

Research Article

Functionally Active Microbiome and Physicochemical Properties of Milk and Sugary Water Kefir from Brazil

Villanueva CNB^{1#}, Rios DL^{2#}, Alvarenga RL³, Acurcio LB¹, Sandes SHC², Nunes AC², Nicoli JR¹ and Neumann E^{1*}

¹Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Brazil

²Departamento de Genética, Ecologia e Evolução, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Brazil

³Laboratório de Bromatologia, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Brazil

*Equal Contribution to the Execution of Experiments

*Corresponding author: Neumann E, Universidade Federal de Minas Gerais, Campus Pampulha, Av. Antônio Carlos, Brazil

Received: February 09, 2021; Accepted: March 09, 2021; Published: March 16, 2021

Abstract

In Brazil, milk kefir, made of milk kefir grains, has identity features defined by Brazilian regulatory agencies but sugary water kefir, made of water kefir grains, has no definition of microbiological and physicochemical standards. We evaluated the microstructure of Brazilian milk and water kefir grains, the Transcriptionally Active Microbiome (TAM) of kefir beverages made of them, and the effect of fermentation and storage period (28 days at 10°C) over microbiological and physicochemical features of these beverages. Milk and water grains are very different between them and similar to other kefir grains worldwide, macroscopically and microscopically. The genus *Leuconostoc*, with the species *L. mesenteroides*, was most frequent in the microbiome of milk kefir while the *Oenococcus* genus was most frequently seen in sugary water kefir, with the species *O. kitaharae*. The genera *Saccharomyces* and *Torulaspota*, with the species *S. cerevisiae* and *T. delbrueckii*, were more recurrent in the microbiome of sugary water kefir, while *Pichia* and *Yarrowia* were more abundant in milk kefir, with the species *P. fermentans* and *Y. lipolytica*. Microbiological and physicochemical parameters of milk kefir were in concordance with features defined by Brazilian legislation. None of the parameters was altered by cold storage for 28 days. Our results reinforce some Brazilian identity requirements for milk kefir and allow us to suggest the inclusion of new ones that are not defined yet. Regarding sugary water kefir, some microbiological and physicochemical parameters are similar to milk kefir during the same storage period, although with a quite different functional microbiome.

Keywords: Brazilian Kefir grains; Brazilian kefir beverages; Sugary water kefir identity; Functional microbiome

Introduction

Kefir is a kind of fermented milk originated from the Caucasian mountains and dispersed worldwide. It is obtained from the fermentation by starter microorganisms present in typical grains. Kefir grains possess a microbiota composed of an association between Lactic Acid Bacteria (LAB), Acetic Acid Bacteria (AAB), and yeast, entrapped by an Exopolysaccharide (EPS) matrix [1-4]. When inoculated in milk, the grains' microbiota produces lactic and acetic acid, ethanol, CO₂, and aromatic compounds, leading to pH reduction with protein precipitation. These microorganisms that give a distinct character to the drink should be viable and abundant until the predetermined expiry date [5-8]. Sugary water kefir is usually produced in water added with brown sugar (around 5% w/v) or fruit juices, by addition of water kefir grains. Fermentation from both kefir occurs for 24 to 48 hours at room temperature, producing turbid, sparkling, acid, and slightly alcoholic beverages [9-11].

Kefir has been consumed due to the health benefits produced by potentially probiotic microorganisms isolated from kefir grains and beverages [12,13]. In addition, EPS production from LAB is a significant feature due to its rheological improvements to the beverages and its potential functional properties [14,15].

In Brazil, there is an incipient industrial production of kefir.

Kefir is often produced at domestic level by using two types of kefir grains that circulate the country, milk kefir grains used to produce the traditional fermented milk, and water kefir grains used to prepare a watery fermented drink with brown sugar. The geographic origin of grains, with different climate conditions, the grains subculture methods and the substrate used for fermentation may result in alterations of the beverage's characteristics. Indeed, there are few data available regarding microbial composition and scientific literature concerning quality and physicochemical standards for the different kefir beverages produced in Brazil [2-4,16-19].

Transcriptomics deals with the complete set of RNA transcripts produced by the microbial cells in a specific time or place using high-throughput NGS technologies called RNA-Seq [20]. Therefore, a more accurate estimative of the abundance of active bacteria and yeasts of kefir community could be achieved seeking mRNA transcripts of housekeeping and ribosomal protein genes, generating a Transcriptionally Active Microbiome (TAM).

Thus, this work aimed to assess the functional microbiome of different milk and water Brazilian kefir and evaluate the effect of fermentation and cold storage period on the microbiological and physicochemical characteristics of those beverages. These results could contribute to define a true identity for both types of beverages produced in Brazil.

Materials and Methods

Kefir grains

Kefir grains used in this study were cultivated in two different food matrixes: milk and water (with brown sugar), according to its original propagation matrix. The water kefir grains were provided from different domestic environments from Brazilian cities of Belo Horizonte (KABH), Curitiba (KACU), and Salvador (KASA). Milk grains were from Curitiba (KLCU), Salvador (KLSA), and Divinópolis (KLDI). Water and milk grains from Viçosa (KAVI and KLVI, respectively) were from the Fermented Dairy Products Laboratory from the Federal University of Viçosa (UFV). All grains were kept at -86°C until their usage.

