* (formerly *Lactobacillus hilgardii*), *Lentilactobacillus farraginis* (formerly *Lactobacillus farraginis*), *Liquorilactobacillus satsumensis* (formerly *Lactobacillus satsumensis*), *Schleiferilactobacillus harbinensis* (formerly *Lactobacillus harbinensis*), *Lentilactobacillus diolovorans* (formerly *Lactobacillus diolivorans*) and *Lacticaseibacillus casei/paracasei* (formerly *Lactobacillus casei/paracasei*), and several AAB species including *Acetobacter tropicalis*, *Acetobacter indonesiensis*, *Acetobacter lovaniensis*, and *Gluconobacter oxydans* as major isolates.

In the present study, using the same three above-described sugar substrates, we analyzed the bacterial microbiota succession during multiple fermentation of sugary kefir using high-throughput 16S ribosomal RNA gene (16S rDNA) sequencing. The microbial composition of kefir varied depending on the three-sugar medium, consistent with our previous result obtained through culture-dependent approach [1]. An interesting trade-off manner of the bacterial composition between specific lactic- and acetic acid bacteria species was observed depending on the sugar substrates, which was not found in our previous culture-dependent approach. Kefir beverage produced from sugar solutions could be beneficial for certain consumers such as vegans, lactose intolerant people, and people who are allergic to dairy products. In our previous study, we found that kefir fermentation improved functional properties of brown sugar-based (BS) kefir, purified molasses-based (PM) kefir and high-test molasses-based (HM) kefir [1]. The detailed bacterial microbiota characterized in this study will provide useful information for improving the quality and probiotic benefits of sugary kefir beverages.

Materials and Methods

Samples and Inoculation of Kefir Grains

Kefir grains and fermentation conditions used in this study were described previously [1]. Briefly, grains stored at −80 °C were activated at 25 °C for 24 h (10 g grains with 100 mL sugar solution). Three sugar solutions were used as growth media: 5% (w/v) brown sugar (BS, Kanbayashi Co., Ltd., Osaka, Japan); 6.5% (w/v) purified molasses (PM, Dai-Nippon Meiji Sugar Co., Ltd., Tokyo, Japan); and 6.5% (w/v) high-test molasses (HM, Dai-Nippon Meiji Sugar Co., Ltd.). PM and HM are by-products from manufacturing of white sugar and raw sugar, respectively. These three solutions have a similar sugar concentration (4.7–4.9°Brix). The media were replaced by fresh solutions three to four times a week to maintain the microbial activity of the grains.

Activated grains were used for fermentation. The grains were incubated at 25 °C for 24 h in respective sugar solutions for seven consecutive fermentations (multiple cycles of fermentation). Each sample was named as sugar solution plus fermentation day (e.g. first day of fermented BS [BSD1] to seventh day of fermented BS [BSD7] and BS kefir grains [BSG]; PM beverages from PMD1 to PMD7 and grains PMG; HM beverages from HMD1 to HMD7 and grains HMG). After final fermentation, the grains were separated from beverage by filtration with a sieve of 1-mm² mesh size. Part of beverages at 1st, 4th and 7th cycles of fermentations and their grains were used for DNA extraction.

Metagenomic DNA Extraction

Two-hundred milliliters of each sugar solution was inoculated with 20 g of kefir grains and incubated at 25 °C for 24 h, the most common time point at which kefir is prepared [14]. To extract DNA from the kefir, 0.5 mL of fermented products were added into tubes from the EZ-extract for DNA kit (AMR Co., Ltd., Tokyo, Japan) containing sterilized glass beads. Samples mixed with beads were shaken by vortex for 10 min. Samples were incubated for 3 min at room temperature and shaken again for 10 min. Aliquots of 175 µL were transferred to new tubes. From here, DNA was extracted using NucleoSpin® tissue kit (Macherey–Nagel GmbH & Co. KG, Düren, Germany) according to manufacturer's instructions. Briefly, 200 µL of lysis buffer was added to samples and incubated at 70 °C for 10 min. Next, 210 µL absolute ethanol was added to each sample. Samples were poured into spin column and centrifuged. Columns were washed twice with wash buffer, and DNA was eluted with elution buffer pre-heated at 70 °C. After elution, pure DNA samples were obtained. Kefir grains were disrupted in a mortar and resuspended in sterilized 0.8% (w/v) NaCl. Samples of 0.5 mL were used for DNA extraction as described above. The quality of extracted DNA was evaluated by 1.0% (w/v) agarose electrophoresis.

