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# Dynamic microbiota in water kefir: microbial shift and ecological selection during fermentation

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## Abstract

This study provides a comprehensive analysis of the bacterial and fungal microbiota in four water kefir grain (WKG) samples of different geographical origins and their corresponding fermented beverages (WK), in order to understand microbial dynamics during fermentation. The findings reveal marked shifts in community composition and diversity, underscoring the selective and dynamic nature of the water kefir ecosystem.

WKG samples exhibited considerable microbial variability. Genera such as *Clostridium*, *Ethanoligenens*, *Acetobacter*, and *Gluconacetobacter* dominated specific grain samples. After fermentation, the microbial landscape became more homogeneous, with *Liquorilactobacillus* emerging as the dominant genus (43.9–65.2%) across all WK samples. Phylogenetic clustering indicated that even low-abundance taxa in grains, such as *Lentilactobacillus*, can proliferate under fermentation conditions, while others, such as *Clostridium*, were undetected post-fermentation—likely filtered by low pH, oxygen exposure, and antimicrobial metabolites produced by dominant yeasts and lactic acid bacteria.

Fungal diversity also decreased significantly. Grain samples displayed varying fungal profiles (e.g., *Brettanomyces* in WKG-A vs. *Saccharomyces* in WKG-MG), yet all fermented beverages were overwhelmingly dominated by *Saccharomyces cerevisiae* (97.5–100%). This dominance reflects *Saccharomyces*' metabolic efficiency, acid and ethanol tolerance, and competitive exclusion of other yeasts such as *Dekkera* and *Candida*, which were initially present in grains.

Overall, the results highlight a transition from taxonomically diverse grain communities to fermentation-adapted microbiota dominated by specialized taxa. These findings emphasize the importance of microbial succession and ecological selection in determining water kefir composition and quality. They also offer valuable insights for optimizing artisanal and industrial production, balancing microbial control with the preservation of functional diversity to enhance product consistency and flavor complexity.

**Keywords** Fermentation dynamics, Microbiota shift, Water kefir

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## Introduction

Water kefir is a fermented beverage, slightly acidic and effervescent, produced by the fermentation of an unrefined sugar solution to which pieces of dried or fresh fruit may be added [19]. Fermentation occurs through the use of kefir grains, gelatinous structures approximately 5 mm in diameter with an irregular shape [29], composed of glucose and fructose polymers in which a wide variety of microorganisms, including both yeasts and bacteria, are embedded [16]. Among yeasts, members of the genera *Saccharomyces*, *Kluyveromyces*, and *Candida* are most frequently found [19], while among bacteria, lactic acid bacteria belonging to the genera *Lactobacillus*, now reclassified into multiple genera [32], *Lactococcus*, *Leuconostoc*, and *Streptococcus* are predominant [25], along with acetic acid bacteria [16]. The exact origin of kefir grains remains unknown, but three different geographical origins have been proposed: the ginger-beer plant from the Caucasus region, the Tibi grains from Mexico—originally isolated from Opuntia cactus—and the sugary kefir grains collected in France [23]. The grains currently in use have been preserved and passed down through generations. While the microbial composition remains generally similar, the exact species composition varies [5].

Water kefir production is carried out through the spontaneous fermentation of a sugar solution by the microorganisms present in the kefir grains. Typically, unrefined sugar is used as a source of carbon, energy, and other nutrients, at a concentration of 100 g/L [19]. In addition to sucrose, unrefined sugar provides nitrogen (approximately 0.64% expressed as protein), minerals, particularly calcium, potassium, magnesium, and phosphorus, as well as micronutrients and vitamins (A, B, C, D, and E), with vitamin E reaching approximately 55 mg per 100 g [14]. Occasionally, fruit peels or pulp from dried or fresh fruits are added as an additional source of nitrogen, calcium, and other macro- and micronutrients [22]. Fermentation is carried out at temperatures between 21 and 30 °C for 4 to 8 days [19]. After fermentation, the grains are recovered and can be reused by placing them in a fresh sugar solution. The microorganisms forming the kefir grains multiply in the unrefined sugar solution, leading to the formation of new grains. The sugar solution must be periodically renewed to supply the necessary nutrients for microbial growth and to remove metabolites that may become toxic when they exceed certain concentrations.

Fermentation of the sugar solutions begins with the action of microorganisms capable of hydrolyzing sucrose, releasing glucose and fructose, which can be utilized by a broader range of microorganisms. Yeasts, particularly those of the genus *Saccharomyces*, are generally responsible for this function. It has also been proposed that yeasts contribute amino acids and essential vitamins required for the growth of lactic acid bacteria [24]. Additionally, interactions with lactic acid bacteria may lead to modifications in yeast membrane permeability, potentially resulting in autolysis and the release of cellular contents, which serve as nutrients for other microorganisms in the system [19, 24]. Fermentation occurs without agitation, leading to low oxygen concentrations, as oxygen is consumed during the initial stages of the process by aerobic microorganisms. Under these conditions, the primary metabolic products of yeast and lactic acid bacteria growth include ethanol, lactic acid, acetic acid, and carbon dioxide. As a byproduct of yeast metabolism, glycerol is also produced. Acetic acid bacteria contribute to the process by carrying out incomplete respiration, using ethanol as an energy source and oxygen as an electron acceptor, thereby producing acetic acid as a primary metabolic product. These interactions indicate that the presence of all mentioned microbial groups is essential for the successful completion of the fermentation process and for imparting the specific organoleptic characteristics of water kefir [1].

Water kefir is predominantly produced at a household level, using grains passed from person to person [15]. The industrial production of this fermented beverage presents challenges that have likely hindered its commercial development [16, 19]. Among these challenges, some authors highlight the instability of the fermentation process, resulting in products of variable quality [16, 19], along with difficulties in maintaining kefir grains with a stable microbial composition. In this context, the present study will analyze the bacterial and fungal microbiota composition of four kefir grain samples from different origins, as well as the corresponding microbiota obtained after the fermentation process.

