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ORIGINAL RESEARCH

Microbial community dynamics of fermented kefir beverages changes over time

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Kefir is a traditional fermented milk associated with several health benefits, such as immune system modulation, as well as antimicrobial, antitumor and antioxidant activity. The aim of the current study was to investigate the microbial dynamics of kefir production based on high-throughput DNA sequencing. Results of sequence analysis have grouped the reads into 303 and 112 amplicon sequence variants (ASV) for bacteria and fungi, respectively. Firmicutes, Bacteroidetes and Proteobacteria were the prevalent bacterial phyla, whereas Lactococcus *was the prevalent bacterial genus. Ascomycota was the main fungal phylum. Data have shown heterogeneity in diversity and abundance distributions between milk kefir samples.*

Keywords Kefir, Probiotics, Health benefits, Microbiota, High-throughput sequencing.

INTRODUCTION

Several studies have indicated reciprocal interaction between gut microbiota and the function of different host organs (Rinninella *et al*. 2019). The incidence of gut dysbiosis leads to the onset or worsening of systemic abnormalities, such as cardiovascular and metabolic diseases (Bourrie *et al*. 2016; Plaza-Diaz *et al*. 2019; van de Wouw *et al*. 2020). The current scenario has resulted in growing interest in probiotics using, since they are known for providing different health benefits, such as gut homeostasis maintenance, intestinal dysbiosis prevention or reversal (Zhong *et al*. 2020), anticarcinogenic effects (Brasiel *et al*. 2020), hypolipidaemic (Sarfraz *et al*. 2019) and antimicrobial and glycemic reduction properties (Shafi *et al*. 2019).

Accordingly, kefir has been considered a promising probiotic. Probiotics are living microorganisms capable of providing health benefits to hosts when they are administrated at adequate amounts (FAO/WHO 2002). New definitions covering 'viable or inviable microbial cell (vegetative or spore; intact or ruptured) potentially healthful to hosts' have been recently suggested (Zendeboodi *et al*. 2020). Kefir is a fermented milk composed of a complex microbiota in polysaccharide and protein matrix. Lactic acid bacteria (LAB), acetic acid bacteria and lactose-fermenting and non-lactose-fermenting yeasts found in its microbiota are an example of a symbiotic community (Farnworth 2005; Nielsen *et al*. 2014), whose physicochemical and sensory properties can undergo changes (Karaçalı *et al*. 2018; Perna *et al*. 2019; Mitra and Ghosh 2020). Kefir grains differ from fermented products and are susceptible to several variations, which may derive from factors such as the origin and storage of kefir grains, milk type (substrate), microbiological composition of grains, processing conditions, grain/milk ratio and environmental conditions like fermentation time and temperature (Garofalo *et al*. 2015; Hatmal *et al*. 2018).

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It is essential in understanding the structure and stability of the microbial community living in kefir milk to assess the safety and health benefits of its consumption. Culture-independent methods, such as the high-throughput sequencing of the 16S rRNA gene, have been recently used to analyse kefir diversity and microbial structure (Nalbantoglu *et al*. 2014; Walsh *et al*. 2016; Dertli and Hilmi 2017). However, it is not yet fully clear whether the microbial composition of fermented milk changes during its production process. Thus, the aims of the present report were to study the microbiota of fermented milk produced from kefir and to assess changes in its microbial composition, both at the beginning and throughout its production process. The 16S rRNA gene and Internal Transcribed Spacer (ITS) region sequencing procedures were performed to access bacterial and fungal communities of the milk kefir.