Electron microscopy of kefir grains

Approximately 0.5g of milk and water kefir grains from Curitiba and Salvador were prepared according to procedures for electron microscopy (scanning and transmission) [22], which were conducted at Microscopy Center from UFMG. Preparation for Scanning (SEM) and Transmission (TEM) electronic microscopic was realized through the Osmium-Tannin-Osmium (OTO) method. SEM samples were analyzed in an electronic scanning microscope FEG - Quanta 200 (Fei Tecnai, Oregon, USA). TEM samples were cut by microtome and analyzed in an electronic transmission microscope - Tecnai Spirit Biotwin G2-12 (Fei Tecnai, Oregon, USA).

Kefir production

Milk kefir was prepared with reconstituted skim milk powder (10% w/v) and sugary water kefir with brown sugar solution (5% w/v). After sterilization, both substrates received a 3% w/v of specific grain inoculum, and they were incubated for 24h at 25°C, followed by maintenance at 10±2°C for 24h, sieving grains and the fermented beverages stored at 10±2°C for 28 days. All experiments were done with four repetitions.

Microbiological and physicochemical properties of kefir-fermented beverages

Microbiological properties were evaluated through LAB enumeration in De Man, Rogosa and Sharpe (MRS, Acumedia, Lansing) and yeast count in Potato Dextrose Agar (PDA, Acumedia). The physicochemical analysis included pH, titratable acidity, fat, protein and lactose measurements [22]. The analysis was conducted, always duplicated, at 1, 2, 7 and 28 days post-inoculation of substrates with the grains.

Identification of the transcriptionally active microorganisms in kefir-fermented beverages by transcriptomic analysis (RNA-seq)

One milliliter of kefir-fermented milk or 40 mL of water kefir was centrifuged for 10min at 10,000xg, the cell pellets were transferred to microtubes containing 0.3g of zirconium beads, ruptured in the FastPrep-24 instrument (MP Biomedicals), and total RNA was extracted using the RNAeasy mini kit (Qiagen), according to the manufacturer's recommendations. The extracted RNA was reversed-transcribed to cDNA to build libraries for NGS sequencing. The samples were divided into two parts, one destined to analyze the bacteria and the other the study of yeasts. The bacterial sample was treated with the Ribo-Zero rRNA removal kit, and the yeast sample was enriched with the capture of mRNAs by the poly-A tail, all the

procedures according to the manufacturer's recommendations (Illumina).

The cDNA libraries were elaborated according to the RNA Sample sequencing protocol from Illumina, and sequencing by bridging PCR in MiSeq sequencer, as stated by the manufacturer (Illumina).

MiSeq reagent kit v3 (600-cycle) was used to enable the highest output of sequenced information (15Gb, 2x300 bp, up to 25 million reads).

Analysis of bioinformatics

The computational issues were developed in the servers Sagarana and Truta, located in the Laboratories of Informatics of the ICB/UFMG and Fiocruz/MG, in GNU Linux/Debian operating system. Some small computational algorithms were developed throughout the project. These scripts were made in Python programming language. The computational strategy used was multithreading, aiming to increase performance and reduce the processing time associated with the programs used.

To perform the first stage of pipeline development for the RNA-seq analysis, Trimmomatic and FastQC were used for pre-processing and quality analysis of the reads. Then, FASTQ-Join [23] merged the sequences forward and reverse to form consensus sequences that were aligned via MegaBLAST with NCBI NT database (nucleotide sequences), by the software HS-BLASTN [24]. In the third step of the pipeline for RNA-seq analysis, assembly in contigs and functional annotation of reads were performed using Trinity software [25], Transdecoder [26], AC-DIAMOND and STAR software. The Transdecoder identifies which contigs are mRNA and what possible ORFs. The AC-DIAMOND aligns by BLASTx the annotated contigs as mRNA against the NCBI NR database (non-redundant protein sequences) and UEKO-UniRef Enriched KEGG Orthology [27]. Finally, STAR software aligns the reads again against the contigs annotated as mRNA for quantifying the gene expression.

The transcriptionally active microbiome of Bacteria by multilocus sequence analysis (bTAM) and yeast by rRNA ITS sequence analysis (fTAM)

BLASTx searched contigs related to the housekeeping and ribosomal protein genes in the UniProt revised protein database for MLSA and rMLSA analysis [28]. Seventy-eight housekeeping markers related to the RNA polymerase core subunits and sigma factors, RNA polymerase-associated proteins, transcription elongation and termination factors, DNA replication initiation, elongation and termination factors, and DNA topoisomerases were chosen for MLSA; and seventy-five genes related to the ribosome-associated proteins and protein translation initiation, elongation, and release factors were selected for rMLSA (Supplementary Table S1).

For the identification of yeasts, it was used a dataset with all ITS (Fungal Internal Transcribed Spacer RNA) sequences available in the RefSeq Targeted Loci Project-Bio Project, of NCBI.