## Materials and methods

### Samples

Four water kefir grains (WKG) (WKG-A, WKG-C, WKG-MN, and WKG-MG) were obtained from different private holders in Uruguay, including households and small local producers. These grains had been propagated and shared for years as part of traditional practices, typically maintained in sucrose-based solutions at room temperature and periodically refreshed Water kefir (WK) was produced from each WKG as follows: Fifty grams of WKG were placed in a glass flask with a non-hermetic cover and supplemented with 500 ml of unrefined sugarcane solution at a concentration of 100 g/L. Fermentation was carried out at room temperature (22 ± 2 °C). After 7 days of incubation, the grains were separated from the WK (WK-A, WK-C, WK-MN, and WK-MG) using a plastic sieve. Both the WKG and the corresponding WK were subjected to amplicon-based metagenomic sequencing. In addition, water kefir samples were analyzed using microbiological and chemical methods.

### Characterization of the bacterial and fungal of WKG and WK microbiota by high-throughput amplicon sequencing

The microbiota of WKG and WK was characterized using amplicon-based metagenomic sequencing. For WKG characterization, 0.1 g of each WKG was resuspended in 900 µL of distilled water and vortexed at maximum speed for 1 min. The resulting WKG suspension was transferred to a new tube and centrifuged at 10,000 rpm for 10 min. The pellet was resuspended in 200 µL of distilled water and used for total DNA extraction. For WK microbiota characterization, 10 mL of each WK sample was centrifuged, and the resulting pellet was resuspended in 200 µL of distilled water and used for total DNA extraction.

Genomic DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research) following the manufacturer's instructions. Total DNA from each sample was quantified using a fluorometer (Qubit, Invitrogen, USA) with the Qubit dsDNA HS Assay Kit (Invitrogen, USA), and the DNA concentration was adjusted to 5.0 ng/µL. DNA samples were sent to Novogene (Beijing, China) for amplification, amplicon library preparation, and sequencing on Illumina platform. The V4 region of bacterial 16 S rRNA was amplified using the universal primers 341 F and 806R [18], while the ITS1–5 F region of fungal rRNA was amplified using the universal primers 1737 F and 2043R [9]. Following 16 S and ITS library preparation, library quality was assessed, and sequencing was performed on a NovaSeq 6000 platform (Illumina, San Diego, CA, USA) using a single-end sequencing approach.

### Data analysis of amplicon sequences

Clean data were imported and preprocessed using the Quantitative Insights into Microbial Ecology 2 (QIIME 2) pipeline (version 2019.1) for demultiplexing and quality filtering. DADA2 [6] was then used to denoise reads, merge paired ends, and generate a table of amplicon sequence variants (ASVs). Taxonomic classification of ASVs was performed using the NCBI reference database (https://www.ncbi.nlm.nih.gov/). Alpha diversity indices, including Chao1 and Shannon metrics, were calculated using the PAST software [13].

Phylogenetic trees were constructed for ASVs corresponding to lactic acid bacteria, acetic acid bacteria, and yeasts using MEGA version X, incorporating sequences from type strains retrieved from NCBI reference database. Maximum likelihood algorithm was used to infer evolutionary relationships, and evolutionary distances were computed using the Tamura-Nei model. Clade stability was assessed using 1,000 bootstrap replicates.

### Chemical and microbiological analysis of water kefir beverages

The concentrations of ethanol, lactic acid, acetic acid, glycerol, and residual sugars were determined by HPLC with refractive index detection. A Shimadzu HPLC system (Model CBM-20) equipped with an LC-20AT pump (Shimadzu Corp.), a SUPELCOGEL C-610 H column (30 cm × 7.8 mm, Supelco Co.), and a refractive index detector were used. The mobile phase consisted of 0.1% phosphoric acid. In each case, pH was measured using a Hanna pH meter (model HI2211, Hanna Instruments, USA). In addition to the chemical analysis, a microbiological evaluation of the fermented samples was performed. The concentrations of lactic acid bacteria and yeasts were determined for each water kefir beverage following the methodology described by Gonda et al. [11]. Briefly, yeast and lactic acid bacteria analyses were performed by plate counting on Potato Dextrose Agar (Oxoid Ltd., England) amended with 0.017% of chloramphenicol (Sigma-Aldrich, Missouri, MO USA) and Man, Rogosa, and Sharpe Agar (Oxoid Ltd., Hampshire, England) with 0.04% cycloheximide (Sigma-Aldrich, Missouri, USA), respectively. Plates were incubated for four days at 28 ∘C, in aerobic and anaerobic conditions, respectively. Results were analyzed by ANOVA and significant differences between means were determined by an LSD test at a significance level of 0.05 using the INFOSTAT software package version 2017 (https://www.infostat.com.ar) (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina, 2009).

## Results

### Characterization of the bacterial microbiota using metabarcoding in WKG and the corresponding WK

The V4 region of bacterial 16 S rDNA from four water kefir grains and their corresponding water kefir beverages was characterized using metabarcoding. The raw sequence data were deposited in the Genome Sequence Archive [30] in National Genomics Data Center [21], China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences under the accession number CRA027966) (https://ngdc.cncb.ac.cn/gsa/browse/CRA027966).

The number of reads, ASVs, percentage of taxonomically classified reads and diversity indexes for WKG and WK are listed in Table 1. In both cases, the average sequence length was approximately 404 nt.

Most bacterial ASVs were assigned at the genus level using the homologous sequence alignment method and clustering with sequences from NCBI reference database (Fig. 1). According to Simpson (1-D) diversity index, among the grains samples, WKG-MN exhibited the highest diversity, while WKG-C showed the lowest. Similarly, the Shannon diversity index indicated that WKG-MN

**Table 1** Sequencing statistics of V4 region from water Kefir grains (WK-G) and water Kefir beverages (WK) samples

| Sample | Reads | ASVs | Reads classified | Shannon index | Simpson index |
|---|---|---|---|---|---|
| Water kefir grains | | | | | |
| WKG-A | 85,646 | 151 | 79,550/92,9% | 1.686 | 0.6325 |
| WKG-C | 82,745 | 332 | 76,516/93,3% | 0.9188 | 0.2552 |
| WKG-MG | 74,487 | 280 | 69,514/93,3% | 2.033 | 0.7512 |
| WKG-MN | 57,491 | 2141 | 20,200/35,1% | 6.024 | 0.9865 |
| Water kefir beverage | | | | | |
| WK-A | 146,443 | 62 | 136,539/93,2% | 2.816 | 0.8995 |
| WK-C | 144,334 | 63 | 139,425/96,6% | 2.318 | 0.818 |
| WK-MG | 143,572 | 76 | 134,138/93,4% | 2.572 | 0.8473 |
| WK-MN | 136,817 | 75 | 124,733/91,2% | 2.26 | 0.7579 |

had the greatest species richness and evenness, while WKG-C had the lowest. This reflects the fact that when abundance is dominated by a single species, the Shannon index approaches zero.