MATERIALS AND METHODS

Kefir production and sampling

Kefir grains were obtained from the Federal University of Viçosa, Department of Nutrition and Health, Viçosa, Brazil. They were inoculated with pasteurised whole milk (Benfica®, Juiz de Fora, Brazil) at the ratio of 1:10 (w/v) and incubated in sterile glass bottle protected with proper paper, at 25 ± 2 °C, for 24 h. Next, they were filtered through a sterilised plastic sieve, washed in sterile distilled water and inoculated with milk again for fermentation purposes. This procedure was repeated for 30 days to enable sample collection and maintenance of active grains. Fermented kefir samples were collected after 24 h of fermentation (KI), and the 10-mL daily collection pool was kept until the 30th day (KP). In other words, KP was prepared by pooling equal amounts from each of the 30 fermented products. Fermented milk beverage samples were immediately stored in sterile container at –80 °C, until analysed.

DNA extraction, PCR amplification and Illumina MiSeq sequencing

Libraries of 16S rDNA and ITS amplicons were generated for each kefir sample by using locus-specific primers accounting for targeting V3-V4 hypervariable regions of 16S rRNA gene and ITS region in order to define bacterial and fungal communities, respectively. Samples were sequenced in Illumina MiSeq platform in GenOne Biotechnologies enterprise (Rio de Janeiro, Brazil), based on standard Illumina sequencing protocols.

Milk kefir samples were homogenised for 1 min; 2 mL of each sample was centrifuged at 1370 *g*, for 15 min. Pellet was used for total DNA extraction in MagaZorb® DNA Mini-Prep Kit (Promega BioSciences, LLC, San Luis Obispo, USA), based on the manufacturer's protocol, with some modifications, as described below. Pellets were resuspended in 1 mL of lysis buffer and incubated at 70 °C, for 5 min. Next, centrifugation at 21 913 *g*, for 2 min, was performed. The supernatant was transferred to a clean tube, and 500 μL of binding buffer and 20 μL of proteinase K were added to the mixture and incubated at 56 °C for 30 min, in compliance with the isolation protocol provided by the manufacturer. DNA quality and quantity measurements were based on Nano-Drop 1000 spectrophotometry (Thermo Scientific, Waltham, MA, EUA), whereas DNA electrophoresis was analysed in DNA Bioanalyzer. Extracted DNA was stored at −20 °C.

The V3-V4 regions of bacteria presenting the 16S rRNA gene were amplified through PCR (95 °C for 2 min, followed by 25 cycles at 95 °C for 30 s, at 55 °C for 30 s, at 72 °C for 30 s and by a final extension at 72 °C for 5 min) by using primers such as 341F (5′-barcode-CCTAY GGGRBGCASCAG)-3′ and 806R (5′-GGACTACNNGGG TATCTAAT-3′); the barcode was an eight-base sequence unique to each sample. Internal Transcribed Spacer (ITS) sequencing process has used specific primers such as ITS3F 5′(GCATCGATGAAGAACGCAGC)3′ and ITS4R 5′ (TCCTCCGCTTATTGATATGC)3′. All PCR products were carried out in Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Next, combined PCR products were purified with the aid of Qiagen Gel Extraction Kit (Qiagen, Germany).

Sequencing libraries were generated by using NEBNext® Ultra™ DNA Library Prep Kit for Illumina, based on manufacturer's recommendations, and index codes were added to them. Library quality was assessed in Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Q32866) and Agilent Bioanalyzer 2100 system. Finally, libraries were paired-ended (2 × 250 bp) sequenced in Illumina Miseq platform.

Data analysis

Illumina demultiplexed sequences were processed based on DADA2 (Divisive Amplicon Denoising Algorithm 2) (1.14) (Callahan *et al*. 2016) in order to fix amplicon errors, identify chimeras, merge paired-end reads and generate an ASV table. Amplicon sequence variant (ASV) pipeline was applied to maximise data resolution and reproducibility. Taxonomy was assigned to ASVs, based on naïve Bayesian classifier method against SILVA database release 132 (Pruesse *et al*. 2007) and UNITE general FASTA release, version 01.12.2017 (UNITE Community 2017) for 16S rRNA and ITS data, respectively. All subsequent analyses were carried out through packages implemented in the R software (R Core Team 2019). Rarefaction curve was plotted with vegan package (Oksanen *et al*. 2019). Community diversity was estimated through Observed Species and Simpson indices by using the phyloseq package (McMurdie and Holmem 2013). Taxonomy and phylogeny plots were also performed through DECIPHER (Wright 2016) by using the phyloseq and ggplot2 packages (Wickham 2016).