Statistical analysis

Statistical analysis was conducted at SAS software 9.2 (SAS Institute Inc., Cary, NC, USA), at a 5% significance level. The effects of storage conditions on the microbiological and physicochemical characteristics of kefir were determined by Analysis of Variance

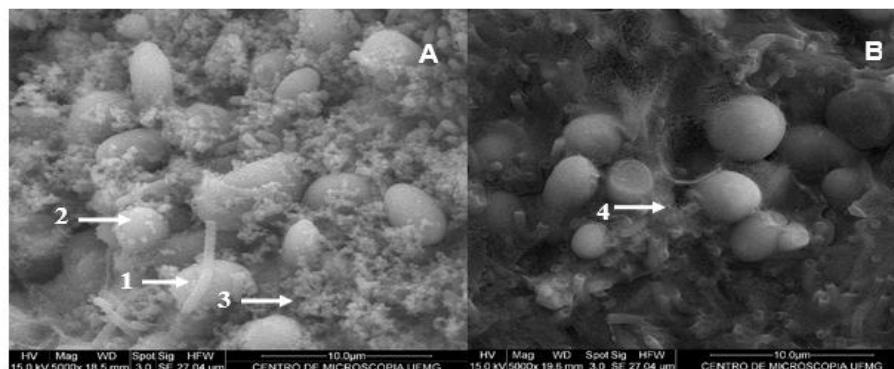


Figure 1: Scanning Electronic Microscopic (SEM) of Brazilian milk kefir grains. Micrograph A: Outer surface of KLSA grains. 1-Bacillus, 2-Yeast, and 3-Granular material (cotted protein). Micrograph B: Inner part of fractured KLSA grains. Presence of bacteria (long and rods; bacilli in pairs) and yeast. 4-Fibrillar material: EPS kefiran.

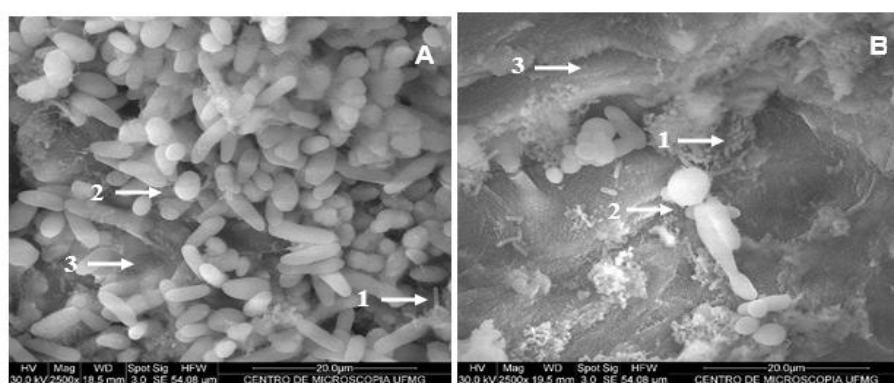


Figure 2: Scanning Electronic Microscopic (SEM) of Brazilian sugary water kefir grains. Micrograph A: Outer surface of KASA grains. Micrograph B: Inner part of fractured KASA grains. 1-Bacillus, 2-Yeast (rod and elongated shapes), and 3-Polysaccharidic matrix.

(ANOVA) and, if necessary, by the Tukey test.

Results and Discussion

Microstructure of Brazilian kefir grains

Brazilian milk kefir grains studied herein were in general small, round, with an irregular shape, white or yellow, similar to cauliflower pieces. Water kefir grains had a consistent gelatinous aspect, yellow and translucent, with irregular shape and size. Both types of grains are very different between them and similar to other kefir grains worldwide [2-4,29-31].

Scanning Electronic Microscopic (SEM) of two Brazilian milk kefir grains whose internal portions were obtained by cryogenic fracture showed, in both of them, presence of yeast at surface level (Figure 1A) and also at the internal part of the grain (Figure 1B). At the grains' surface, it was possible to observe rod-shaped bacteria (Figure 1A-1), a large number of yeasts with short and long shape (Figure 1A-2), and granular material (Figure 1A-3), which has been described as clotted protein [2]. At the internal portion, bacilli and yeast, all involved by a fibrillar and porous structure to which microorganisms are attached to, can be observed as described by other authors [4,21,29,31].

Brazilian water kefir grains presented bacteria and yeast adhered

to a matrix that covers the whole grain surface (Figure 2A) and the internal surface (Figure 2B), as it was seen in milk grains. This matrix is smooth and poreless on the outside and spongy on the inside. Yeast shape was similar to the previously described for Brazilian milk kefir grains. Microbial density was lower than the observed in milk kefir grains, especially at the core of the grain. These characteristics was similar to the observed for others water grains studied worldwide [3,32].

The SEM findings were confirmed by Transmission Electron Microscopy (TEM) (Figure 3A). Microbiota from grains was active at the moment of fixation since it could be observed duplicating bacteria and budding yeasts (Figure 3B and 3C).