In contrast, the beverage samples exhibited comparable levels of diversity and abundance across all samples, as indicated by both diversity indices.

In water kefir grains, the bacterial microbiota composition was characteristic of each sample. Although all samples shared the predominant genera (*Acetobacter*, *Bifidobacterium*, *Clostridium*, *Ethanoligenens*, *Gluconacetobacter*, *Gluconobacter*, *Komagataeibacter*, *Lentilactobacillus*, *and Liquorilactobacillus*), their relative abundances varied among samples. For example, the

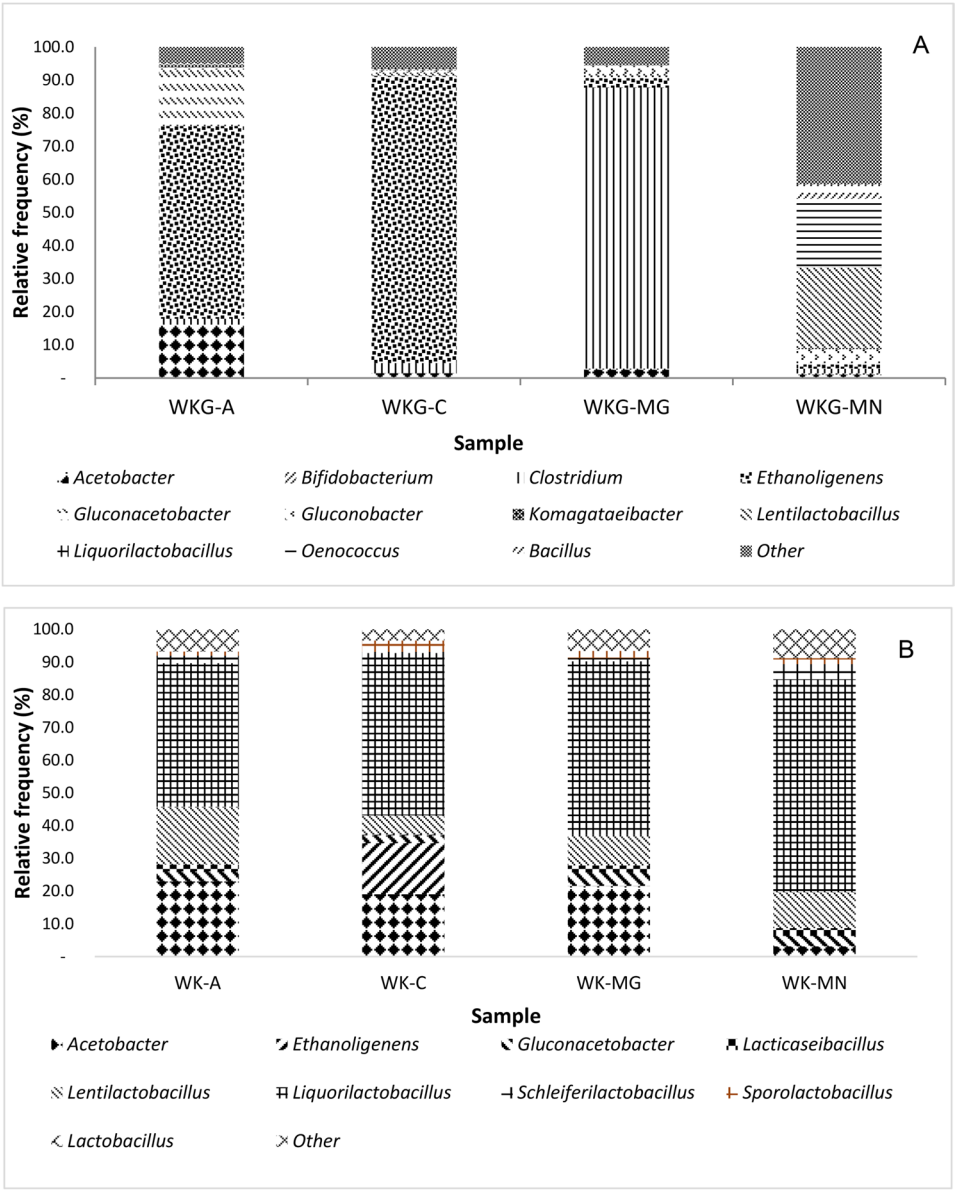



**Fig. 1** The most abundant genera and their relative abundance in each water kefir grain samples (**A**) and water kefir beverage samples (**B**)

genus *Ethanoligenens* was detected in all samples; however, it was the most abundant genus only in WKG-A and WKG-C, with 49,727 (58.1%) and 71,700 (86.7%) reads, respectively, assigned to this genus. A similar pattern was observed for the genus *Clostridium*, which was detected in all grain samples; however, its relative abundance varied from 1.5% in WKG-A to 84.8% in WKG-MG. To further investigate the *Clostridium* species associated with the grain samples, a phylogenetic tree was constructed using ASVs corresponding to clostridial bacteria (Fig. 2). Phylogenetic analysis revealed that the clostridial ASVs in granules clustered with *C. arbusti*, *C. pasteurianum*, and *C. acidisoli*.

Interestingly, although *Lentilactobacillus* and *Liquorilactobacillus* were present in all samples, their abundances were very low, ranging from 0.01% to 0.3%. Less abundant genera, representing at least 0.01% of the total sequences obtained, were grouped as "Other." In WKG-A, WKG-C, and WKG-MG, the "Other" category consisted of *Cutibacterium*, *Ruminococcus*, *Wolbachia*, *Sphingobium*, *Roseburia*, *Sphingomonas*, *Gluceribacter*, and *Blautia*. In contrast, in the WKG-MN sample, the "Other" category was much more diverse, comprising 374 ASVs with relative abundances ranging from 0.00086% to 1.07%.