DNA Amplification and Sequencing

Metagenomic DNA was used as template for PCR amplification with KOD FX DNA polymerase (Toyobo, Tokyo, Japan). The V3 and V4 variable regions of the bacterial 16S rDNA were amplified by PCR. The primers for 1st PCR amplification were designed according to Illumina library construction protocol, 341F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG<u>CCTACGGGNGGCMGCAG</u>-3′, forward) and 806R (5′- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG<u>GACTACHVGGGTATCTAATCC</u>-3′, reverse). Each primer contains the specific adaptor sequence for Illumina MiSeq apparatus on 5′-ends, and the sequences annealing to 16S rDNA were underlined. The

conditions used for PCR amplification were 94 °C denaturation for 2 min, 35 cycles of 98 °C for 10 s (denaturation), 50 °C for 30 s (annealing) and 68 °C for 60 s (extension). All DNA was subject to a hot start at 94 °C prior to PCR amplification. Amplicons generated were purified using the Agencourt AMPure XP purification system (Beckman Coulter Genomics, Indianapolis, USA). Second PCR was performed using the primers in Nextera XT Index Kit v2 (Illumina®, San Diego, USA). These primers contain the 8-bp multiplexing indexes, in addition to the Illumina MiSeq adaptor sequences, to facilitate the pooling and subsequent differentiation of samples. Purified PCR products were quantified using the Nanodrop 3300 Fluorospectrometer (Thermo Scientific, MA, USA). Equal amount of 16S rDNA V3-V4 amplicons from each sample were pooled and sequenced on an Illumina MiSeq (Illumina®) according to Illumina protocols.

16S rDNA-Based Taxonomic Analysis

Data obtained from the MiSeq analysis were processed and analyzed using QIIME pipeline software (version 1.8.0) [15]. The non-chimeric reads were clustered into operational taxonomic units at a 97% cutoff threshold. The taxonomic identification at the genus level was performed by the Ribosomal Database Project (RDP) classifier program (version 2.2) [16] equipped with the Greengenes 16S rDNA database (version gg_13_8) [17]. In order to identify the closest species for representative sequences, we used the Basic Local Alignment Search Tool (BLAST) (version 2.2.20). Diversity (Shannon) and richness (Chao1) indices were calculated by sampling the obtained reads up to 53,580 sequences.

Accession Number(s)

The sequence data obtained from the MiSeq analyses were deposited under the DDBJ DRA database with the accession number DRA008253.

Results and Discussion

Richness and Diversity Observed for Bacterial Microbiota of Sugary Kefirs

In this study, a total of 631,104 reads were obtained, after chimeric reads and reads shorter than 250 bp were filtered out. Rarefaction curves showed that enough sequencing reads have been obtained for downstream analysis (Fig. S1). Chao1 and Shannon indices were calculated to evaluate the bacterial richness and diversity, respectively, in sugary kefir and their grains (Fig. 1). Chao 1 richness was similar in BS (25.5, an average of 1st, 4th, 7th cycles of fermentation) and

PM kefirs (25.2), whereas it was higher in HM kefir (36.07) (Fig. 1a), and relatively higher value was observed from the early fermentation stage of HM kefir beverage (HMD1). Shannon index showed that the diversity of middle-fermentation stage of BS kefir beverage (BSD4) was relatively high (Fig. 1b). Species richness and diversity of grains and corresponding kefir beverages changed during the multiple cycles of fermentation. The richness and diversity indices of grains were either higher or lower than those of respective beverages. Marsh et al. [10] previously reported slightly different results, in that almost all four grains had higher richness and lower diversity than their respective sugary kefir.