Sequence data were subjected to the GenBank database; they are accessible under Bioproject no. PRJNA672176. Bacterial 16S rRNA gene data are available under accession numbers SAMN16560548 and SAMN16560549, whereas fungi ITS data are accessible under accession nos. SAMN16560550 and SAMN16560551.

RESULTS

Bacterial composition of kefir milk

Sequencing of both kefir milk samples has generated 131 689 paired-end readings, which were subjected to stringent quality filtering and assigned to 303 bacterial ASVs. Rarefaction curve (Figure 1a) has indicated that the sequencing effort was enough to represent bacterial diversity, since the curve has reached the plateau. Observed ASV (species richness estimator) values were 180 and 211 for initial and pool kefir milk, respectively. Lower microbial diversity level was observed for KP at Simpson diversity index of 0.51; this very same index recorded value of 0.62 for KI (Figure 1b).

The bacterial community observed in milk kefir comprised the three main phyla, Firmicutes, Bacteroidetes and Proteobacteria (Figure 2a). There were significant changes in kefir microbiota throughout its production process. Firmicutes' prevalence has increased from 65% to 75.8% from the initial kefir sample to the pool. Its subpopulation mainly comprised Streptococcaceae (KI – 61.2%; KP – 72.1%) and Lactobacillaceae (KI – 2%; KP – 3.1%) (Figure 2b). It is important highlighting the high incidence of a single ASV assigned to *Lactococcus* within family Streptococcaceae. These bacteria represent approximately 72% of kefir pool microbiota and 62% of microbiota in initial kefir. Family Lactobacillaceae mainly comprised ASVs assigned to *Lactobacillus* (Figure 2c).

Relative Bacteroidetes abundance has decreased during milk kefir fermentation (Figure 2a). Bacteroidetes rate in initial kefir (31.1%) has decreased to 11.6% in kefir pool. Bacteroidetes' composition mainly comprised Bacteroidaceae and Prevotellaceae (Figure 3b). Genus *Bacteroides* has decreased from 10.6% in initial kefir to 3.9% in kefir pool. With respect to family Prevotellaceae, there was decrease in genera *Prevotella*, *Alloprevotella* and in other 'unclassified Prevotellaceae' (Figure 2c). Another shift in kefir microbiota was observed in phylum Proteobacteria. Proteobacteria incidence has increased from 4.8% in initial kefir to 12.6% in kefir pool (Figure 3a). The major change was observed within family Pseudomonadaceae, mainly in genus *Pseudomonas*, which changed from 0.2% (KI) to 7.3% in kefir pool (Figure 2c and Figure S1 in Supplementary Information). There was also increase (KI – 0.1%; KP – 2.5%) in genus *Rahnella* (Enterobacteriaceae). On the other hand, genera *Acinetobacter* (Moraxellaceae) and *Suturella* (Burkholderiaceae) have decreased over fermentation time.

Fungal composition of milk kefir

ITS sequencing of kefir samples has generated 97 565 high-quality reads with 48 782 sequences per sample, on average. Overall, 112 ASVs were detected through DADA2 pipeline. Rarefaction curve was generated to assess whether the sampling process provided sufficient coverage to accurately describe the fungal composition of each kefir sample (Figure 3a). The number of Observed ASVs and the Simpson diversity index was higher in KP than in KI (Figure 3a,b).