In the case of water kefir, the bacterial community was highly similar across samples, with the genus *Liquorilactobacillus* being predominant in all of them (43.9%–65.2%). *Acetobacter*, *Gluconacetobacter*, *Lentilactobacillus*, *Lacticaseibacillus*, *Schleiferilactobacillus*, and *Sporolactobacillus* were present in all samples, with slight variations in their relative abundance. Notably, the genus *Ethanoligenes* was detected only in sample WK-C, while *Clostridium* genus was not detected in any sample.

Phylogenetic trees were constructed for ASVs corresponding to lactic acid bacteria and acetic acid bacteria present in grains and beverages, using MEGA version X, incorporating sequences from type strains retrieved from NCBI reference database. (Fig. 3).

Since the ASVs corresponding to lactic acid bacteria were predominant in the beverages, whereas they were found in very low proportions in the granules, only two ASVs associated with this type of bacteria originated from granules. As expected, these ASVs cluster with ASVs from beverages associated with the same taxonomic group, which could indicate that, although they were initially present in low proportions at the beginning of fermentation, they were able to proliferate throughout the process and eventually became the dominant microbiota, as mentioned above.

As previously stated, some ASVs were detected exclusively in the beverages, such as those associated with the genera *Lacticaseibacillus*, *Schleiferilactobacillus*, and *Sporolactobacillus*. According to the phylogenetic analysis, ASVs associated with the latter two genera may belong to the species *Schleiferilactobacillus harbinensis* and *Sporolactobacillus spathodeae*.

Regarding acetic acid bacteria, with the exception of the genera *Komagataeibacter* and *Gluconobacter*, which were found exclusively in granules and beverages, respectively, it can be observed that ASVs present in granules were also detected in the beverages. Based on the analysis conducted, the ASVs associated with acetic acid bacteria found in both granules and beverages clustered with the species *Acetobacter garniciae*, *A. falalx*, *A. lovaniensis*, *A. surathaniensis*, and *Gluconoacetobacter dulcium*. The ASV associated with the genus *Komagataeibacter* clustered with the species *K. saccharivorans*, whereas the ASVs associated with the genus *Gluconoacetobacter* clustered with the species *G. vitians* and *G. cerinus*.

### Characterization of the fungal microbiota using metabarcoding

Fungal community was characterized by analysis of the ITS1–5 F region of fungal rARN using metabarcoding. In addition, the raw sequence data were deposited in the Genome Sequence Archive [30] in National Genomics Data Center [21], China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences under the accession number CRA027966) (https://ngdc.cncb.ac.cn/gsa/browse/CRA027966).

Table 2 show the number of reads, ASVs, and the percentage of taxonomically classified reads for WKG and WK. In both cases, the average sequence length was approximately 318 nt.

As performed above, most fungal ASVs were assigned at the genus level using the homologous sequence alignment method and clustering with sequences from NCBI reference database (Fig. 4).

In water kefir grains, the fungal microbiota composition was also characteristic of each sample. Similar to the bacterial microbiota composition, the predominant genera *(Brettanomyces*, *Candida*, *Pichia*, *Saccharomyces*, *and Zygotorulaspora*) were shared across all samples, but their relative abundance was specific to each one. For instance, the relative abundance of the genus *Brettanomyces* ranged from 1.2% in WKG-MG to 93.5% in WKG-A, while *Saccharomyces* varied from 0.4% in WKG-A to 78.3% in WKG-MG. According to Simpson (1-D) a slight difference in diversity was observed, with the samples WKG-A and WKG-MN as the most and less diverse, respectively. Regarding Shannon index, all grain samples were similar in abundance between each other. In case of beverages, all samples were similar in diversity and abundance between each other. In all beverages, Shannon index was close to zero, indicating that abundance is primarily concentrated into one genera, which was *Saccharomyces*.