Succession of Acetic Acid Bacteria in Sugary Kefir

Firmicutes, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* were detected in kefir beverages and grains, with *Firmicutes* (21–99%) and *Proteobacteria* (0–78%) being at relatively higher proportions (Fig. 2). Difference at the phylum level was observed for PM kefir beverages (PMD), since this sugar substrate does not maintain *Proteobacteria* during the multiple cycles of fermentation, despite similar Brix degree (4.7–4.9°) in all three sugar solutions. The majority of *Proteobacteria* comprised AAB (*Acetobacter* and *Gluconobacter*) as shown in Fig. 3. Therefore, the PM is a poor substrate for acetic acid fermentation. Contents of fat, nitrogen and carbohydrates in PM and HM are similar (data not shown). However, some minor essential components in PM might not be sufficient, compared to HM, as nutritionally poorer white sugar is used as starting material for PM. By contrast, HM was evidently suitable for growth of *Proteobacteria*, which drastically increased from 22% (HMD1) to 78% (HMD7) of total bacteria population during seven cycles of fermentations. *Acetobacter lovaniensis* constituted the majority of bacterial inhabitants (62% for HMD4 and 75% for HMD7, Fig. 3), indicating that this species contributes to majority of acetic acid production in this sugar substrate. This species was also dominant (approximately 10^7 CFU/mL) after seven cycles of fermentation of HM kefir in culture-dependent analysis [1]. Brown sugar kefir could not sufficiently maintain *A. lovaniensis*, as this species decreased from 14% (BSD1) to 1% (BDS7) through multiple fermentations (Fig. 3). In summary, the high-throughput 16S rDNA sequencing analysis revealed that only HM supports the viability of AAB, indicating that the sugary kefir beverages are microbially diverse depending on the sugar substrates, as shown in our previous culture-dependent study [1].

Diversity of Lactic Acid Bacteria in Sugary Kefir

Among other phyla of bacterial microbiota, such as *Firmicutes*, specific species of LAB were identified at stable proportions. *Liquorilactobacillus nagelii* was the major LAB

Fig. 1 Chao1 (**a**) and Shannon (**b**) index of kefir grains (black spots) and resulting beverages (white spots) after consecutive fermentations in brown sugar (BS), purified molasses (PM), and high-test molasses (HM). Consecutive fermentation was carried out for 7 days and samples were taken from 1st, 4th and 7th cycles of fermentation under the condition described in materials and methods. BSG, PMG and HMG indicate kefir grains of each substrate
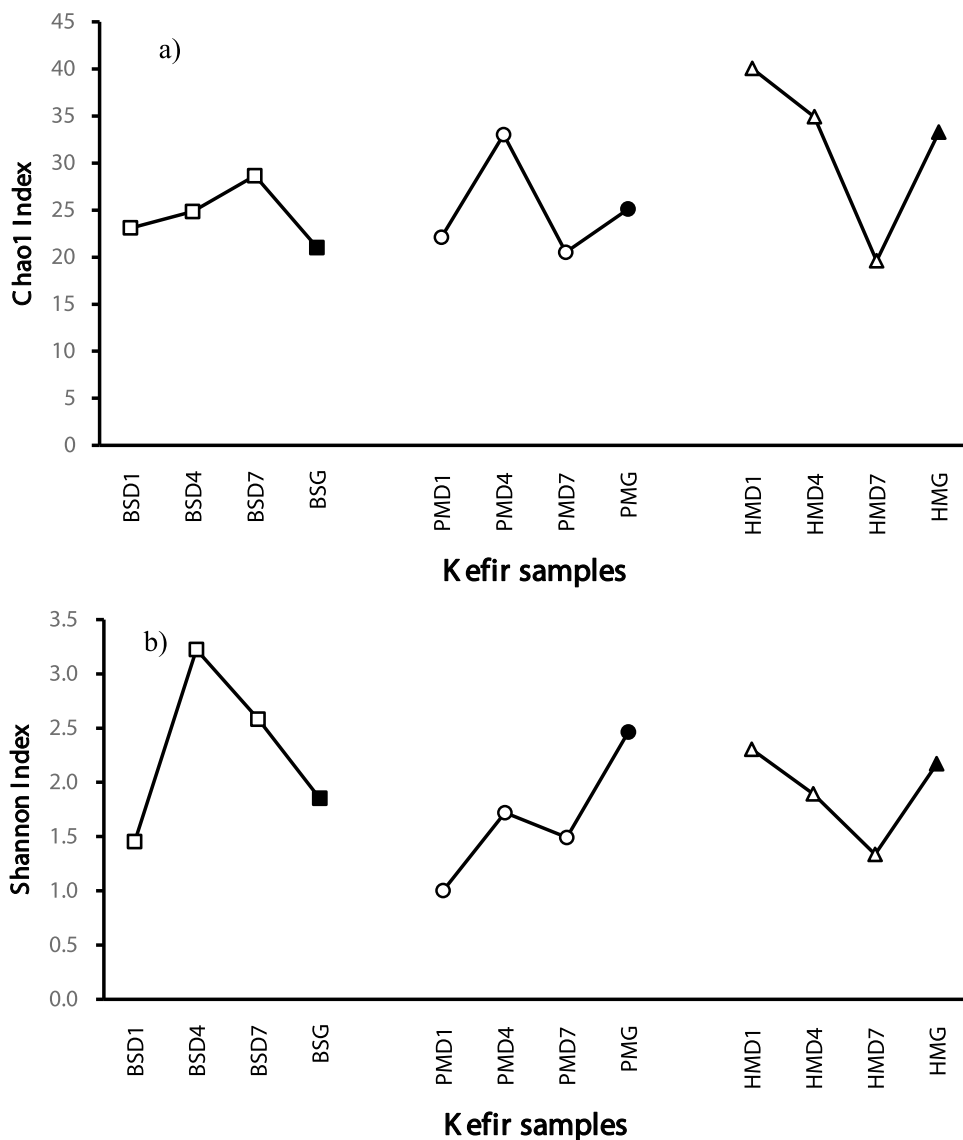