Phylum Ascomycota has prevailed in milk kefir fungal community; it represented more than 99% in both samples. These communities have shown changes in fermentation time. *Aspergillus* (*Aspergillaceae*) was the most prevalent genus in both samples; however, the incidence of this fungus has decreased from 71.9% in initial kefir to 65.3% in kefir pool (Figure 4a,b). It is worth emphasising that a single ASV assigned to *Aspergillus amstelodami* has prevailed in the community. *Cordyceps* (Cordycipitaceae) was another abundant genus (Figure 4a,b), which was mainly represented by a single ASV assigned to *Cordyceps bassiana*. This species incidence was higher in kefir pool (24%) than in initial kefir (16.9%). Genera *Saccharomyces* and *Saroclaadium* were observed at lower abundance, although more enriched in initial kefir (KI – 5.1% and 2.1%; KP – 2.5% and 0.8%, respectively) (Figure 4b). Genus *Cladosporium* was mostly abundant in kefir pool (KI – 2% and KP – 4.9%), whereas genus *Fusarium*, represented by species *Fusarium solani*, recorded similar abundance in both samples (approximately 1% abundance).

DISCUSSION

The current study has investigated how the bacterial and fungal communities composing kefir beverages behaved throughout their cultivation based on using the same matrix (starter kefir grains). This investigation is particularly important because fermented beverages are mainly consumed at household level; thus, understanding the composition of their microbiota during production can help establishing safety evaluations and recommendations for cultivation, as well as determining its health benefits resulting from specific microbial compositions. Although other studies have already evaluated milk kefir, to the best of our knowledge, this is the first study focused on investigating the dynamics of this microbial community found in fermented beverage after a long period-of-time (30 days).

Nowadays, there is the trend to move away from OTU-based methods towards DNA sequences capable of representing single-nucleotide variations to reduce methodological failures at the time to analyse microbiota data. DADA2 was one of the ASV-clustering methods capable of building quality-based models to filter errors and identify variations in 16S rRNA gene sequences (Callahan *et al*. 2016;

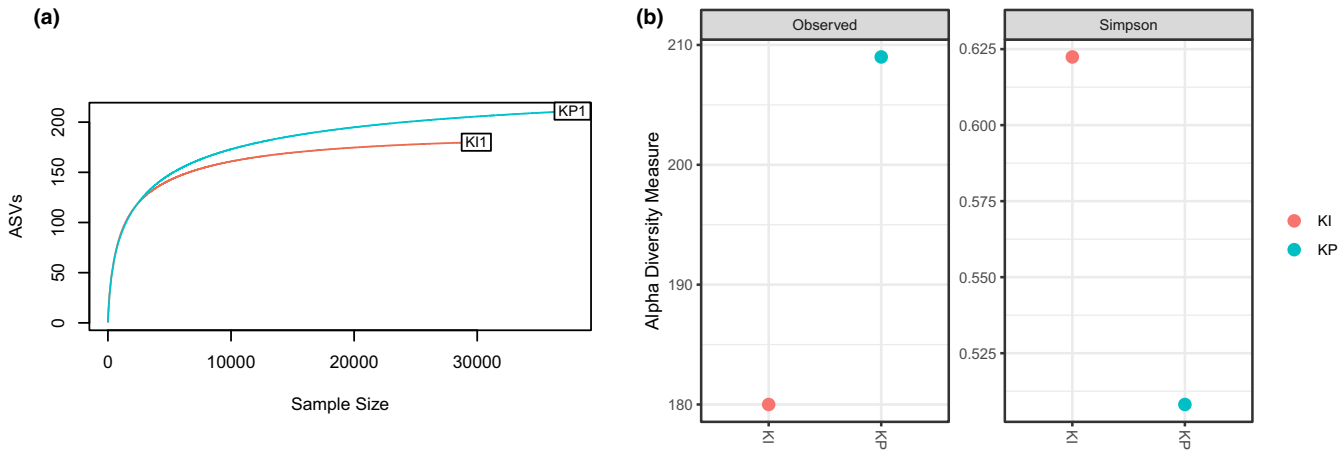

Figure 1 Rarefaction curves (a), number of Observed ASVs and Simpson diversity index (b) based on 16S rRNA sequence. KI: initial kefir; KP: pool kefir.