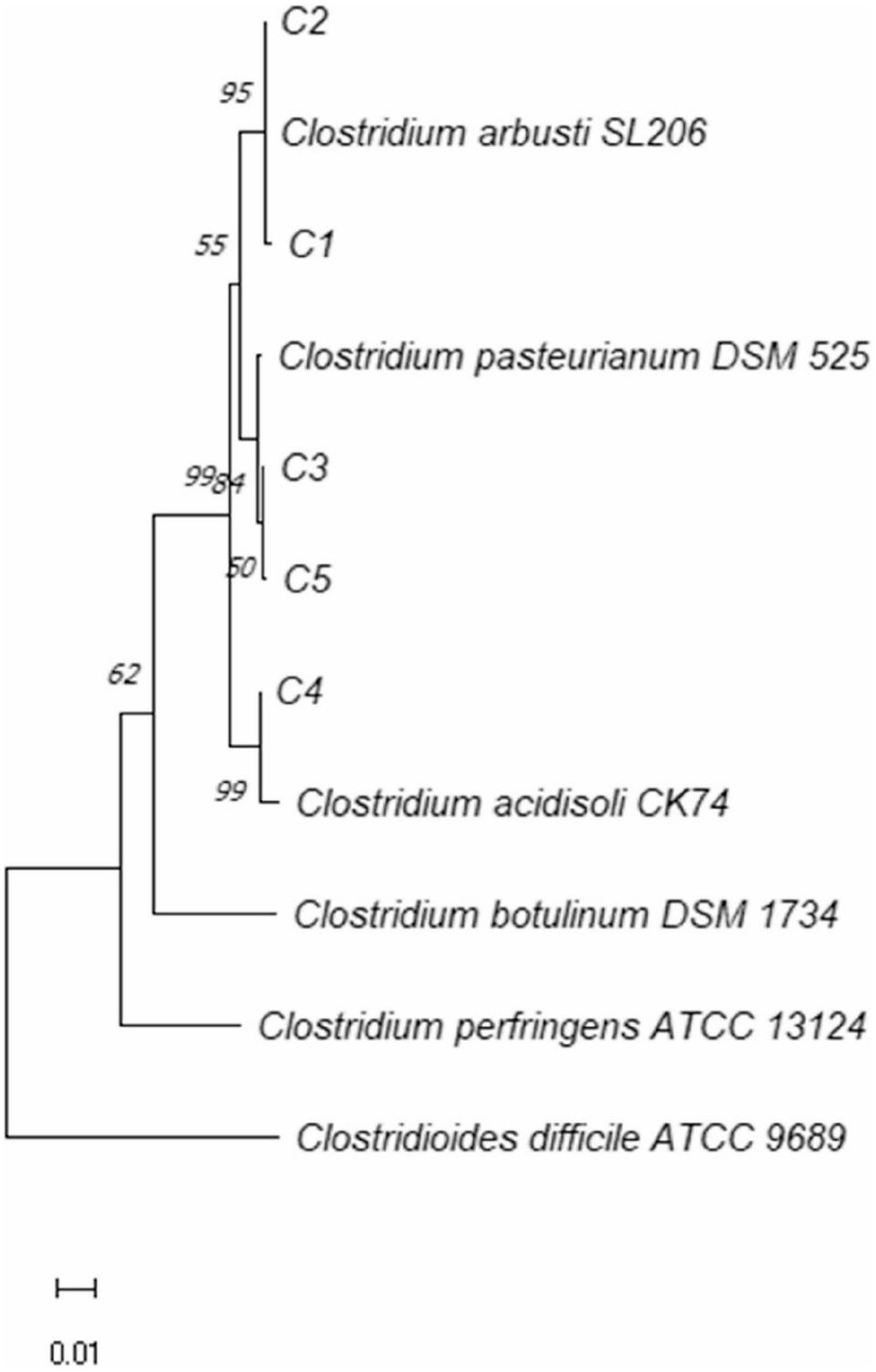



**Fig. 2** Phylogenetic tree based on sequences of ASVs corresponding to clostridia bacteria present in grains The phylogenetic tree was constructed with 15 ASVs and type-strain sequences retrieved from NCBI reference database. The tree was constructed using the Maximum likelihood algorithm. Bootstrap values (1000 replicates) are indicated at the nodes

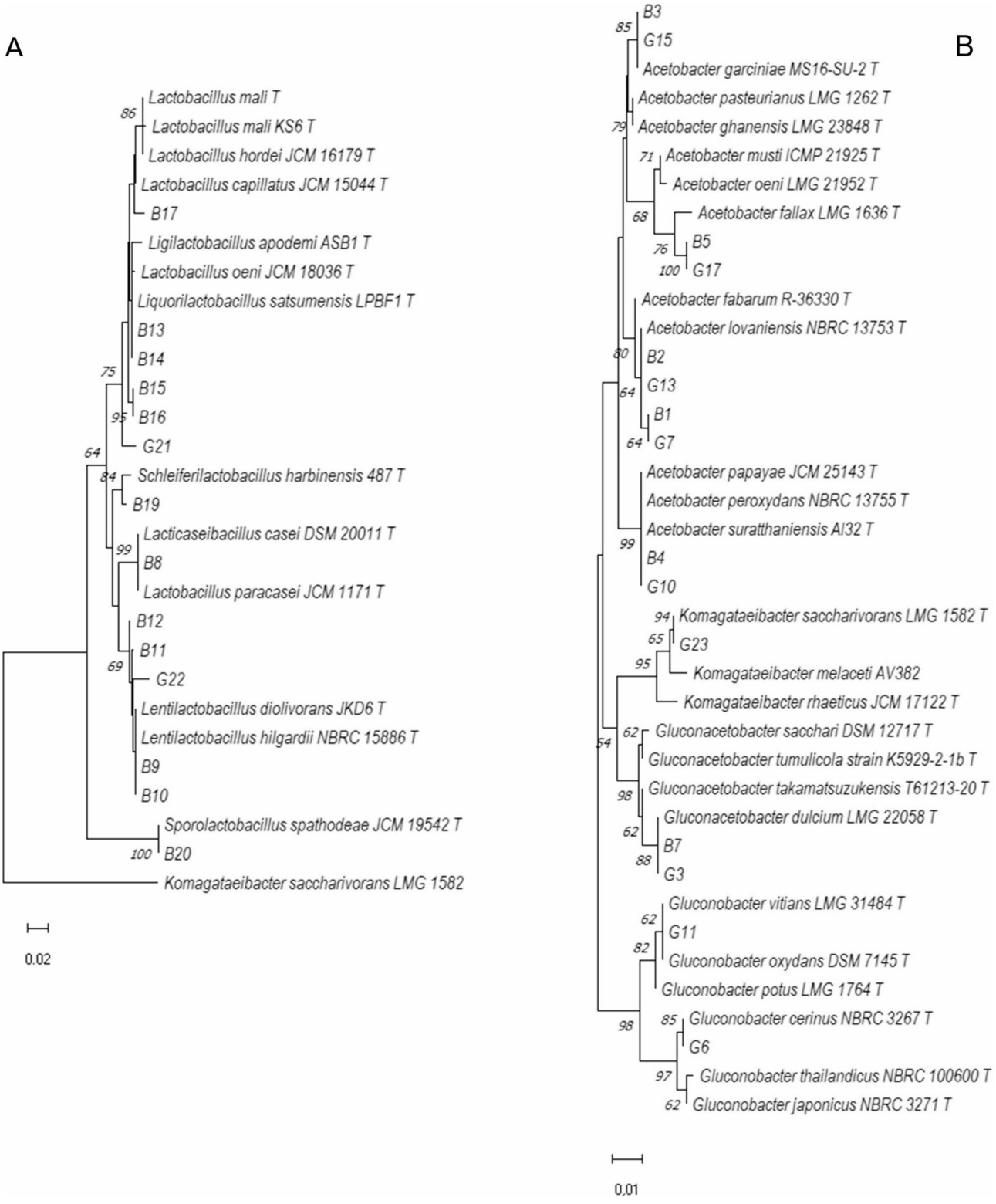



**Fig. 3** Phylogenetic tree based on sequences of ASVs corresponding to lactic acid bacteria present in grains and beverages (**A**). Phylogenetic tree based on sequences of ASVs corresponding to acetic acid bacteria present in grains and beverages (**B**). The phylogenetic trees were constructed with 14 and 15 ASVs and type-strain sequences retrieved from NCBI reference database, respectively. The trees were constructed using the Maximum likelihood algorithm. Bootstrap values (1000 replicates) are indicated at the nodes