Fig. 2 16S phylogenetic composition of the bacteria of the sugary kefir and grains grown in brown sugar (BS), purified molasses (PM), and high-test molasses (HM) at phylum level. Consecutive fermentation was carried out for 7 days and samples were taken from 1st, 4th and 7th cycles of fermentation under the condition described in materials and methods. BSG, PMG and HMG indicate kefir grains of each substrate
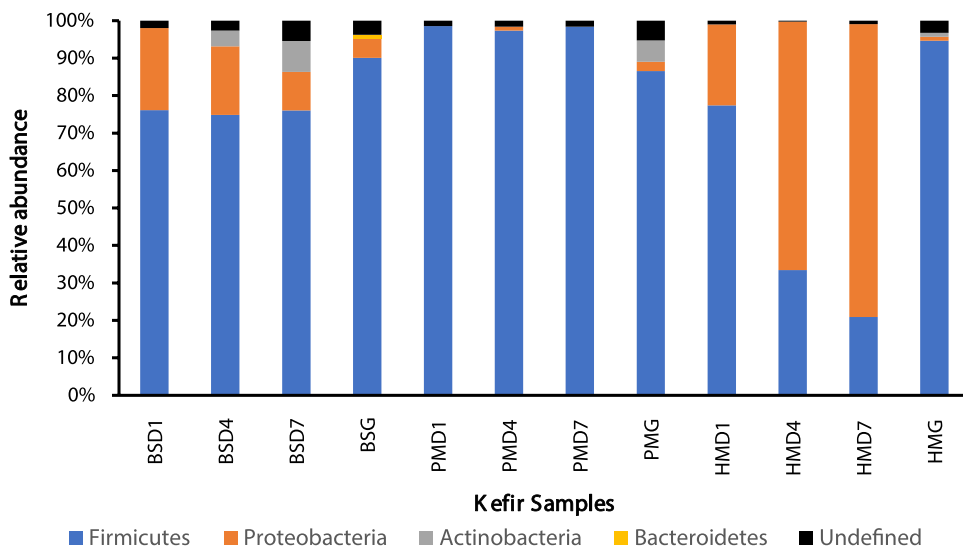
Fig. 3 16S phylogenetic composition of the bacteria of the sugary kefir and grains grown in brown sugar (BS), purified molasses (PM) and high-test molasses (HM) at genera/(species) level. The closest species dependent on the partially analyzed 16S rDNA region was deduced using BLAST. Consecutive fermentation was carried out for 7 days and samples were taken from 1st, 4th and 7th cycles of fermentation under the condition described in materials and methods. BSG, PMG and HMG indicate kefir grains of each substrate
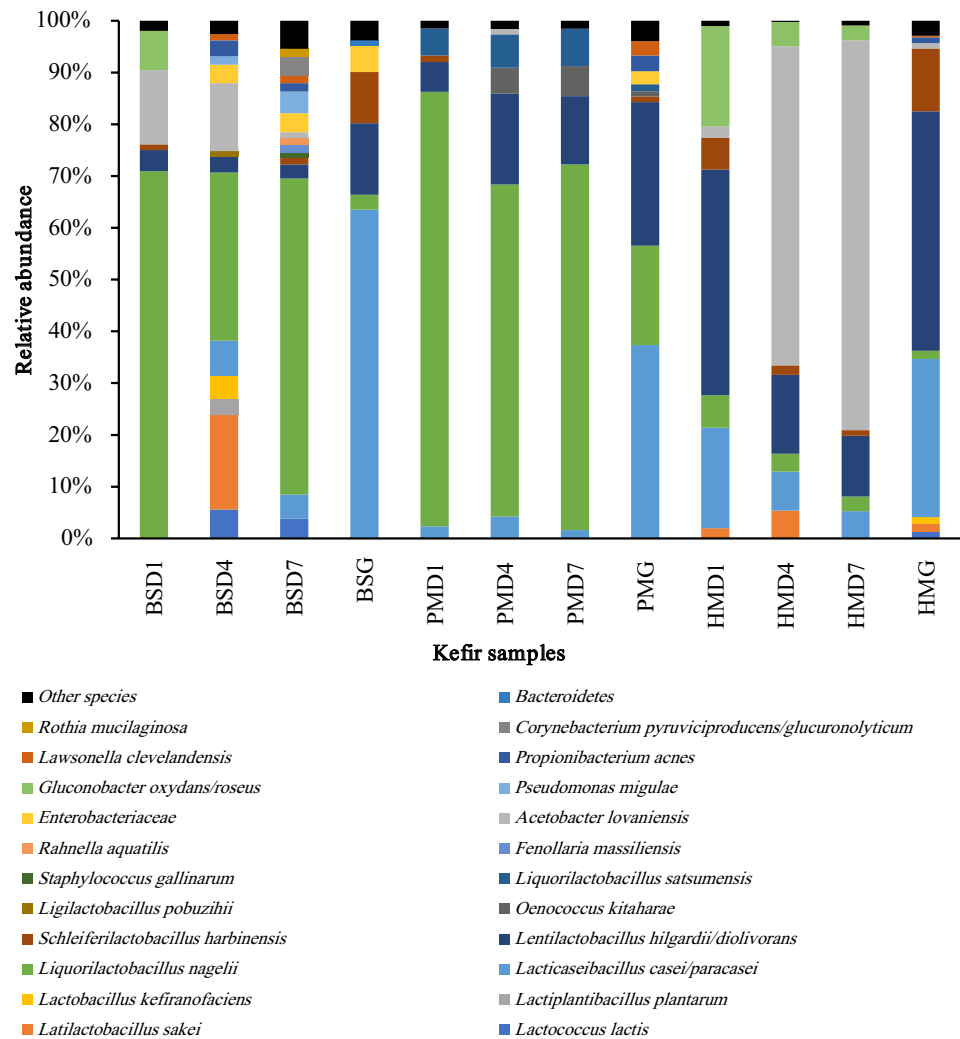