Callahan *et al*. 2017). This method was used in the current study, which represented an important methodological advantage in comparison with other studies.

According to high-throughput sequencing data, the current study has captured the whole microbial diversity observed for bacterial and fungal communities in milk kefir samples,

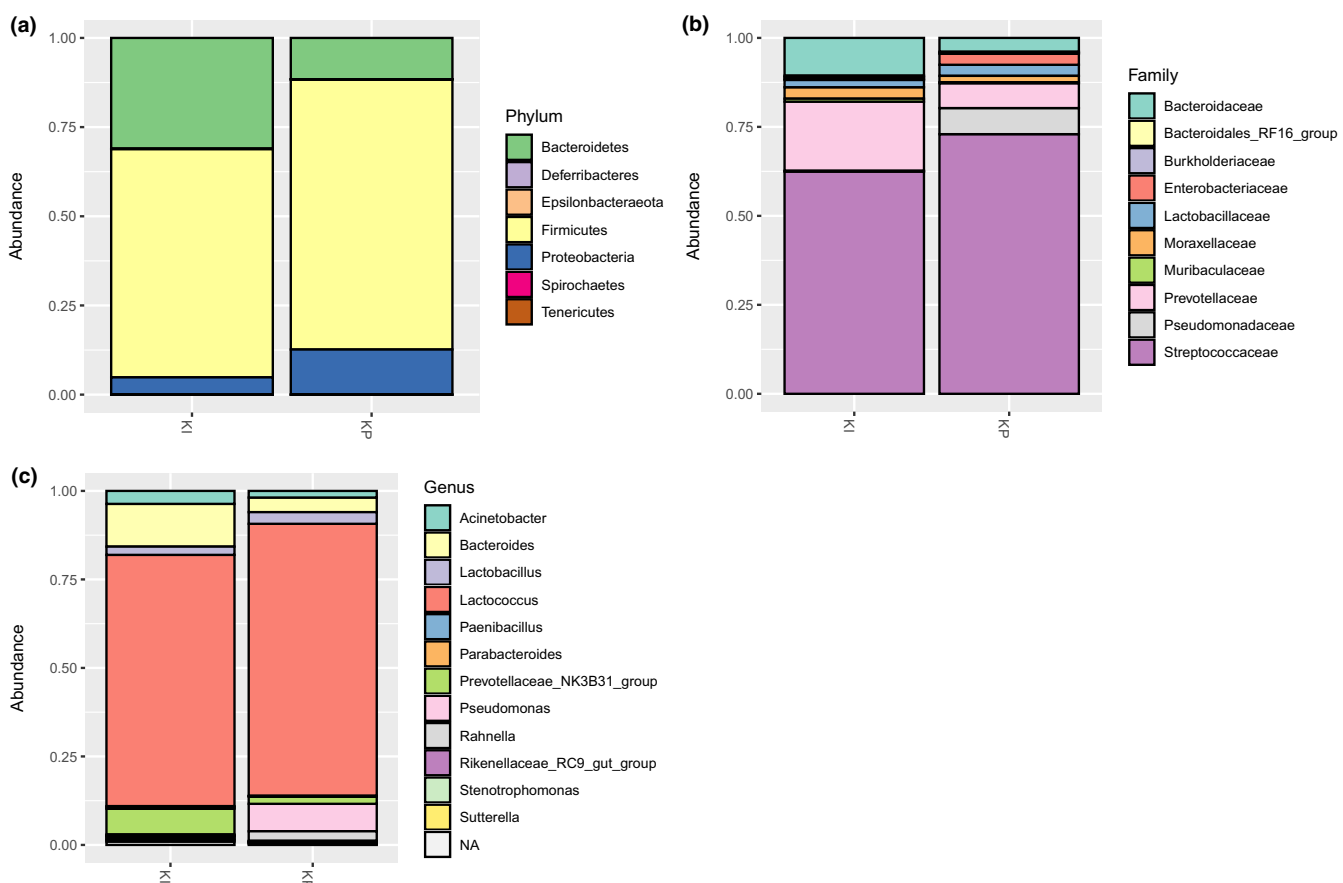

Figure 2 Relative abundance analysis at Phylum level (a), Family level (b) and Genus level (c) based on 16S rRNA data. KI: initial kefir; KP: pool kefir.

