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Metagenomic analysis of the microbial community in kefir grains from different milk sources

JUNI SUMARMONO[1,♥], RIO JATI KUSUMA[2,3], NOVIA RAHAYU[4], ARI SURYA SUKARNO[5], PUTRI DIAN WULANSARI[4]

[1]Faculty of Animal Science, Universitas Jenderal Soedirman. Jl. Dr. Soeparno No. 60, Purwokerto Utara, Banyumas 53122, Central Java, Indonesia. ♥email: juni.sumarmono@unsoed.ac.id

[2]Department of Nutrition and Health, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada. Jl. Farmako, Sekip Utara, Sleman 55281, Yogyakarta, Indonesia

[3]Center of Herbal Medicine, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada. Jl. Farmako Sekip Utara, Sleman 55281, Yogyakarta, Indonesia

[4]Department of Animal Science, Faculty of Agriculture, Universitas Perjuangan Tasikmalaya. Jl. Peta 177, Tasikmalaya 46115, West Java, Indonesia

[5]Faculty of Animal Science, Universitas Gadjah Mada. Jl. Fauna 3 Bulaksumur, Sleman 55281, Yogyakarta, Indonesia

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Abstract. *Sumarmono J, Kusuma RJ, Rahayu N, Sukarno AS, Wulansari PD. 2023. Metagenomic analysis of the microbial community in kefir grains from different milk sources. Biodiversitas 24: 5302-5308.* Kefir is a type of traditional fermented milk made from kefir grains. The quality of kefir grain depends on the milk medium, which impacts microbial diversity and population. This study uses metagenomic analysis to assess the microbial populations of kefir grains prepared from cow's milk and goat milk using the 16s rRNA method. The results revealed that >99% Relative Abundance (RA) of the sample's total bacteria belonged to the phylum Firmicutes and the phylum Bacteroidota, respectively. However, most of the phylum Firmicutes was found in the sample of goat milk kefir. Further investigation revealed that goat milk samples had *Lactobacillus*, *Streptococcus*, and *Staphylococcus* genera, while the cow milk samples contained >99% RA of *Lactobacillus*, *Bacteroides*, and *Muribaculaceae*. Regarding yeast, the family *Saccharomycetaceae* predominated in cow's milk, while goat milk had 2% of the family *Saccharomycetaceae* and 97% of unassigned microorganisms. The functional analysis revealed that kefir grains from the two sources had a variety of amino acid metabolisms, secondary metabolite metabolisms, and vitamin production. This metagenomic analysis shows that the type of milk affects the diversity of the population of both bacteria and yeast. This study opens up opportunities to further investigate the potential of kefir as a functional food beverage, a source of probiotics, and an antibacterial agent property.

Keywords: Kefir grain, kefir, lactic acid bacteria, metagenomic, yeast

INTRODUCTION

Kefir is a traditional milk-based food fermented by microorganisms originating from kefir grain. Kefir grain is a complex combination of bacteria, yeast, and polysaccharides produced by their respective microflora. Various lactic acid bacteria in kefir grain show probiotic characteristics (Ganatsios et al. 2021). These complex microbial communities undergo consistent reproduction and pass on their characteristics to the subsequent production of kefir (Ganatsios et al. 2021; Alraddadi et al. 2023). Kefir grain is protected by an exopolysaccharide structure known as kefiran, and it is mainly produced by *Lactobacillus kefiranofaciens* and yeast (Gentry et al. 2023). A comprehensive understanding of the structure and diversity of microflora in the kefir grain must support the successful strategy of producing and utilizing milk kefir as a functional food. Several efforts have identified bacterial communities in kefir grain from different locations/regions and methods (Leite et al. 2012; Marsh et al. 2013; Nalbantoglu et al. 2014; Yegin et al. 2022). Investigations of bacterial and yeast diversities in kefir grain have been undertaken in many countries, such as China (Gao et al. 2013), Turkey (Ilıkkan and Bağdat 2021; Yegin et al. 2022), Tibet (Du et al. 2021), and Greece (Kazou et al. 2021), the results show that there is diversity in each sample. In Indonesia, milk-based kefir has gained significant popularity. However, to our knowledge, comprehensive information on microflora diversities in kefir grain in the country is scarce. Previous investigations showed that many factors, such as the type of milk and environment, highly influence the structure and diversity of microflora in kefir grain (Schwan et al. 2016). Milk sources affected microbial diversity after that described metagenomic tools already used to identify microbial diversity in kefir or other sources such as rumen (Daning et al. 2022).