**Table 2** Sequencing statistics of ITS-5 F from water Kefir grains (WK-G) and water Kefir beverages (WK) samples

| Sample | Reads | ASVs | Reads classified | Shannon index | Simpson index |
|---|---|---|---|---|---|
| Water kefir grains | | | | | |
| WKG-A | 63,423 | 35 | 61,848/97,8% | 1.715 | 0.7272 |
| WKG-C | 75,136 | 29 | 74,072/98,6% | 1.44 | 0.6538 |
| WKG-MG | 53,853 | 47 | 48,710/90,5% | 1.149 | 0.4701 |
| WKG-MN | 78,785 | 35 | 77,802/98,8% | 0.6842 | 0.3737 |
| Water kefir beverage | | | | | |
| WK-A | 112,486 | 8 | 112,486/100% | 0.052 | 0.014 |
| WK-C | 101,456 | 4 | 101,456/100% | 0.021 | 0.006 |
| WK-MG | 63,007 | 9 | 63,007/100% | 0.234 | 0.085 |
| WK-MN | 130,602 | 10 | 130,602/100% | 0.078 | 0.021 |

Despite the differences in the relative abundance of fungal genera in water kefir grains, after fermentation, *Saccharomyces* was the predominant genus in all samples, with a relative abundance ranging from 97.5% to 100%. The only exception was sample WK-C, where *Saccharomyces* was the sole detected genus. In all other samples, *Candida* was the second genus present in water kefir, but at a very low percentage (0.35–2.5%).

Phylogenetic trees were constructed for ASVs corresponding yeasts present in grains and beverages, using MEGA version X, incorporating sequences from type strains retrieved from NCBI reference database. (Fig. 5).

Similar to what was observed with bacterial ASVs, for the genera present in both granules and beverages, the ASVs originating from granules and associated with the genera *Candida* and *Saccharomyces* clustered with the ASVs from beverages associated with these genera. The ASVs associated with the genus *Candida* clustered with the species *C. boidinii*, whereas all ASVs associated with *Saccharomyces* clustered to *S. cerevisiae*.

According to the phylogenetic analysis, among the ASVs detected exclusively in granules, these clustered with *Pichia membranifaciens* and *Brettanomyces bruxellensis*, while the ASV associated with the genus *Zygotorulaspora* clustered with *Z. chibaensis* or *Z. florentina*.

### Chemical and microbiological analysis of water kefir beverages

According to the chemical and microbiological analyses, no significant differences were observed among the water kefir beverages. The pH values of all samples were around 3.5. With respect to residual sugars, glucose, fructose, and sucrose concentrations ranged between 0.16 and 0.26%, 0.43–0.51%, and 0.02–0.05%, respectively. The concentrations of ethanol, lactic acid, acetic acid, and glycerol varied between 0.94 and 1.32%, 0.14–0.17%, 0.10–0.17%. 0.04–0.05%, respectively. Lactic acid bacteria counts ranged from 2.8 to $3.0 \times 10^7$ CFU/mL, while yeast counts ranged from 1.6 to $2.1 \times 10^7$ CFU/mL.

## Discussion

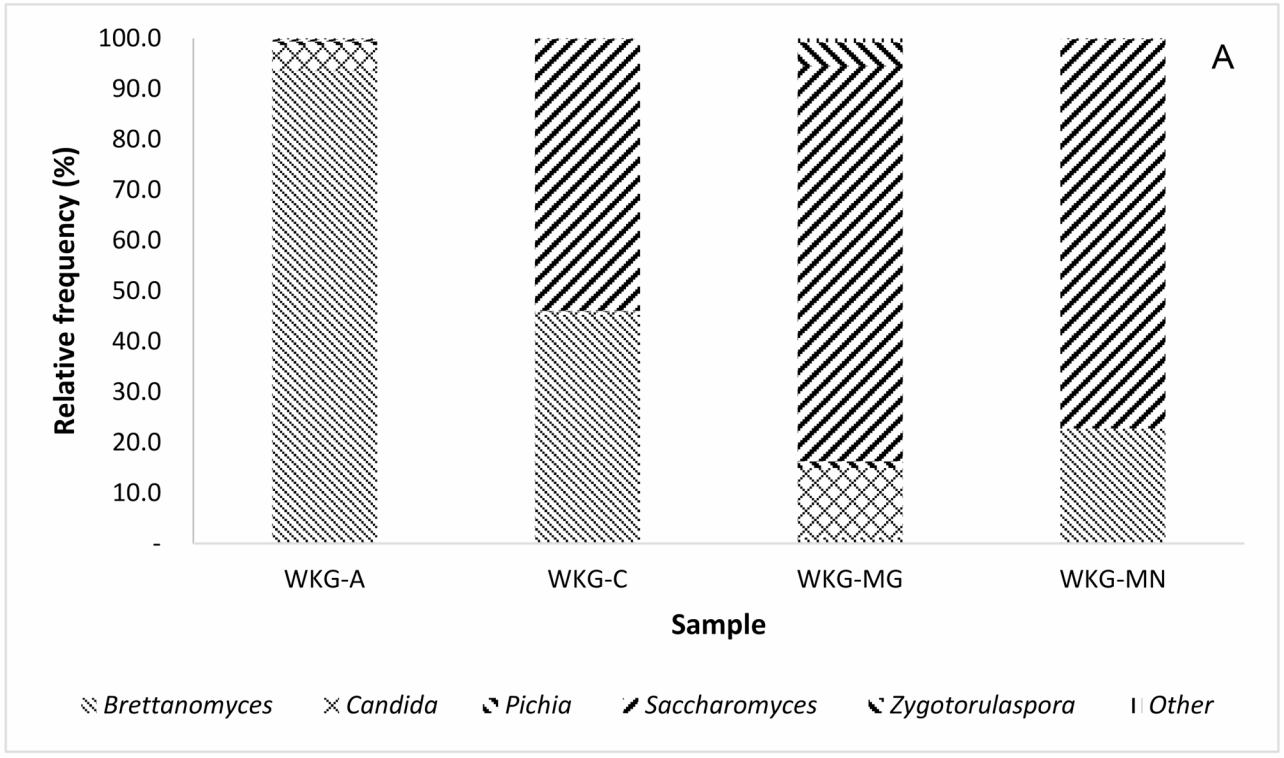
The present study provides a comprehensive analysis of the bacterial and fungal microbiota composition in four water kefir grain (WKG) samples from different origins and their corresponding fermented beverages (WK). The findings highlight significant shifts in microbial communities during fermentation, emphasizing the dynamic nature of water kefir ecosystems and their implications for product quality and stability.

The grain samples exhibited differential diversity patterns for both bacterial and fungal communities. While some genera were present in all samples, each one displayed distinct relative abundance profiles of these microbial taxa. Notably, not all microbial genera present in the grains were subsequently detected in the fermented beverage, which was obtained after 7 days of fermentation.