Milk kefir microbiological and physicochemical characteristics

There was no significant difference in average viable counts of LAB and yeast in milk kefir during 28 days of cold storage (10°C) for almost all the beverages made with grains from different Brazil places (Table 1). The maintenance of at least 10^7 CFU/ml of LAB and 10^4 CFU/ml of Yeast for 28 days is in accordance with national and international standards for kefir [6,7]. Since LAB and yeast counts did not change significantly between the first and the second day of fermentation, it could be established 24h at 25°C as fermentation



Figure 3: Transmission Electron Microscopy (TEM) of Brazilian kefir grains. Micrograph A: KLSA grain. Micrograph B and C: Metabolically active grains (presence of bacteria in the process of duplication and budding yeasts, respectively).

Table 1: LAB and yeast counts of milk kefir produced with grains of different origins, over time (days) (n=4).

| Microorganism | Sample | Time (Days) | | | | P-Value |
|-----------------------|--------|--------------------------|--------------------------|--------------------------|--------------------------|---------|
| | | 1 | 2 | 7 | 28 | |
| LAB (Log CFU/mL) | KLCU | 7.00 ± 0.82 ^a | 7.58 ± 0.49 ^a | 7.67 ± 0.47 ^a | 7.18 ± 0.88 ^a | 0.489 |
| | KLDI | 7.22 ± 1.12 ^a | 7.57 ± 1.00 ^a | 7.33 ± 1.22 ^a | 6.93 ± 0.95 ^a | 0.8677 |
| | KLSA | 6.77 ± 0.37 ^a | 7.89 ± 0.88 ^a | 7.55 ± 1.00 ^a | 7.58 ± 0.87 ^a | 0.3009 |
| | KLVI | 6.84 ± 0.66 ^a | 7.35 ± 0.93 ^a | 7.47 ± 1.33 ^a | 6.78 ± 0.79 ^a | 0.6691 |
| Yeast (Log CFU/mL) | KLCU | 5.41 ± 0.19 ^b | 5.97 ± 0.28 ^a | 6.05 ± 0.17 ^a | 6.25 ± 0.27 ^a | 0.0016 |
| | KLDI | 5.55 ± 0.32 ^b | 6.22 ± 0.22 ^a | 6.23 ± 0.25 ^a | 6.36 ± 0.25 ^a | 0.0039 |
| | KLSA | 5.78 ± 0.46 ^a | 6.12 ± 0.27 ^a | 6.19 ± 0.19 ^a | 6.13 ± 0.44 ^a | 0.4139 |
| | KLVI | 5.70 ± 0.60 ^a | 6.00 ± 0.47 ^a | 6.38 ± 0.43 ^a | 6.42 ± 0.55 ^a | 0.2097 |

Different lowercase letters in the lines indicate a significant difference by the Tukey test (P <0.05). KLCU-Curitiba; KLDI-Divinópolis; KLSA-Salvador; KLVI-Viçosa.

Table 2: Physicochemical features of milk kefir produced with grains of different origins, over time (days) (n=4).

| Parameter | Sample | Time (Days) | | | | P-Value |
|---------------------------------|--------|--------------------------|---------------------------|---------------------------|--------------------------|---------|
| | | 1 | 2 | 7 | 28 | |
| pH | KLCU | 4.59 ± 0.43 ^a | 4.68 ± 0.44 ^a | 4.62 ± 0.30 ^a | 4.54 ± 0.26 ^a | 0.9443 |
| | KLDI | 4.81 ± 0.27 ^a | 4.68 ± 0.30 ^a | 4.59 ± 0.26 ^a | 4.54 ± 0.19 ^a | 0.4948 |
| | KLSA | 5.05 ± 0.12 ^a | 4.97 ± 0.14 ^a | 4.86 ± 0.15 ^a | 4.76 ± 0.20 ^a | 0.0922 |
| | KLVI | 4.95 ± 0.19 ^a | 4.86 ± 0.23 ^a | 4.74 ± 0.16 ^a | 4.64 ± 0.22 ^a | 0.1975 |
| Titratable acidity ^a | KLCU | 0.74 ± 0.37 ^a | 0.83 ± 0.26 ^a | 1.02 ± 0.33 ^a | 1.30 ± 0.39 ^a | 0.1611 |
| | KLDI | 0.70 ± 0.20 ^b | 0.85 ± 0.16 ^{ab} | 0.98 ± 0.24 ^{ab} | 1.19 ± 0.25 ^a | 0.0416 |
| | KLSA | 0.59 ± 0.15 ^c | 0.70 ± 0.11 ^{bc} | 0.84 ± 0.03 ^{ab} | 1.02 ± 0.08 ^a | 0.0004 |
| | KLVI | 0.61 ± 0.14 ^c | 0.75 ± 0.11 ^{bc} | 0.88 ± 0.10 ^{ab} | 1.07 ± 0.13 ^a | 0.0012 |
| Lactose ^b | KLCU | ND | 3.57 ± 0.40 ^a | 3.30 ± 0.34 ^a | 2.77 ± 0.49 ^a | 0.0623 |
| | KLDI | ND | 3.32 ± 0.47 ^a | 3.15 ± 0.32 ^a | 2.82 ± 0.28 ^a | 0.1966 |
| | KLSA | ND | 3.61 ± 0.41 ^a | 3.44 ± 0.23 ^{ab} | 2.87 ± 0.42 ^b | 0.0444 |
| | KLVI | ND | 3.55 ± 0.50 ^a | 3.29 ± 0.35 ^a | 2.90 ± 0.43 ^a | 0.148 |
| Fat ^b | KLCU | ND | 0.11 ± 0.03 ^a | 0.10 ± 0.01 ^a | 0.13 ± 0.01 ^a | 0.1792 |
| | KLDI | ND | 0.11 ± 0.04 ^a | 0.11 ± 0.01 ^a | 0.12 ± 0.02 ^a | 0.9995 |
| | KLSA | ND | 0.11 ± 0.04 ^a | 0.10 ± 0.01 ^a | 0.13 ± 0.01 ^a | 0.5332 |
| | KLVI | ND | 0.11 ± 0.02 ^a | 0.11 ± 0.02 ^a | 0.12 ± 0.01 ^a | 0.2009 |
| Protein ^b | KLCU | ND | 3.70 ± 0.09 ^a | 3.61 ± 0.08 ^a | 3.67 ± 0.22 ^a | 0.6778 |
| | KLDI | ND | 3.67 ± 0.17 ^a | 3.79 ± 0.09 ^a | 3.74 ± 0.09 ^a | 0.4275 |
| | KLSA | ND | 3.68 ± 0.07 ^a | 3.64 ± 0.13 ^a | 3.69 ± 0.12 ^a | 0.7602 |
| | KLVI | ND | 3.73 ± 0.13 ^a | 3.67 ± 0.08 ^a | 3.60 ± 0.16 ^a | 0.3938 |