Metagenomic sequencing has been tested to be an effective instrument for describing the microbiota of fermented foods, supplying taxonomic decisions on the species and strains of microorganisms (You et al. 2022). Metagenomic analysis has provided valuable insights into many fermented milk products' microbial diversity and functional characteristics. For example, kefir samples from two distant localities in Mexico were found to have a high bacterial diversity, with an abundance of Actinobacteria in both samples (Tenorio-Salgado et al. 2021). Metagenomic analysis is also helpful in detecting the presence of

pathogens such as *Klebsiella* spp. in the traditional fermented milk of Gambia (Baldeh et al. 2022). Furthermore, metagenomic analysis can be used to identify bioactive compounds, as has been demonstrated by Tenorio-Salgado et al. (2021) in kefir samples from Mexico. Cow's milk is extensively produced and consumed in Indonesia. On the other hand, goat's milk production continue to increase and is widely known for its distinct taste and nutritional values (Sumarmono 2022). Cow's milk is distinguishable from goat milk for its composition, digestibility, alkalinity, buffering capacity, and therapeutic properties (Yadav et al. 2016; Nayik et al. 2021). Diverse types of milk for kefir production and re-activation are likely to affect bacterial and yeast communities. Previous studies reported that microbial diversities in kefir grain may be affected by factors like the origins of kefir, storage, microbiological composition, production process, fermentation time and temperature, the origin of microbiota, maintenance, storage condition, and types of milk (Garofalo et al. 2015; Schwan et al. 2016; Ma'mon et al. 2018).

The thriving commercial production of milk kefir as milk-based healthy food from cow's and goat milk requires a good understanding of microbial communities within the kefir grain. Metagenomic analysis is a powerful technique to analyze food products' microbial diversity effectively. Therefore, the objective of this study was to evaluate the microbial diversities of kefir grain manufactured from cow's milk and goat milk using metagenomic analysis.

MATERIALS AND METHODS

Kefir grain and kefir development

Traditional cow kefir grain belongs to the Biojaya Kefir (Tasikmalaya, West Java) collection, and goat Kefira's (Sleman, Yogyakarta) Indonesia as a commercial product. We cultivated both kefir grains at a laboratory using sterilized cow milk (for Biojaya Kefir) and goat milk (for Kefira) at a concentration of 10% (w/v). We want to keep a natural microflora from both grains, so we reculture the grains following the type of milk they used before. Cultivated kefir grains were prepared by heating goat milk/cow milk at 85°C for 15 min, letting it cool to room temperature, and then inoculating it with 10% (v/v) of kefir grain. The inoculated goat/cow milk was incubated at room temperature for 18 h, and then the kefir grains were sieved to separate them from the fermented product. When kefir grain biomass reached 10%, it was sieved to separate kefir grain from kefir; it was stored (-20^{o}C) or directly used for total DNA isolation (Nalbantoglu et al. 2014).

DNA extraction from kefir grain

According to manufacturer instructions, total DNA was extracted from kefir grains using a ZymoBIOMICS DNA Miniprep kit (Zymo Research, USA). The quality and quantity of the extracted DNA were measured using nanodrop and validated by gel electrophoresis on 1% agarose gel. For bacterial analysis, the hypervariable region V3-V4 of bacterial 16S rRNA was amplified using forward primer 341 (5-CCTACGGGAGGCAGCAG-3) and 806 reverse (5-GGACTACHVGGGTTCTAAT-3) (Morris et al. 2018) with Hiseq Rapid V2 Kit for 2∗250 base pair (bp) sequence. Tagged universal primers were also used to amplify fungal DNA from the variable ITS-1 rRNA region. In this instance, the forward primer of ITS1F (5-CTTGGTCATTTAGAGGAAGTAA-3) and ITS2 reverse (5-GCTGCGTTCTTCATCGATGC-3) generated PCR products of 410 bp. The amplified product was purified using Qiagen Gel (Qiagen, Germany), and the TruSeq DNA PCR kit was used to design the sequence library. The sequencing was performed in HiSeq2500 PE250.

