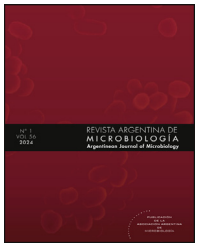
Revista Argentina de Microbiología 56 (2024) 191–197

asociación argentina de microbiología

REVISTA ARGENTINA DE MICROBIOLOGÍA

www.elsevier.es/ram



ORIGINAL ARTICLE

Bacterial diversity using metagenomics of 16s rDNA in water kefir, an innovative source of probiotics for bee nutrition

María A. Rodríguez[a,c], Leticia A. Fernández[a,b,d], Marina L. Díaz[b,e], Cristian A. Gallo[e], Miguel Corona[f], Jay D. Evans[f], Francisco J. Reynaldi[d,g,*]

[a] *Laboratorio de Estudios Apícolas (LabEA-CIC), Departamento de Agronomía, Universidad Nacional del Sur (UNS), Bahía Blanca, Buenos Aires, Argentina*
[b] *Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur (UNS), Bahía Blanca, Buenos Aires, Argentina*
[c] *Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC), Argentina*
[d] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina*
[e] *Centro de Recursos Naturales Renovables de la Zona Semiárida (CERZOS), Universidad Nacional del Sur-CONICET, Bahía Blanca, Argentina*
[f] *United States Department of Agriculture, Bee Research Laboratory, Beltsville, MD, USA*
[g] *Centro de Microbiología Básica y Aplicada (CEMIBA), Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata (UNLP), La Plata, Buenos Aires, Argentina*

Received 3 May 2023; accepted 5 December 2023
Available online 24 January 2024

KEYWORDS
Water kefir;
Bacteria;
High-throughput sequencing;
Beverages;
Anaerocolumna

Abstract Water kefir is a sparkling, slightly acidic fermented beverage made from sugar, water, and water kefir grains, which are a mixture of yeast and bacteria. These grains produce a variety of fermentation compounds such as lactic acid, acetaldehyde, acetoin, ethanol and carbon dioxide. In this study, a high-throughput sequencing technique was used to characterize the bacterial composition of the original water kefir from which potential probiotics were obtained. We studied the bacterial diversity of both water kefir grains and beverages. DNA was extracted from three replicate samples of both grains and beverages using the Powerlyzer Microbial Kit. The hypervariable V1–V2 region of the bacterial 16S ribosomal RNA gene was amplified to prepare six DNA libraries. Between 1.4 M and 2.4 M base-pairs were sequenced for the library. In total, 28 721 971 raw reads were obtained from all the samples. Estimated species richness was higher in kefir beverage samples compared to grain samples. Moreover, a higher level of microbial alpha diversity was observed in the beverage samples. Particularly, the predominant bacteria in beverages were *Anaerocolumna* and *Ralstonia*, while in grains *Liquorilactobacillus* dominated, with lower levels of *Leuconostoc* and *Oenococcus*.

* Corresponding author.
E-mail address: freynaldi@yahoo.com (F.J. Reynaldi).

https://doi.org/10.1016/j.ram.2023.12.002

Although the bacterial diversity in kefir grains was low because only three genera were the most represented, all of them are LAB bacteria with the potential to serve as probiotics in the artificial feeding of bees.

PALABRAS CLAVE
Kéfir de agua;
Bacteria;
Secuenciación de alto rendimiento;
Sobrenadante;
Anaerocolumna

Uso de la metagenómica del ARNr 16S para analizar la diversidad bacteriana del kéfir de agua, una fuente innovadora de probióticos para abejas