The bacterial composition in WKG samples exhibited high variability, with genera such as *Ethanoligenens*, *Clostridium*, *Acetobacter*, and *Gluconacetobacter* dominating specific samples. In contrast, the fermented beverages showed a more uniform microbiota, with *Liquorilactobacillus* becoming predominant (43.9–65.2%). This shift suggests that *Liquorilactobacillus* and related lactic acid bacteria (LAB) thrive under fermentation conditions, outcompeting other genera.

The phylogenetic analysis revealed that ASVs from grains clustered with those in beverages, indicating that even low-abundant bacteria in grains (e.g., *Lentilactobacillus*) can proliferate during fermentation. Notably, genera like *Schleiferilactobacillus* and *Sporolactobacillus* were detected only in beverages, suggesting their preponderant role in later fermentation stages. In contrast, the detection of *Ethanoligenens* in grains but its absence in beverages (except WK-C) supports its role as an early fermenter, producing ethanol later utilized by acetic acid bacteria (e.g., *Acetobacter*). This metabolic handoff underscores the importance of microbial succession in water kefir.

The selective presence of *Clostridium* species in water kefir grains and their absence in the final fermented beverage highlights the complex ecological dynamics of this symbiotic culture. The grain's polysaccharide matrix creates oxygen gradients that permit anaerobic microenvironments where *Clostridium* spores may persist quiescently, particularly in the grain's core [10]. While modern metabarcoding analyses confirm these findings, their significance requires careful interpretation. Although the genus *Clostridium* includes notorious pathogens (e.g., *C. botulinum*), it also encompasses commensal species and emerging probiotics [12]. For example, the non-pathogenic species *Clostridium pasteurianum* participates in butyric fermentation, producing butyric acid, small amounts of acetic acid, as well as



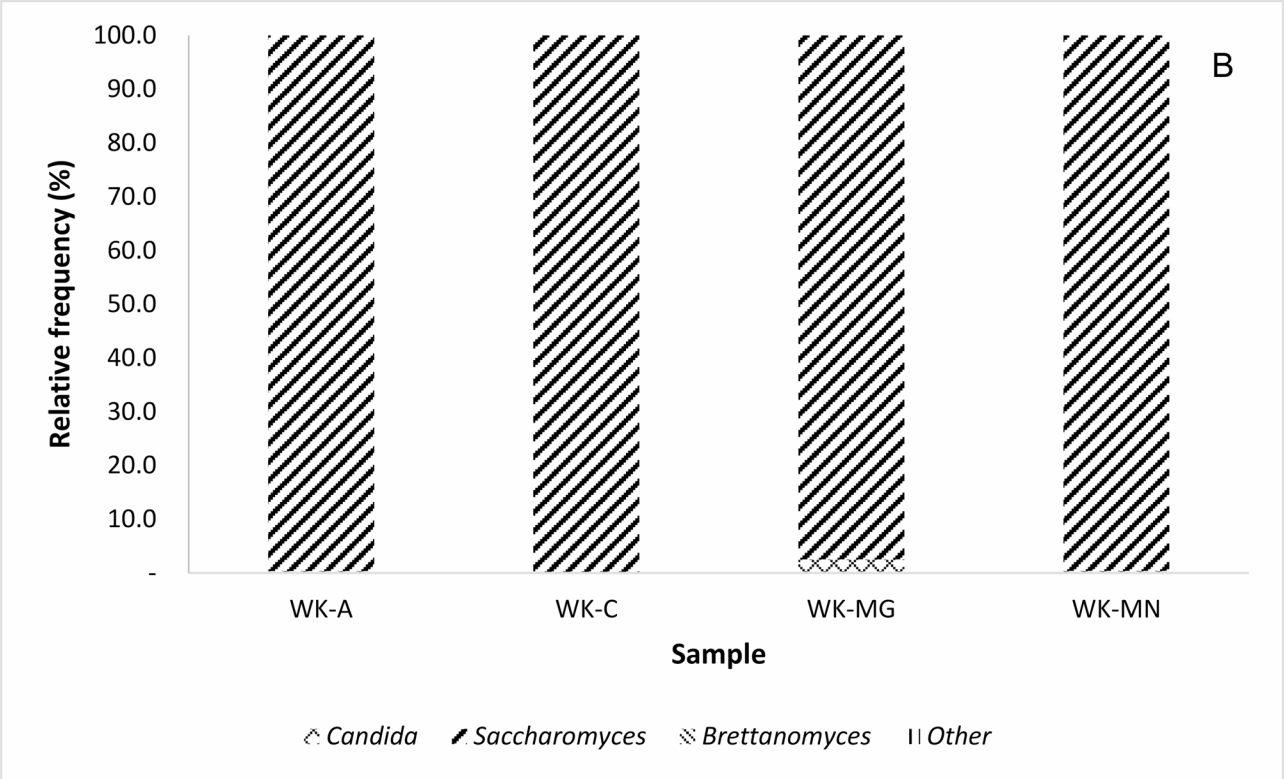




**Fig. 4** The most abundant genera and their relative abundance in each water kefir grain samples (**A**) and water kefir beverage samples (**B**)