Data analysis

The downstream processing of DNA sequences was performed by many software. First, the paired final reads are combined using FLASH V1.2.7 (Magoč and Salzberg, 2011). Then, Qiime V1.7.0 was used to filter the raw tags to obtain higher quality and clean tags (Bokulich et al. 2013). The generated tags were compared with the database via the Gold database. Chimera sequences were detected using the Edgar et al. (2011) algorithm. The effective tags were obtained after chimera removal via Chimera formation (Haas et al. 2011). The effective tags were compared with the database using Uparse v7.0.1001 (Edgar 2013) to obtain an operation taxonomic unit (OTU). The Mothur software was used for OTU sequence annotation from the SSUrRNA database in the SILVA Database (Taib et al. 2013). Further phylogenetic relationships of all OTUs are annotated using MUSCLE Version 3.8.31 (Zambounis et al. 2019). Functional analysis was performed using the Tax4Fun function. The OTU table was generated, and the diversity (alpha diversity) Functional analysis was performed using the Tax4Fun function. The replicates were performed thrice for functional and diversity analysis by copying the current data. All analyses were performed in microbiome analysis.

RESULTS AND DISCUSSION

Bacterial composition of kefir

We collected 171,871 and 160,436 high-quality tags from cow and goat milk kefir, respectively. After chimera removal, there remained 171,026 tags and 159,967 tags for OTU analysis. Before bacterial analysis, data was filtered using median abundance value and interquartile range for low variance data filtering. Firmicutes were the dominant phyla at the phylum level, accounting for 96.77% of cow milk kefir, followed by Bacteroidota (2.67%). The goat milk kefir was composed mainly of Firmicutes, accounting for more than 99% of total bacteria. At the family level, *Lactobacillaceae* accounted for 95-96% of both milk kefir, followed by *Staphylococcaceae* and *Streptococcaceae* for goat's milk kefir and *Muribaculaceae* for cow's milk kefir (Figure 1).