Resumen El kéfir de agua es una bebida fermentada con gas, ligeramente ácida, hecha de azúcar, agua y granos de kéfir de agua, que son una mezcla de levadura y bacterias. Estos granos producen una variedad de compuestos de fermentación como ácido láctico, acetaldehído, acetona, etanol y dióxido de carbono. En este estudio se utilizó una técnica de secuenciación de alto rendimiento para caracterizar la composición bacteriana del kéfir de agua original del que se obtuvieron posibles probióticos. Estudiamos la diversidad bacteriana tanto de los granos de kéfir de agua como de las bebidas. Se extrajo ADN de muestras de granos y sobrenadante (tres réplicas) utilizando el Powerlyzer Microbial Kit. Se amplificó la región V1-V2 conservada del gen del ARN ribosómico 16S bacteriano para preparar seis bibliotecas de ADN. Se secuenciaron entre 1,4 M y 2,4 M de pares de bases para la biblioteca. En total, se obtuvieron 28.721.971 lecturas sin procesar de todas las muestras. La riqueza de especies estimada fue mayor en las muestras de sobrenadante de kéfir en comparación con las muestras de granos. Además, se observó un mayor nivel de diversidad alfa microbiana en las muestras de sobrenadante. En particular, las bacterias predominantes en el sobrenadante fueron *Anaerocolumna* y *Ralstonia*, mientras que en los granos dominó *Liquorilactobacillus*, con niveles más bajos que *Leuconostoc* y *Oenococcus*. Si bien la diversidad bacteriana en los granos de kéfir fue baja debido a que solo tres géneros fueron los más representados, todos ellos fueron bacterias ácido lácticas (BAL) con potencial como probióticos en la alimentación artificial de las abejas.

Introduction

Water kefir is a sparkling, slightly acidic fermented beverage, typically produced by fermenting a sucrose solution to which fresh or dried fruits, and sometimes a slice of lemon, have been added, using kefir 'grains'[36]. This beverage is similar to but distinct from milk or dairy kefir which is produced typically with bovine milk using milk kefir grains[25]. The gelatinous grains of water kefir are a symbiotic mixture of bacteria and yeast embedded in a primarily polysaccharide matrix[19]. Water kefir is obtained by fermentation of a sugar solution with kefir grains after 48 h. The microorganisms produce a variety of fermentation compounds such as lactic acid, acetaldehyde, acetoin, ethanol and carbon dioxide[5].

The microbial populations of water kefir comprise lactic acid bacteria (LAB) such as *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Streptococcus*, yeasts, such as *Kluyveromyces*, *Candida*, *Pichia* and *Saccharomyces* and some acetic acid bacterial species[10,20,21]. The diversity, abundance, and interactions between the microbial species, estimated at around 300 species, vary according to the culture medium, origin of the grains, manufacturing techniques, temperature, substrate used and storage conditions[29,37].

It is well-documented that kefir is a source of potential probiotics[4,22,24]. In our laboratory, we have isolated bacteria and yeasts from water kefir with inhibitory *in vitro* activity against *Paenibacillus larvae* and *Ascosphaera apis*, two important bee pathogens[28]. We have also evaluated the microbial composition of water kefir grains and beverages with culture-dependent techniques to determine whether the number and type of microorganisms changed over a period of one year[14,28].

In this study, a high-throughput sequencing technique was used to characterize the microbial composition of the original water kefir from which potential probiotics were obtained. Thus, the bacterial diversity of water kefir grains and beverage was analyzed and compared to obtain a comprehensive insight into the microbiological profile of this innovative source of probiotics for bee nutrition. This analysis provides various opportunities to better understand the functional role of the microbial consortia, microbial diversity and their functional profiles within kefir-fermented beverage systems.

Materials and methods

Preparation of water kefir

Three replicates of water kefir were prepared. For each replicate, 60 g of grains were inoculated in 400 ml of sugar solution (14 g/l of unrefined sugar) with some raisins and a slice of lemon. Both, sugar and water, were previously sterilized. The beverage was contained in a glass jar with a non-hermetic cover. The fermentation process was conducted at room temperature (23 ± 2 °C) for 48 h[31,32]. After fermentation, the grains were separated from the beverage and washed with distilled water. Three replicates of water kefir were prepared for characterization.