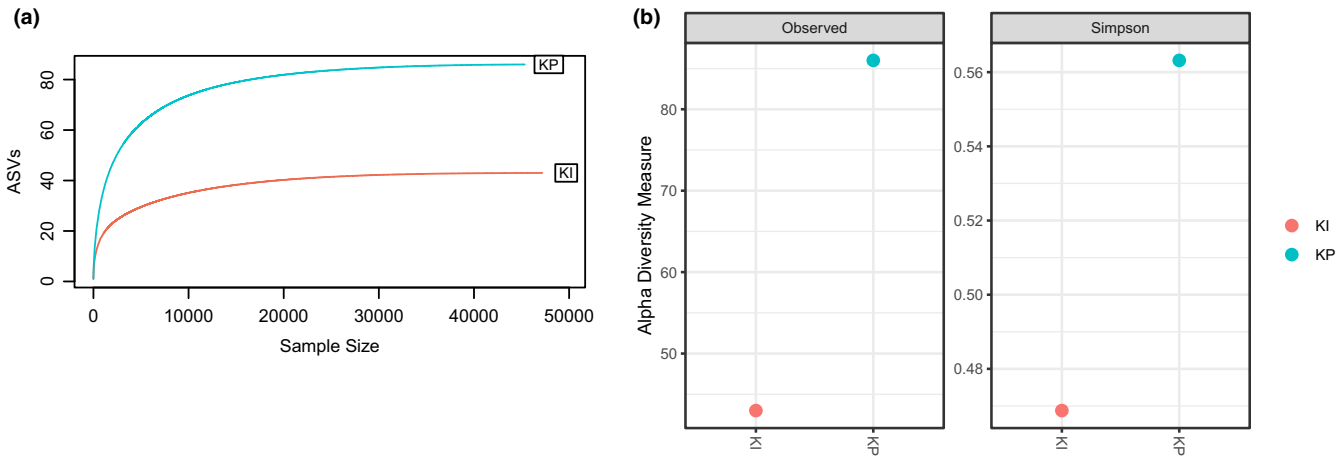

Figure 3 Rarefaction curves (a), number of Observed ASVs and Simpson diversity index (b) based on internal transcribed spacer (ITS) sequences. KI: initial kefir; KP: pool kefir.