Legend:
- *Other species*
- *Rothia mucilaginosa*
- *Lawsonella clevelandensis*
- *Gluconobacter oxydans/roseus*
- *Enterobacteriaceae*
- *Rahnella aquatilis*
- *Staphylococcus gallinarum*
- *Ligilactobacillus pobuzihii*
- *Schleiferilactobacillus harbinensis*
- *Liquorilactobacillus nagelii*
- *Lactobacillus kefiranofaciens*
- *Latilactobacillus sakei*
- *Bacteroidetes*
- *Corynebacterium pyruviciproducens/glucuronolyticum*
- *Propionibacterium acnes*
- *Pseudomonas migulae*
- *Acetobacter lovaniensis*
- *Fenollaria massiliensis*
- *Liquorilactobacillus satsumensis*
- *Oenococcus kitaharae*
- *Lentilactobacillus hilgardii/dioliivorans*
- *Lacticaseibacillus casei/paracasei*
- *Lactiplantibacillus plantarum*
- *Lactococcus lactis*

in BS kefir (33–71% of total bacteria, BSD) and PM kefir (64–84%, PMD) beverages. The result was comparable to that from our previous culture-dependent investigation [1]. A different pattern was observed for HM kefir beverages, which contained *Lentilactobacillus hilgardii/dioliivorans* (12–44%) and *Lacticaseibacillus casei/paracasei* (5–19%) at higher abundance than *L. nagelii* (3–6%). Although the major LAB species varied depending on sugar substrates, our result showed that *L. nagelii, L. hilgardii/dioliivorans* and *L. casei/paracasei* played an important role in lactic acid formation in sugary kefir beverages. Previous studies reported that similar bacteria were detected in sugary kefirs from different countries [18], though different substrates were used such as brown sugar solution (6 g/100 mL [19] and 5 g/100 mL [20, 21]), sucrose solution (10 g/100 mL) with figs and a sliced lemon [22].

There was a trade-off manner of ratio between *L. nagelii* and AAB in microbiota of kefir from HM and other two sugar substrates. This might be explained by (i) acetic acid

produced by AAB represses the viability of *L. nagelii*, or alternatively (ii) *L. nagelii* is more resistant than *L. hilgardii/dioliivorans* and *L. casei/paracasei* to ethanol, which is produced by yeasts and consumed by AAB coexisting in kefir. In either case, the growth of AAB would increase the acetic acid level and decrease the ethanol level, which might be disadvantageous for *L. nagelii*. We detected 10^5–10^6 CFU/mL of several yeast species (*Saccharomyces cerevisiae* and *Pichia* species) in culture-dependent analysis [1], indicating that the ethanol level in kefir beverage could affect growth of LAB with various ethanol tolerance. It is notable that *Oenococcus kitaharae* resided in PM kefir beverages (5–6%), even though at a low percentage. Since the genus *Oenococcus* is a known ethanol-tolerant LAB, it is expected to grow in sugary kefir in a trade-off manner with alcohol-degrading AAB. Indeed, *O. kitaharae* was quite scarce (no more than 1%) in HM kefir beverage. We also isolated this species from PM kefir beverage in the previous culture-dependent analysis [1]. Another species *Latilactobacillus sakei* (formerly

Lactobacillus sakei) was observed in BSD4, HMD1 and HMD4, but it was only detected occasionally with lower stability than the above three major LAB species (*L. nagelii*, *L. hilgardii/diolivorans* and *L. casei/paracasei*). *Lentilactobacillus farraginis* detected at high population (detected at 10^5-10^7 CFU/mL in BS and PM kefir beverages) in the culture-dependent analysis was not observed as major constituents in the present study. This may be a selection bias due to either of the culture-dependent or PCR methodologies.

Bacterial Microbiota in Different Sugary Kefir Grains

Interestingly, all grains taken after seven cycles multiple fermentations comprised *L. casei/paracasei* (31–63%) and *L. hilgardii/diolivorans* (14–46%) at higher abundance than *L. nagelii* (2–19%), showing a different trend between beverages and grains of sugary kefir. This difference was previously seen in milk kefir [12, 13]. One notable exception was

reported by Marsh et al. [10], who found high abundance of ethanol-producing bacteria *Zymomonas* in both grains and beverages of sugary kefir. Although different dominant bacteria have been reported in various studies, it is conceivable that the level of ethanol (produced by yeasts or *Zymomonas*) and/or acetic acid in kefir modulates the microbiota stability and later succession of specific LAB and other species mentioned above. Microbiota composition of sugary kefir could be affected by different sugar substrates as shown in this study, thus chemical characteristics in final beverage products could also be different. Future studies should include detailed analysis of chemical components and fungal microbiota including yeasts in these sugary kefirs.