Purification of total DNA

DNA was extracted from three replicate samples of grains and beverage which were lyophilized. For freeze-dry purposes, fresh and cleaned kefir grains were frozen at −20 °C for a period of 48 h in Petri dishes covered with aluminum foil. Samples were spray-dried in a pilot scale freeze dryer apparatus (Heto FD 1.0, Copenhagen, Denmark). Spray drying conditions were: freeze-temperature −20 °C, condenser temperature −20 to −40 °C and chamber air pressure 100–300 uHg. Final products were kept in airtight bags at room temperature until their use[26].

For recovery of the total DNA from granules, 3 g of lyophilized grains were resuspended in 40 ml of water (molecular grade) using 50 ml plastic tubes as containers. To recover DNA from the lyophilized supernatant, 14 ml of water were added to the 15 ml tubes containing the lyophilized samples. For both types of lyophilized materials, the tubes were heated for 45 min at 65 °C and vortexed every 15 min.

DNA extractions were performed using the Powerlyzer Microbial Kit (Qiagen 12255), with the following modifications: for each sample, 2 ml of the resuspended solutions were placed in 2 ml tubes and centrifuged at 10 000 RPM for 2 min. The supernatant was discarded. For each sample, the resulting pellets were resuspended in 350 μl of PowerBead solution and transferred into a Powerbead tubes containing 0.1 mm glass beads. Fifty microliter of solution SL were added to each tube and the samples were homogenized using a FastPrep®-24 5G homogenizer (MP Biomedicals) at six m/s for 2 min, three times (total 6 min). DNA purification was continued following the manufacturer's protocol without further modifications. The samples were recovered in 50 μl of elution buffer and stored at −20 °C.

PCR amplification and DNA sequencing

A hypervariable region spanning from the V1–V2 region of the bacterial 16S ribosomal RNA gene was amplified using the universal primer set and thermal protocol described by Floyd et al (2020). PCR amplification was performed using Bio-Rad master mix reagents (166509EDU) and a Bio-Rad CFX96 Real-Time System C1000 Touch Thermal Cycler. After confirmation by gel electrophoresis of the expected 359 base-pair amplicons, samples were purified using Ultracel 100K 0.5 ml centrifugal filters (Amicon). Purified PCR products were used to prepare six DNA libraries for ILLUMINA MiSeq sequencing using the TruSeq DNA library protocol. Between 1.4 M and 2.4 M base-pairs were sequenced for the library (University of Maryland, Institute for Genome Sciences sequencing facility, Baltimore, MD).

Taxonomic assignment and relative abundance

Read quality was assessed using FASTQC software[1]. Subsequently, reads were trimmed using Trimmomatic software[3] in order to remove the primers. The resulting reads were processed using MOTHUR v1.48 software[30]. Pair end reads were combined using the make.contigs command, and the result was filtered using the screen.seqs command. To remove duplicate sequences, unique.seqs command was used. Next, the resulting sequences were aligned to the SILVA reference database v. 138.1 with the align.seqs command. Further de-noise of the sequences was performed by means of the pre-cluster command, allowing for up to two differences between sequences. In order to remove chimeric sequences, the chimera.vsearch command was used. The resulting sequences were classified with the classify.seqs command, and those sequences not belonging to bacteria were removed with the remove.lineage command. Finally, the sequences were clustered into OTUs using the cluster.split command with a taxlevel=4 and a cutoff=0.03, and the resulting OTUs were classified by means of the classify.otu command. Low abundance OTUs with less than 10 reads on all samples were removed. The relative abundance of each OTU was calculated as a proportion of the sum of sequences for each sample based on annotation. The microbial community structure was estimated by the Shannon Diversity Index and Simpson's Diversity Index and were used to calculate α diversity using the NAMCO software[7] https://exbio.wzw.tum.de/namco/. The samples were normalized by the Rarefaction.

Results

Sequencing coverage

The bacterial diversity of water kefir consortia was determined by a metagenomic approach. The V1–V2 hypervariable region of the bacterial 16S ribosomal RNA gene was sequenced from six samples, three corresponding to grain samples and three to beverage samples. In total, 28 721 971 raw reads were obtained from all the samples. After processing the libraries using MOTHUR software, 2975 unique OTUs were inferred with a minimum abundance of 10 reads across all samples (Table 1).