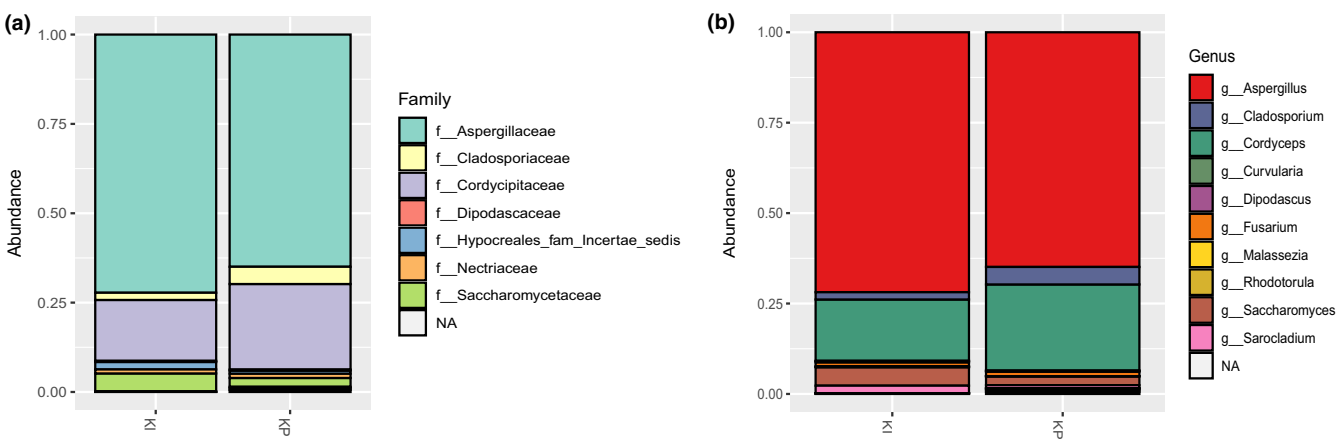

Figure 4 Relative abundance analysis at Family level (a) and Genus level (b) based on internal transcribed spacer (ITS) sequences. KI: initial kefir; KP: pool kefir.

since the rarefaction curves have reached the asymptote. We observed contrasting bacterial diversity result based on the used metrics. Based on the Observed ASV index, kefir pool presented the richest microbiota. However, results of Simpson index (richness and evenness index) have indicated that initial kefir was the most diverse sample. These contradictory results can be explained by the prevalence of a specific bacterial group (*Lactococcus lactis*), mainly in kefir pool. The Simpson index takes into account sample equitability (Kim *et al*. 2017); initial kefir microbiota was slightly more equally abundant.

KI and KP milk kefir presented similar microbial community composition, although with different relative abundance. The 16S rRNA profile has shown that the bacterial population in the tested milk kefir was mostly composed of Firmicutes, Bacteroidetes and Proteobacteria. *Streptococcaceae* has already been identified as the prevalent family in fermented milk, whereas genera *Leuconostoc*, *Lactococcus*, *Lactobacillus* and *Acetobacter* were the most abundant ones (Dobson *et al*. 2011; Marsh *et al*. 2013; Garofalo *et al*. 2015). According to Marsh *et al*. (2013), bacterial population in fermented milk presented lower species diversity than that of corresponding grains. The current study has also found prevalence of family *Streptococcaceae*, as well as relevant contribution from LAB, *Lactococcus* and *Lactobacillus*, which had been associated with kefir in previous studies (Magalhães *et al*. 2011; Marsh *et al*. 2013; Korsak *et al*. 2015; Walsh *et al*. 2016). The herein presented data have shown that the ratio of these genera has considerably increased in milk kefir during its production process. The aforementioned can affect the improvement of kefir probiotic effects, whose benefits, such as anti-proliferative, pro-apoptotic and anti-oxidative activity; serum cholesterol level reduction; and anti-inflammatory and anti-mutagenic

properties are well-known (de Moreno de LeBlanc *et al*. 2008; Nowak *et al*. 2018; Slattery *et al*. 2019).

Lactococcus was the most prevalent bacterial genus in fermented milk (relative abundance higher than 60%), whereas kefir grains recorded much lower values for it (Gao and Zhang 2018). Some strains belonging to this genus were described as potentially probiotic, as well as associated with fermented dairy products. Their ability to ferment lactose is of paramount importance, mainly when they are used as starter cultures in the dairy industry (Vieira *et al*. 2017; Yerlikaya 2019). Lee *et al*. (2015) have investigated the effect of *Lactococcus lactis* KC24 isolates and identified reduced nitric oxide in lipopolysaccharide-induced production, as well as antimicrobial effects on the manufacture of multifunctional probiotic products. It is important mentioning that *Lactococcus* was enriched in the herein investigated milk kefir, and its abundance has increased depending on the cultivation period; it went from 62.43% in initial kefir to 72.62% in kefir pool (+10.19%). On the other hand, relative abundances recorded for genus *Lactobacillus* reached just over 3%. Although this bacterial genus has already been well described in kefir production processes, its abundance was significantly more expressive in kefir grains than in fermented milk. This outcome has indicated low *Lactobacillus* growth capacity in milk (Dobson *et al*. 2011; Gao and Zhang 2018). The great microbial diversity observed in kefir leads to competition among microorganisms. Competition by bacteria, yeasts and between fungal species is affected by substrate microenvironment and determines the organism or group of organisms that will prevail (Bullerman 2003).