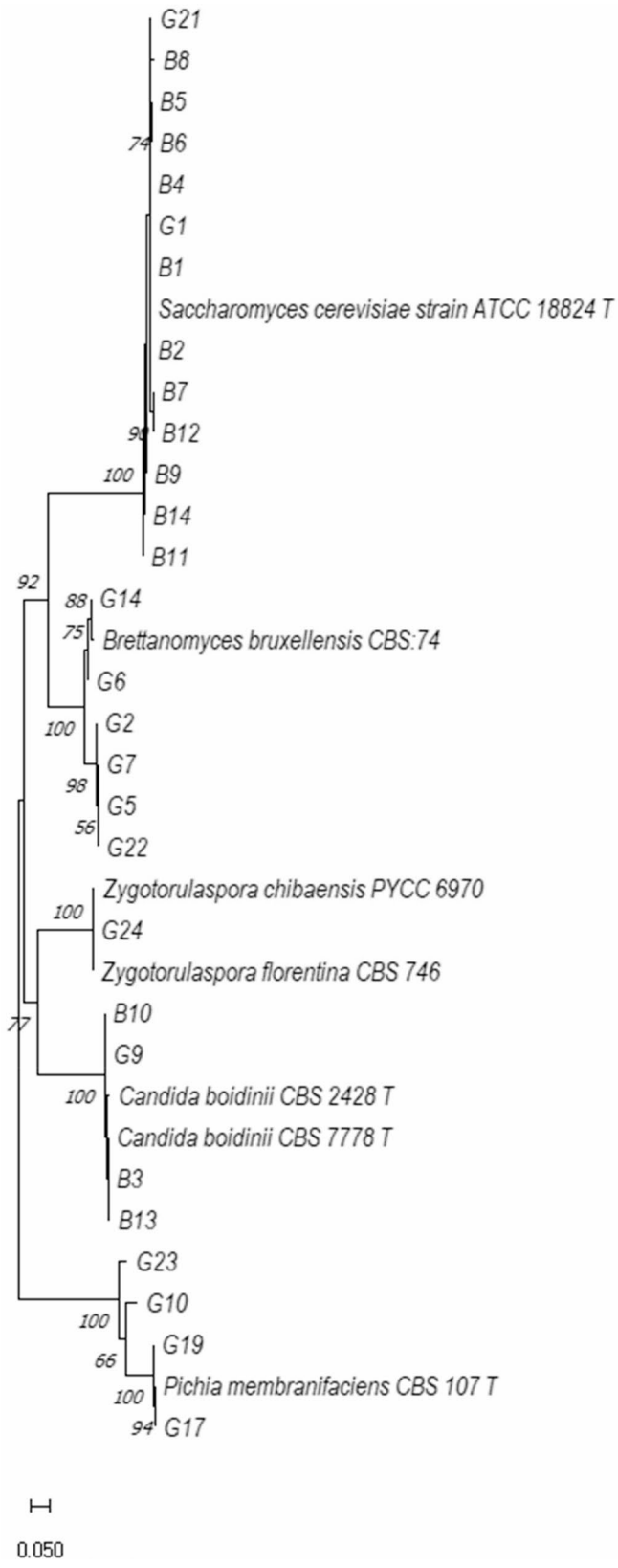



**Fig. 5** Phylogenetic tree based on sequences of ASVs corresponding to yeasts present in grains and beverages. The phylogenetic tree was constructed with 28 ASVs and type-strain sequences retrieved from NCBI reference database. The tree was constructed using the Maximum likelihood algorithm. Bootstrap values (1000 replicates) are indicated at the nodes

$CO_2$ and $H_2$ [20]. Some species may also form lactic acid and/or ethanol as well. Current food safety paradigms rightly treat most clostridia as undesirable contaminants due to toxigenic potential [3], yet their mere detection in grains does not necessarily indicate risk. The beverage pH (3.5–4.5), the relative presence of oxygen [28] and some antimicrobial metabolites generated by dominant LAB and yeasts [1] creates an effective biological barrier against *Clostridium* proliferation and toxin production. This ecological filtering underscores why properly fermented water kefir remains safe despite the grains' transient harboring of anaerobic taxa.

Fungal diversity in grains varied significantly (e.g., *Brettanomyces* in WKG-A vs. *Saccharomyces* in WKG-MG), while the beverages were overwhelmingly dominated by *Saccharomyces cerevisiae* (97.5–100%). This uniformity underscores *Saccharomyces*'s adaptability and competitive advantage in fermentative environments, likely due to its efficient sugar metabolism and ethanol and low pH tolerance. Other yeast genera (*Dekkera*, *Candida*, *Pichia*) that were detectable in grains became undetectable in the final beverage, demonstrating the stringent selective pressure of the fermentation environment. This pattern exemplifies competitive exclusion in microbial ecosystems, where a combination of metabolic efficiency and stress tolerance enables *Saccharomyces* to outcompete less-adapted taxa. In particular, the absence of *Dekkera* in fermentations could be considered beneficial, as *D. bruxellensis* is known to produce phenolic off-flavors in other fermented beverages [8].

The higher Shannon and Simpson indices in WKG-MN (bacteria) and WKG-A (fungi) reflect greater microbial diversity in these grains, possibly linked to their origin or handling. The drastic reduction in diversity post-fermentation (especially for fungi) highlights the selective pressure of the fermentation process.

Our results align with prior studies noting the variability of kefir grain microbiota [31] and the dominance of LAB and *Saccharomyces* in fermented beverages [26]. However, the near-complete dominance of *Saccharomyces* in WK contrasts with reports of more diverse fungal communities in other fermented products, possibly due to differences in substrate or fermentation duration [2, 4, 27].

While previous investigations have characterized either bacterial or fungal components of kefir grains, our study provides novel insights by simultaneously tracking the shifts in both prokaryotic and eukaryotic communities before and after the fermentation process. This study elucidates microbial shift from diverse kefir grains to uniform fermented beverages, driven by environmental and nutritional selection. The uniformity of the final beverages was demonstrated not only in terms of bacterial and fungal community composition, but also through consistent microbiological and chemical parameters The dominance of *Liquorilactobacillus* and *Saccharomyces* in WK suggests their pivotal roles in fermentation, while the loss of diversity highlights the need to balance microbial control with functional complexity for product flavor. In addition, non-*Saccharomyces* yeasts, such as *Brettanomyces* (*Dekkera*) species, may also contribute to the development of complex flavour and aroma profiles and, under aerobic conditions, can produce acetic acid [7]. Furthermore, bacteria of the genus *Ethanoligenens* are known to engage in acetate-ethanol fermentation, producing acetic acid alongside ethanol under anaerobic conditions [17], which supports the hypothesis that in water kefir fermentation *Ethanoligenens* may contribute to acetic acid formation from fermentable sugars or ethanol.These insights pave the way for optimizing water kefir production, both artisanal and industrial.

## Supplementary Information

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Supplementary Material 1.

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**Authors' contributions**
All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by GG, SV, FG, AA, EA, MG and SG. The first draft of the manuscript was written by SV and GG and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript."

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**Data availability**
The data are available at: [https://ngdc.cncb.ac.cn/gsa/browse/CRA027966](https://ngdc.cncb.ac.cn/gsa/browse/CRA027966).

## Declarations

**Ethics approval and consent to participate**
This article does not contain any studies with human participants or animals performed by any of the authors.
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

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