Diversity of the microbial communities

Diversity was calculated for each data set (Table 1). The estimation of species richness was higher in kefir beverage samples (1315, an average of the three samples) compared to grain samples (667, an average of the three samples). A higher level of microbial alpha diversity, estimated by the Shannon Index (which is used to estimate the micro-

Table 1 Sequencing results for Argentinian water kefir: estimations of diversity between kefir grain (G) and beverage (B) samples.

Sample*	Number of sequences (raw reads)	OTUs	Shannon Index	Simpson Index
G-F12	3921280	678	1.59	0.35
G-G12	2969701	666	1.6	0.35
G-H12	2421456	657	1.65	0.33
Average	3104145	667	1.61	0.34
B-F12	7887669	1496	4.95	0.02
B-G12	6000839	1242	4.95	0.02
B-H12	5521026	1207	4.95	0.03
Average	6469844	1315	4.95	0.02

* Kefir grain samples: G-F12, G-G12 and G-H12 and beverage samples: B-F12, B-G12 and B-H12.

bial diversity in the sample) and by the Simpson (which is used to study diversity between samples) was observed in the beverage samples (Table 1).

Taxonomic analysis and relative abundances of the bacterial communities in each sample

In order to determine the bacterial relative abundance in each group, the inferred OTUs were used to interrogate the SILVA reference database. In the beverage, the analysis at genus level showed that the microorganisms are predominantly *Anaerocolumna* and *Ralstonia*. In grains, the most represented genus was *Liquorilactobacillus* although the genera *Leuconostoc* and *Oenococcus* are also well represented (Fig. 1).

Discussion

In our study, we used a genotype sequencing technique to describe the complex bacterial community of an Argentinian water kefir to get insights into the microbiological profile of this source of potential bee probiotics. Similar to other studies, we found that the richness and diversity indices of kefir grains were lower than those of the respective beverages[8,11,16] (Table 1). We consider two hypotheses to explain the lower levels of microbial diversity observed in the grain samples compared to beverage samples. First, an inefficient adhesion of microorganisms to the grain surface results in an increased of bacterial load in the beverage[8]. Second, while yeast and bacteria could be equally represented in the grains, bacteria could be more abundant in the beverage[22]. It is also possible that the unidentified yeasts, which are a great part of the microbial composition of water kefir, led to an underestimation of the real diversity of kefir[11,23].

In previous studies, we compared the microbial composition of water kefir grains and beverage[28]. We found that the number of bacteria and yeasts from kefir grains was significantly higher ($p < 0.01$) compared with that of the beverage. These results show that the culture-dependent techniques and the culture-independent methods, such as the high-throughput sequencing of the 16S rRNA gene, can lead to different results. Thus, it is important to utilize both, the traditional and the modern techniques to evaluate the complex microbial community present in products such as water kefir.

The analysis of the complex microbial community of water kefir revealed three predominant genera in the grain samples: *Leuconostoc* (18%) and *Oenococcus* (15%), which belong to the Leuconostocaceae family, and *Liquorilactobacillus* (55%), a member of the Lactobacilllaceae family. To our knowledge, this latter genus had not been isolated from the hive, but was previously reported in water kefir[2]. *Liquorilactobacillus* is of great importance because it secretes dextran-like EPS, which are of particular interest as potential prebiotics[33]. On the contrary, in the beverage samples, the group ''others'' achieved almost 50%, reflecting the highest diversity observed. The other genera that were sequenced in the beverage samples were not suitable candidates to be probiotics as they were not LAB or were deemed unsafe to be used as probiotics. Thus, despite the fact that the bacterial diversity in the kefir grains was lower because only three genera were the most represented, all three of them include species that are potential probiotics to be used in the artificial feeding of bees.