^aTitratable acidity expressed as mg lactic acid/mL; ^bLactose, fat, and protein expressed as g/100g. Different lowercase letters in the lines indicate a significant difference by the Tukey test (P <0.05). KLCU-Curitiba; KLDI-Divinópolis; KLSA-Salvador; KLVI-Viçosa.

condition. In terms of industrial production, reducing the time of fermentation means reduced costs overall.

Beverages' pH values ranged from 4.54 to 5.05, which is close to criteria used to stop the fermentation process of fermented milk [33], and did not significantly vary during 28 days of cold storage in all milk kefir. Rapid pH reduction is essential to alter the drink consistency [34,35]. The pH of the kefir also influences its microbiome, since it favours lactic acid bacteria and reduces most pathogenic bacteria [5,34]. Titratable acidity increased in all beverages during the cold storage. Acidity rise can be explained by the metabolic activity of acetic and lactic acid bacteria in kefir and lactose fermentation by some yeast strains [4].

As expected, lactose concentration reduced significantly ($P < 0.05$) in all beverages along the storage time (Table 2). The lower lactose content is desirable considering that lactose intolerance affects, worldwide, a significant portion of the adult population, and fermented products, such as kefir, appears as a great alternative to dairy consumption [36,37]. Protein content did not change during fermentation or cold storage. Regarding fat content, our milk kefir can be classified as skim, as it was used milk with 0.5% of fat as substrate (Table 2).

Fermented milk tested herein attended national [6] and international [7] requirements after 48h of fermentation (Table 3). Since there is no standard criteria on pH and lactose content, we suggest 4.6 for pH and 3.5g/100g of lactose, considering the average values for milk kefir analyzed.

Sugary water kefir microbiological and physicochemical characteristics

Table 3: Microbiological and physicochemical features of milk kefir with 48 hours of fermentation (n=4) and current standards.

| Parameter | KLCU | KLDI | KLSA | KLVI | RTIQ Brasil (2007) | Codex Alimentarius (2018) |
|---------------------|--------------------|--------------------|--------------------|--------------------|--------------------|---------------------------|
| LAB (Log CFU/mL) | 3.80×10^7 | 3.71×10^7 | 7.76×10^7 | 2.24×10^7 | Min. 10^7 | Min. 10^7 |
| Yeast (Log CFU/mL) | 9.33×10^5 | 1.67×10^6 | 1.32×10^6 | 1.00×10^6 | Min. 10^4 | Min. 10^4 |
| Titrate acidity (%) | 0.83 ± 0.26 | 0.85 ± 0.16 | 0.70 ± 0.11 | 0.75 ± 0.11 | 0.5 a 1.5 | Min. 0.6 |
| Protein (%) | 3.70 ± 0.09 | 3.67 ± 0.17 | 3.68 ± 0.07 | 3.73 ± 0.13 | Min. 2.9 | Min. 2.7 |
| Fat (%) | 0.11 ± 0.03 | 0.11 ± 0.04 | 0.11 ± 0.04 | 0.11 ± 0.02 | v | < 10 |
| Lactose (%) | 3.39 ± 0.42 | 3.60 ± 0.39 | 3.57 ± 0.41 | 3.56 ± 0.47 | n.s. | n.s. |
| pH | 4.68 ± 0.28 | 4.98 ± 0.15 | 4.69 ± 0.41 | 4.85 ± 0.22 | n.s. | n.s. |

RTIQ: Technical Regulation of Identity and Quality of Fermented Milk; V: According to the fat content of the raw material; NS: Not Specified; KLCU-Curitiba; KLDI-Divinópolis; KLSA-Salvador; KLVI-Viçosa.