Moreover, we found other bacterial groups with low abundance, as summarized in Table 1. Several species including *Pseudomonas migulae* (less than 4.21% of the population) were detected in kefir for the first time. Some of these minor species are considered opportunistic pathogens that affect

Table 1 Species detected in kefir for the first time

Phylum	Species	Abundance (% of total bacterial microbiota) in grains and beverages		
		BS	PM	HM
Proteobacteria	*Pseudomonas migulae*	4.21		
Proteobacteria	*Rahnella aquatilis*	1.28		
Firmicutes	*Ligilactobacillus pobuzihii*	1.07		
Firmicutes	*Staphylococcus gallinarum*	1.11		
Firmicutes	*Fenollaria massiliensis*	1.41		
Actinobacteria	*Lawsonella clevelandensis*		2.78	
Actinobacteria	*Corynebacterium pyruviciproducens/glucurono-lyticum*	3.48		
Actinobacteria	*Rothia mucilaginosa*	1.60		
Phylum	Species	Lower reads (<1%)		
Firmicutes	*Loigolactobacillus rennini*			0.04
Firmicutes	*Companilactobacillus farciminis/ heilongjiangensis*			0.04
Firmicutes	*Sporolactobacillus vineae*		0.53	
Firmicutes	*Streptococcus parauberis*			0.17
Firmicutes	*Enterococcus raffinosus*		0.20	
Firmicutes	*Enterococcus faecalis*	0.55		
Firmicutes	*Kurthia gibsonii*			0.20
Firmicutes	*Carnobacterium* sp.			0.03
Firmicutes	*Anaerococcus tetradius/prevotii*	0.37		
Firmicutes	*Peptoniphilus* sp.	0.54		
Firmicutes	*Finegoldia magna*	0.56		
Proteobacteria	*Moraxella osloens*	0.78		
Proteobacteria	*Methylobacterium* sp.	0.75		
Actinobacteria	*Corynebacterium accolens*	0.84		
Actinobacteria	*Corynebacterium thomssenii*			0.03
Bacteroidetes	*Prevotella melaninogenica*		0.43	

BS brown sugar, *PM* purified molasses, *HM* high-test molasses. Highest percentages of mentioned bacterial species were indicated

immunocompromised people, such as *Rothia mucilaginosa, Fenollaria massiliensis, Staphylococcus gallinarum, Lawsonella clevelandensis, Rahnella aquatilis*, and different *Corynebacterium* species [23–30]. The definite safety of kefir, however, has been established in the long history of this beverage. Therefore, a robust mechanism to suppress the activity of these harmful bacteria must exist intrinsically in kefir. Actually, we detected considerable antibacterial activity of the BS, PM, and HM kefir beverages on food-borne harmful bacteria *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella enterica* subsp. *enterica* serovar Typhimurium in the previous report [1]. As mentioned above, a detailed analysis on chemical components and their relationship with microbiota will reveal more deepened mechanism of this antibacterial effect in the future studies. The great potential of sugary kefir as a good niche for probiotic LAB was also confirmed in this study. Contents of main sugars (glucose, fructose and sucrose) in substrates were enough consumed after fermentation (from 3.4 to 1.1% in BS kefir, from 2.5 to 0.099% in PM kefir and from 2.2 to 0.023% in HM kefir) (Table S1), therefore sugar contents should not be an obstacle to encourage the consumption of these kefirs. Further study is needed to apply the potential of above probiotic microbes to the food industry.

Conclusions

This report provides a detailed analysis on the successive fermentations of sugary kefir using high-throughput 16S rDNA sequencing. We found that bacterial composition differs significantly between grains and beverages, and different type of sugars affect diversity of bacterial microbiota. One remarkable example is diversity in the succession of AAB. Three main LAB species, *L. nagelii*, *L. hilgardii/diolivorans*, and *L. casei/paracasei*, contribute to sugary kefir fermentation, although their abundance differs depending on the types of sugar substrate. We reasoned that the ethanol or acetic acid levels in fermentation medium and the tolerance of respective LAB species toward them influence the later succession of dominant species. It would be important to study the chemical components and fungal populations in sugary kefirs to obtain a thorough understanding of this millenium-old fermented product.

Declarations

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