The metagenomic composition of water kefir microbiota has been investigated by several authors[6,13,15,17,18]. Yerlikaya et al.[38] analyzed the microbial community of commercial water kefir grains and found that they were dominated by the bacterial species *Lactobacillus ruminis* and *Bacillus methanolicus*, while the most common species were *Lactococcus lactis* and *Enterococcus faecium*. Kumar et al.[17] examined the microbial composition of water kefir from Malaysia and, similar to our study, they described the genera *Lactobacillus* and *Oenococcus* as the most abundant. They also reported *Acetobacter* as one of the most common species. Water kefir from Belgium was studied by Verce et al.[35]. They analyzed four samples using shotgun metagenomics and found evidence of a novel *Oenococcus* species related to *Oenococcus oeni* and *Oenococcus kitaharae*. It is important to notice, that *Lentilactobacillus kefiri*, is one of the main lactic acid bacteria species in kefir. Carasi et al.[4] discussed the potential of *L. kefiri* as a probiotic strain. The authors proposed that certain *L. kefiri* strains are excellent candidates for use in the development of food supplements

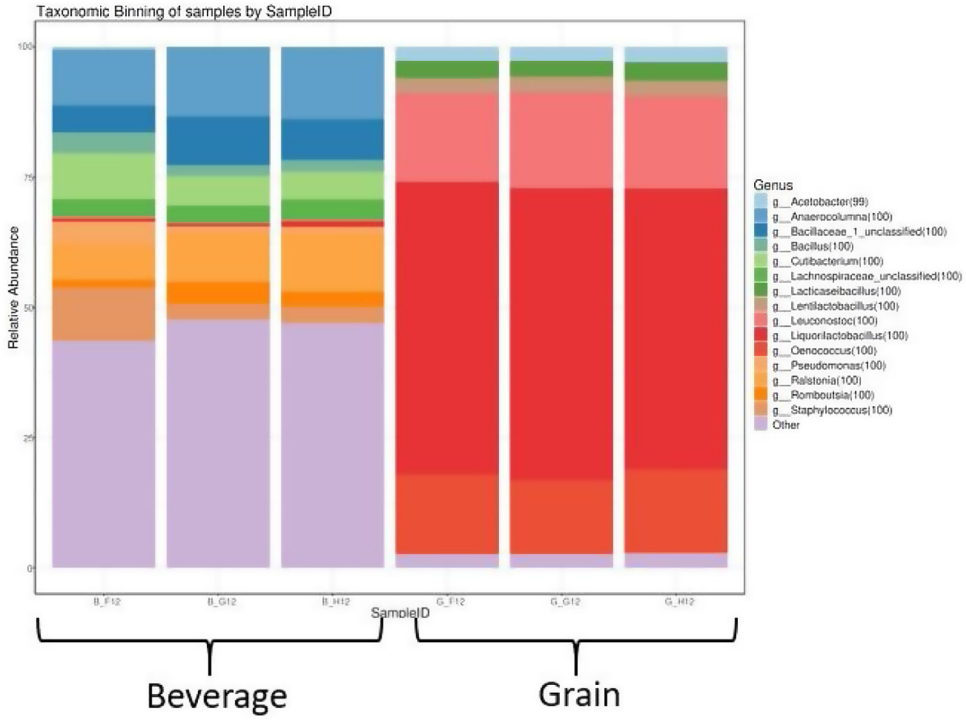


Figure 1 Relative abundance of the top 15 genera of bacteria in beverage and grain samples.

and new fermented foods with health-promoting properties in human beings.

LAB are mainly used as probiotics to improve animal health and their productive capacity[9]. Ramos et al.[27] highlighted the importance of beehives as a wide LAB reservoir, with at least 43 identified bacterial species. In particular, *Oenococcus*, *Bifidobacterium* and *Lactobacillus* have been widely studied in this regard[27]. A novel flora composed of *Lactobacillus* and *Bifidobacterium* has been identified in the honey stomach of honey bees[34]: *Lactobacillus kunkeei* (Fhon2), *Bifidobacterium asteroides* related phylotypes (Hma3, Bin7 and Bin2), *Bifidobacterium coryneforme* (Bma6) and seven *Lactobacillus* phylotypes (Hon2, Hma2, Biut2, Bma5, Hma8, Hma11 and Bin4). More recently, Hoda Mahmoud et al.[12] isolated some LAB from the intestinal tract of *Apis mellifera carnica* collected in El Cairo (Egypt). These isolates were identified as *Enterococcus faecalis* MG890204, *Enterococcus faecalis* KX073783, *Enterococcus faecalis* EU594564, *Lactobacillus brevis* MH191230 and *Lactobacillus casei* KT273339. The high-throughput sequencing of this study revealed both alpha and beta diversity (Table 1) of the bacterial population in water kefir. In this scenario, even though the beverage showed a higher level of microbial alpha diversity, the grains could be a potential probiotic tool to be used in the artificial feeding of bees because the three most represented bacteria were LAB.