Table 4: LAB and yeast counts of sugary water kefir produced with grains of different origins, over time (days) (n=3).

| Microorganism | Sample | Time (Days) | | | | P-Value |
|--------------------|--------|----------------------|----------------------|----------------------|----------------------|---------|
| | | 1 | 2 | 7 | 28 | |
| LAB (Log CFU/mL) | KABH | 6.25 ± 1.02^a | 7.41 ± 0.30^a | 6.43 ± 0.19^a | 6.13 ± 0.37^a | 0.0885 |
| | KACU | 5.84 ± 0.76^a | 7.19 ± 0.17^a | 6.56 ± 0.54^a | 6.15 ± 1.03^a | 0.1821 |
| | KASA | 6.44 ± 0.18^c | 7.59 ± 0.25^a | 7.13 ± 0.23^{ab} | 6.64 ± 0.15^{bc} | 0.0006 |
| | KAVI | 6.27 ± 0.13^{ab} | 7.32 ± 0.41^a | 7.12 ± 0.70^a | 6.00 ± 0.10^b | 0.0112 |
| Yeast (Log CFU/mL) | KABH | 6.13 ± 0.49^b | 6.46 ± 0.17^{ab} | 6.70 ± 0.14^{ab} | 6.87 ± 0.21^a | 0.0559 |
| | KACU | 6.27 ± 0.38^a | 6.46 ± 0.22^a | 6.69 ± 0.09^a | 6.84 ± 0.33^a | 0.1351 |
| | KASA | 6.86 ± 0.23^a | 7.11 ± 0.53^a | 6.99 ± 0.46^a | 7.13 ± 0.54^a | 0.8727 |
| | KAVI | 5.89 ± 0.80^a | 6.27 ± 0.43^a | 6.58 ± 0.55^a | 6.82 ± 0.52^a | 0.3143 |

Different lowercase letters in the lines indicate a significant difference by the Tukey test ($P < 0.05$). KABH-Belo Horizonte; KACU-Curitiba; KASA-Salvador; KAVI-Viçosa.

All sugary water kefir beverages reached 10^7 CFU/ml of LAB on 48h, and drop to 10^6 after 28 days of cold storage (Table 4). Yeast population suffered no significant alterations during storage, maintaining an average level of 10^6 CFU/ml until the end of the experimental period. Titratable acidity has slightly increased during storage (Table 5). The pH reached 4.5 in all sugary water kefir beverages after 24h of fermentation and around 4.0 after storage. Other authors observed a similar drop in pH [3], but lower pH has already found in delayed fermentation time [10].

Currently, there are no identity and quality standards defined for sugary water kefir by Brazilian regulatory agencies. According to results of this study and the scientific literature available to this date, standards of 10^6 CFU/mL for lactic acid bacteria and yeast and 0.12mg of lactic acid/mL and pH range of 3.8 to 4.4 could be proposed.

The transcriptionally active microbiome (TAM) of milk and sugary water kefir

In the Bacterial Transcriptionally Active Microbiome (bTAM) of milk kefir (KLSA and KLCU) and sugary water kefir (KASA and KACU), Firmicutes and Proteobacteria phyla comprise more than 99.99% of protein-related reads. However, the relevance of each phylum in milk kefir or sugary water kefir is dissimilar, with Firmicutes and Proteobacteria counting 92% to 97% and 3% to 8%, respectively, of total reads in milk kefir, and 58% to 70% and 30% to 42%, in sugary water kefir (Figure 4A). The families most recurrent were Leuconostocaceae, Lactobacillaceae and Acetobacteraceae (Figure 4B). The bacterial genera most often seen in milk kefir KLSA and KLCU were *Leuconostoc* (56% and 82%) and *Lactobacillus* (7% and 39%), and in sugary water kefir KASA and KACU were

Table 5: Physicochemical features of water kefir produced with grains of different origins, over time (days) (n=3).

| Parameter | Sample | Time (Days) | | | | P-Value |
|---|--------|--------------------------|---------------------------|---------------------------|--------------------------|---------|
| | | 1 | 2 | 7 | 28 | |
| Titratable acidity (mg lactic acid/100mL) | KABH | 0.08 ± 0.03 ^b | 0.10 ± 0.02 ^b | 0.13 ± 0.03 ^b | 0.22 ± 0.03 ^a | 0.0025 |
| | KACU | 0.06 ± 0.02 ^a | 0.08 ± 0.01 ^a | 0.11 ± 0.01 ^a | 0.17 ± 0.03 ^a | 0.4921 |
| | KASA | 0.05 ± 0.01 ^b | 0.08 ± 0.02 ^{ab} | 0.13 ± 0.04 ^{ab} | 0.18 ± 0.02 ^a | 0.0397 |
| | KAVI | 0.06 ± 0.03 ^a | 0.09 ± 0.03 ^a | 0.11 ± 0.02 ^a | 0.14 ± 0.02 ^a | 0.1666 |
| pH | KABH | 4.33 ± 0.50 ^a | 4.02 ± 0.19 ^a | 3.82 ± 0.08 ^a | 3.71 ± 0.21 ^a | 0.1141 |
| | KACU | 4.57 ± 0.22 ^a | 4.34 ± 0.12 ^{ab} | 4.12 ± 0.13 ^{ab} | 3.99 ± 0.24 ^b | 0.0206 |
| | KASA | 4.57 ± 0.32 ^a | 4.35 ± 0.24 ^a | 4.06 ± 0.38 ^a | 3.91 ± 0.39 ^a | 0.1551 |
| | KAVI | 4.61 ± 0.55 ^a | 4.39 ± 0.45 ^a | 4.12 ± 0.34 ^a | 4.00 ± 0.40 ^a | 0.3848 |