Genus *Pseudomonas*, which is known for its ubiquity, can be found in several fermented food items (Tamang *et al*. 2016); it was already found in kefir grains (Chen *et al*. 2008; Wang *et al*. 2012), although there is no emphasis on its presence in fermented milk. Thus, KI recorded low *Pseudomonas* rate (0.24%), although it was substantially higher in KP (7.33%). This genus plays key role in food spoilage (Raposo *et al*. 2017), and it may explain its increase after 30 cultivation days. However, attention must be given to *Pseudomonas* increase in kefir since some species are opportunistic pathogens capable of affecting humans (Raposo *et al*. 2017).

Results of fungal analysis based on ITS sequencing have indicated that the fungal community was the most diverse at 30 fermentation days. Unlike previous studies, *Aspergillus* sp., *Cordyceps* sp. and *Saccharomyces* sp. were the most prevalent yeast populations found in the current study. These species had already been found in fermented food and can be explored by the food industry (Bourdichon *et al*. 2012). Although genus *Aspergillus* is not often reported in kefir, it is often found in fermented drinks and accounts for producing several carbohydrases such as amylase, amyloglucosidase and maltase, among others (Tamang *et al*. 2016a; Tamang *et al*. 2016b). *Aspergillus* was also recently identified as the prevalent genus in kefir grains in a study carried out in Nyingchi (China), which showed great variation among different regions (Liu *et al*. 2019). It is worth mentioning that none of the previous studies has used ASV-clustering methods, whose results are the most accurate representation of the communities.

Interestingly, the relative abundance of *Saccharomyces* sp. has decreased from 5.1% to 2.5% during the milk kefir cultivation period, which represented decrease by 51% in KP. Members of genus *Saccharomyces* have been used as probiotics to treat antibiotic-associated diarrhoea. Several studies have indicated that some species can perform immunomodulatory, gastrointestinal modulatory and antioxidant functions. They also show stability in gastric conditions and play key role in the production of β-glucan, glutathione, protein, fibre, vitamin B and folic acid deriving from cell wall (Fakruddin *et al*. 2017; Hong *et al*. 2018; Hong *et al*. 2019).

CONCLUSIONS

Sequencing data have confirmed that milk kefir microbiota changes over time. In addition, microbial diversity underwent variations during the cultivation period; there was greater bacterial diversity associated with the initial kefir than with pooled-kefir. Moreover, microbial community succession and quality changed during the kefir fermentation process. Accordingly, the prevalence of the genus *Lactococcus* in fermented milk was confirmed. Understanding the complex microbial community found in kefir can help identifying the origin of its beneficial health properties and guiding its domestic use. The current results help to improving the knowledge of microorganisms acting in fermentation processes; they can be used to help controll the quality of fermented beverages.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Poliana Guiomar de Almeida Brasiel: Conceptualization, formal analysis, methodology, supervision, writing-original

draft. **Julliane Dutra Medeiros**: Methodology, software, writing-review & editing. **Alessandra Barbosa Ferreira Machado**: Writing-review & editing. **Maíra Schuchter Ferreira**: Formal analysis, investigation. **Maria do Carmo Gouveia Peluzio**: Supervision, writing-review & editing. **Sheila Cristina Potente Dutra Luquetti**: Conceptualization, data curation, funding acquisition, investigation, project administration, writing-original draft.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Sequence Read Archive (SRA) database from NCBI at https://www.ncbi.nlm.nih.gov/sra/PRJNA672176, reference numbers: SAMN16560548, SAMN16560549, SAMN16560550, SAMN16560551 (Brasiel *et al*., 2020).

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SUPPORTING INFORMATION

The following supporting information is available for this article:

Figure S1. Phylogenetic relationship of ASVs assigned to phylum Proteobacteria deriving from kefir samples. KI: initial kefir; KP: pool kefir.