In conclusion, the microbial community composition of this Argentinian water kefir studied by high-throughput sequencing technology revealed that the beverage and the grain were significantly different. The bacterial diversity and species richness of water kefir grains will be valuable to isolate beneficial strains of probiotics to control honeybee diseases. Additional studies focused on the yeast population are still necessary to further enhance our understanding of the microbial composition of this millennium-old fermented product.

Conflict of interest

The authors declare that they have no conflicts of interest.

References

1. Andrews S. FastQC: a quality control tool for high throughput sequence data; 2010. Available from: http://www.bioinformatics.babraham.ac.uk/projects/fastqc [accessed 01.12.21].
2. Badel S, Bernardi T, Michaud P. New perspectives for *Lactobacilli* exopolysaccharides. Biotechnol Adv. 2011;29:54–66, http://dx.doi.org/10.1016/J.BIOTECHADV.2010.08.011.
3. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for illumina sequence data. Bioinformatics. 2014;30:2114–20.
4. Carasi P, Malamud M, Serradell MA. Potentiality of food-isolated *Lentilactobacillus kefiri* strains as probiotics: state-of-art and perspectives. Curr Microbiol. 2022;79, http://dx.doi.org/10.1007/s00284-021-02728-x.
5. Çevik T, Aydoğdu NS, Özdemir N, Kök Taş T. The effect of different sugars on water kefir grains. Turk Tarim Gida Bilim Teknol Derg. 2019;7:40–5, http://dx.doi.org/10.24925/turjaf.v7isp1.40-45.2687.
6. De Almeida Brasiel PG, Dutra Medeiros J, Barbosa Ferreira Machado A, Schuchter Ferreira M, Do Carmo Gouveia Peluzio M, Potente Dutra Luquetti SC. Microbial community dynamics of fermented kefir beverages changes over time. Int J Dairy Technol. 2021;74, http://dx.doi.org/10.1111/1471-0307.12759.