Different lowercase letters in the lines indicate a significant difference by the Tukey test (P<0.05). KABH-Belo Horizonte; KACU-Curitiba; KASA-Salvador; KAVI-Viçosa.

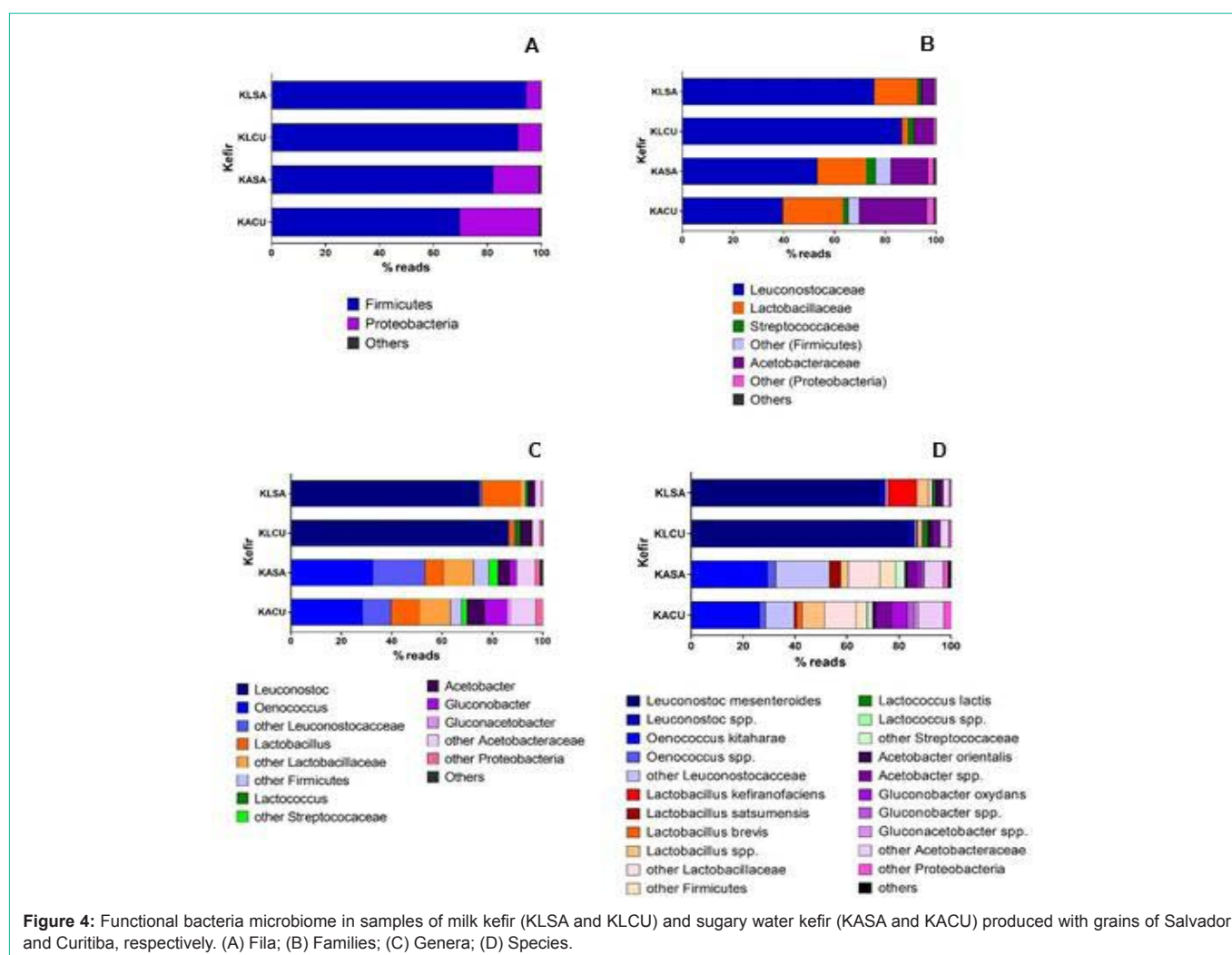


Figure 4: Functional bacteria microbiome in samples of milk kefir (KLSA and KLCU) and sugary water kefir (KASA and KACU) produced with grains of Salvador and Curitiba, respectively. (A) Filia; (B) Families; (C) Genera; (D) Species.