7. Dietrich A, Steffi Matchado M, Zwiebel M, Ölke B, Lauber M, Lagkouvardos I, Baumbach J, Haller D, Brandl B, Skurk T, Hauner H, Reitmeier S. Namco: a microbiome explorer. Microb Genom. 2022;8, http://dx.doi.org/10.1099/mgen.0.000852, mgen000852.
8. Dobson A, O'Sullivan O, Cotter PD, Ross P, Hill C. High-throughput sequence-based analysis of the bacterial composition of kefir and an associated kefir grain. FEMS Microbiol Lett. 2011;320:56–62, http://dx.doi.org/10.1111/j.1574-6968.2011.02290.x.
9. Forsgren E, Olofsson TC, Vásquez A, Ingemar F. Novel lactic acid bacteria inhibiting *Paenibacillus larvae* in honey bee larvae. Apidologie. 2010;41:99–108, http://dx.doi.org/10.1051/apido/2009065.
10. Franzetti L, Galli A, Pagani MA, Noni I. Microbiological and chemical investigations on sugar kefir drink. Ann Microbiol Enzimol. 1998;48:67–80.
11. Gamba RR, Koyanagi T, León Peláez A, De Antoni G, Enomoto T. Changes in microbiota during multiple fermentation of kefir in different sugar solutions revealed by high-throughput sequencing. Curr Microbiol. 2021;78:2406–13, http://dx.doi.org/10.1007/s00284-021-02501-0.
12. Hoda Mahmoud E, Abdel-rhman Abdel-atti A, Nasr Fawzy N, Yasser Essam E, Ashwak Abdel Moneim H. Isolation and identification of lactic acid bacteria from the intestinal tracts of honey bees, *Apis mellifera* L., in Egypt. J Apicul Res. 2020;60:1–9, http://dx.doi.org/10.1080/00218839.2020.1746019.
13. Ibacache-Quiroga C, González-Pizarro K, Charifeh M, Canales C, Díaz-Viciedo R, Schmachtenberg O, Dinamarca MA. Metagenomic and functional characterization of two Chilean kefir beverages reveals a dairy beverage containing active enzymes, short-chain fatty acids, microbial β-amyloids, and bio-film inhibitors. Foods. 2022;11:900, http://dx.doi.org/10.3390/foods11070900.
14. Ibáñez Battiston TE, Rodríguez MA, Fernández LA. Probióticos y abejas: aliados en la apicultura. Ciencia Hoy. 2022;181:50–4.
15. Kalamaki MS, Angelidis AS. High-throughput, sequence-based analysis of the microbiota of Greek kefir grains from two geographic regions. Food Technol Biotechnol. 2020;58:138–46, http://dx.doi.org/10.17113/ftb.58.02.20.6581.
16. Kazou M, Grafakou A, Tsakalidou E, Georgalaki M. Zooming into the microbiota of home-made and industrial kefir produced in Greece using classical microbiological and amplicon based metagenomics analyses. Front Microbiol. 2021;12:621069, http://dx.doi.org/10.3389/fmicb.2021.621069.
17. Kumar MR, Yeap KS, Mohamad NE, Abdullah JO, Khalid M, Leow AT, Alitheen NB. Metagenomic and phytochemical analyses of kefir water and its subchronic toxicity study in BALB/c mice. BMC Complement Med Ther. 2021;21:183, http://dx.doi.org/10.1186/s12906-021-03358-3.
18. Liu W, Zhang M, Xie J, Wang H, Zhao X, Chen B, Suo H. Comparative analyses of microbial community diversities of Tibetan kefir grains from three geographic regions. Int J Dairy Technol. 2019;72, http://dx.doi.org/10.1111/1471-0307.12616.
19. Lynch Kieran M, Wilkinson S, Daenen Luk, Arendt Elke K. An update on water kefir: microbiology, composition and production. Int J Food Microbiol. 2021;345:109128, http://dx.doi.org/10.1016/j.ijfoodmicro.2021.109128.
20. Magalhães KT, de Melo Pereir GV, Ribeiro Dias D, Freitas Schwan R. Microbial communities and chemical changes during fermentation of sugary Brazilian kefir. World J Microbiol Biotechnol. 2010;26:1241–50, http://dx.doi.org/10.1007/s11274-009-0294-x.
21. Miguel MG, Cardoso PG, Lago LA, Schwan RF. Diversity of bacteria present in milk kefir grains using culture-dependent and culture-independent methods. Food Res Int. 2011;43:1523–8, http://dx.doi.org/10.1016/j.foodres.2010.04.031.
22. Moretti AF, Moure MC, Quiñoy F, Esposito F, Simonelli N, Medrano M, Peláez LA. Water kefir, a fermented beverage containing probiotic microorganisms: from ancient and artisanal manufacture to industrialized and regulated commercialization. Future Foods. 2022;5:100123, http://dx.doi.org/10.1016/j.fufo.2022.100123.
23. Marsh AJ, O'Sullivan O, Hill C, Ross RP, Cotter PD. Sequence-based analysis of the microbial composition of water kefir from multiple sources. FEMS Microbiol Lett. 2013;48:79–85, http://dx.doi.org/10.1111/1574-6968.12248.
24. Plessas S, Kiousi DE, Rathosi M, Alexopoulos A, Kourkoutas A, Mantzourani I, Galanis A, Bezirtzoglou E. Isolation of a *Lactobacillus paracasei* strain with probiotic attributes from kefir grains. Biomedicines. 2020;8:594, http://dx.doi.org/10.3390/biomedicines8120594.
25. Prado MR, Blandón LM, Vandenberghe LPS, Rodríguez C, Castro GR, Thomaz-Soccol V, Soccol CR. Milk kefir: composition, microbial cultures, biological activities, and related products. Front Microbiol. 2015;6:422, http://dx.doi.org/10.3389/fmicb.2015.01177.
26. Ramírez Huaman A. Actividad metabólica, viabilidad y exopolisacáridos de bacterias lácticas de granos de kéfir liofilizado con potencial tecnológico en la región central. Tesis para optar el título profesional de: Ingeniero en Industrias Alimentarias. Facultad de Ingeniería en Industrias Alimentarias, Universidad Nacional del Centro del Perú, Huancayo – Perú; 2020.
27. Ramos OI, Basualdo M, Linonatti C, Vega MF. Current status and application of lactic acid bacteria in animal production systems with a focus on bacteria from honey bee colonies. J Appl Microbiol. 2019;128:1248–60, http://dx.doi.org/10.1111/jam.14469.
28. Rodríguez MA, Fernández LA, Díaz ML, Pérez M, Corona M, Reynaldi FJ. Microbiological and chemical characterization of water kefir: an innovative source of potential probiotics for bee nutrition. Rev Argen Microbiol. 2023;55:176–80, http://dx.doi.org/10.1016/j.ram.2022.09.003.
29. Rosa DD, Días MMS, Grześkowiak ŁM, Reis SA, Conceição LL, Do CG, Peluzio M. Milk kefir: nutritional, microbiological and health benefits. Nutr Res Rev. 2017;30:82–96, http://dx.doi.org/10.1017/S0954422416000275.
30. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009;75:7537–41, http://dx.doi.org/10.1128/AEM.01541-09.
31. Simova E, Beshkova D, Angelov A, Hristozova T, Frengova G, Spasov Z. Lactic acid bacteria and yeasts in kefir grains and kefir made from them. J Ind Microbiol Biotechnol. 2002;28:1–6, http://dx.doi.org/10.1038/sj/jim/7000186.
32. Stadie J, Gulitz A, Ehrmann MA, Vogel RF. Metabolic activity and symbiotic interactions of lactic acid bacteria and yeasts isolated from water kefir. Food Microbiol. 2013;35:92–8, http://dx.doi.org/10.1016/j.fm.2013.03.009.
33. Tan LL, Ngiam JJ, Edric SZ, Conway PL, Joachim Loo S. Ch. *Liquorilactobacillus satsumensis* from water kefir yields a-glucan polysaccharides with prebiotic and synbiotic qualities. Carbohydr Polym. 2022;290:119515, http://dx.doi.org/10.1016/j.carbpol.2022.119515.
34. Vásquez A, Olofsson T. The lactic acid bacteria involved in the production of bee pollen and bee bread. J Apicul Res. 2009;48:189–95, http://dx.doi.org/10.3896/IBRA.1.48.3.07.
35. Verce M, De Vuyst L, Weckx S. Shotgun metagenomics of a water kefir fermentation ecosystem reveals a novel *Oenococcus* species. Front Microbiol. 2019;10:479, http://dx.doi.org/10.3389/fmicb.2019.00479.

36. Wang X, Li W, Xu M, Tian J, Li W. The microbial diversity and biofilm-forming characteristic of two traditional Tibetan kefir grains. Foods. 2022;11:12, http://dx.doi.org/10.3390/foods11010012.
37. Witthuhn RC, Cilliers A, Britz TJ. Evaluation of different techniques on the storage potential of kefir grains. J Dairy Res. 2005;72:125–8, http://dx.doi.org/10.1017/s0022029904000652.
38. Yerlikaya O, Akan E, Kinik O. The metagenomic composition of water kefir microbiota. Int J Gastron Food Sci. 2022;30:100621, http://dx.doi.org/10.1186/s12906-021-03358-3.