Oenococcus (26% and 32%), *Gluconobacter* (15% and 22%) and *Lactobacillus* (11% and 15%), despite the less accuracy of the description at the genus level in these last kefir samples (Figure 4C). Analyzing bacterial libraries, *Leuconostoc mesenteroides* (54 and 80%) and *Lactobacillus kefiranofaciens* (5 and 30%) dominated the milk kefir, while *Oenococcus kitaharae* (20 and 25%) and *Gluconobacter oxydans* (15% and 22%) were conspicuous in sugary water kefir, as

seen in Figure 4D.

Regarding Fungal Transcriptionally Active Microbiome (fTAM) of the milk kefir samples, families Pichiaceae, Dipodascaceae, Saccharomycetaceae, and Debaryomycetaceae were the most represented (KLCU, 41%, 38%, 13%, 5%; KLSA, 56%, 22%, 10%, 9%, respectively). In the microbiome of sugary water kefir, Saccharomycetaceae dominated with 92% prevalence in both samples

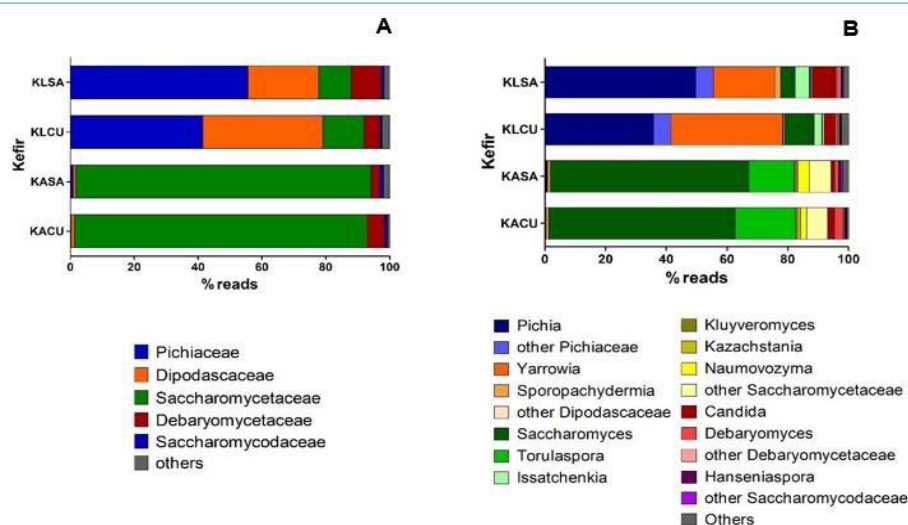


Figure 5: Functional yeast microbiome in samples of milk kefir (KLSA and KLCU) and water kefir (KASA and KACU) produced with grains of Salvador and Curitiba, respectively. (A) Families; (B) Genera.

(Figure 5A). The most common genera in the microbiome of milk kefir were *Pichia* and *Yarrowia* (KLCU, 36% and 37%; KLSA, 50% and 20%, respectively) (Figure 5B), represented by the species *P. fermentans* and *Y. lipolytica* (KLCU, 11% and 35%; KLSA, 25% and 19%, respectively). Analyzing libraries of sugary water kefir, the most found genera were *Saccharomyces* and *Torulaspora* (KACU, 61% and 20%; KASA, 65% and 15%, respectively), and the species *S. cerevisiae* and *T. delbrueckii* (KACU, 51% and 4%; KASA, 56% and 8%, respectively).

The microbial diversity of milk and sugary water kefir has been studied by culture-dependent and culture-independent approaches [2-4,8,10,29,38-40]. This knowledge is essential to understand the community dynamics, metabolite and flavour production beyond such beverages' rheological characteristics. However, the transcriptomic approach used herein allowed us to elucidate the lactic acid bacteria and yeast species metabolically active in Brazilian kefir and, for instance, effectively responsible for physicochemical characteristics of each beverage.

Conclusion

Brazilian's milk and sugary water kefir grains possess a matrix associated with its microbiota composed by bacteria and yeasts, but are macroscopically and microscopically distinct, in a similar way to other milk and sugary water kefir grains worldwide. Fermented beverages produced with four Brazilian milk kefir grains from different locations presented similar characteristics that were in accordance with the *Codex Alimentarius* and Brazilian legislation. This study suggests standards for pH and lactose content values since there is no current official definition for these milk kefir parameters. Fermented beverages produced with four Brazilian water kefir grains from different locations were also similar. This study presented microbiological and physicochemical parameters that can be used as identity standards for this type of fermented beverage since there is no Brazilian legislation that concerns sugary water kefir identity and quality. Differences in the functional microbiome of Brazilian milk and sugary water kefir allow us to conclude that they are different

fermented beverages and require establishing specific identity standards for each one by regulatory agencies. Beyond that, knowing the functional microbiome might contribute to the development of appropriate kefir starter cultures.

Acknowledgment

The authors are grateful for financial support of CAPES and FAPEMIG.

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