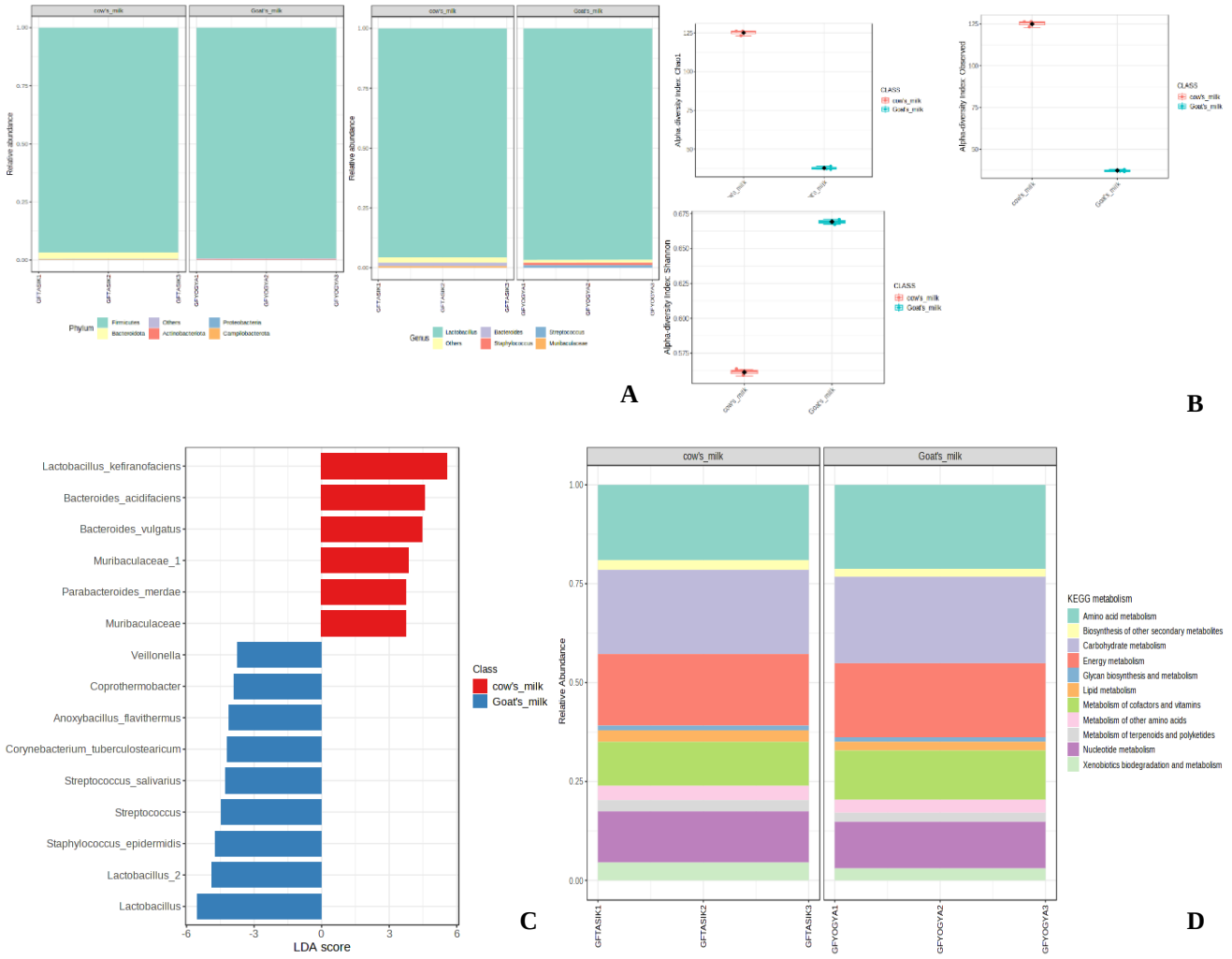

Figure 1. Bacterial composition of milk kefir developed from cows and goat's milk. (A) Both kefirs samples' relative abundance (%) of bacteria phyla and genus level. (B) Comparison of the alpha diversity in cow's and goat's milk kefir. The Chao1 and Observed indexes reflect the abundance of microbiota, while the Shannon reflects the alpha diversity. (C) The linear discriminant analysis (LEfSe) of cow's and goat's milk kefir. (D) Functional prediction analysis of microbiota in cow's and goat's milk kefir

Therefore, several alpha diversity indices (observed by Shannon, Simpson, and Chao1) were performed to assess bacterial diversity between cow milk kefir and goat milk kefir. Cow milk kefir has higher observed Chao1 diversity indexes, while the higher value of Shannon and Simpson indexes indicates goat's milk kefir. These conditions indicated that although goat milk kefir has a lower bacteria composition than cow milk kefir, the bacterial distribution in goat milk was more diverse than cow milk kefir (Table 1).

Next, we performed a linear discriminant analysis (LEfSe) to identify bacteria differently enriched from two different kefirs. There were 15 bacteria with an LDA score of >4 and a false discovery rate (FDR) of >0.05. *Lactobacillus kefiranofaciens*, *Bacteroides acidifaciens*, *Bacteroides vulgatus*, *Muribaculaceae*, and *Parabacteroides merdae* were enriched in cow milk kefir. In contrast, *Veillonella*, *Coprothermobacter*, *Anoxybacillus flavithermus*, *Corynebacterium tuberculostearicum*, *Streptococcus salivarius*, *Staphylococcus epidermis*, and *Lactobacillus* spp. were enriched in goat milk kefir.

Functional prediction of the microbiota composition between two kefirs was performed using Tax4Fun. Amino acid metabolism, carbohydrate metabolism, energy metabolism, glycan biosynthesis, metabolism, and metabolism of co-factors and vitamins were more abundant in goat's milk kefir. Conversely, biosynthesis of secondary metabolites, lipid metabolism, and xenobiotic biodegradation were enriched in cow milk kefir.

The Venn diagram (Figure 2) illustrates the result of classifying bacteria using OTUs analysis to analyze general and unique information in different samples. Figure 2 shows that the classification of bacteria in cow milk produced wider OTUs than that of goat milk. As we can see from the circular area in the Venn diagram, the values in the overlapping areas of two circles (41 UTUs) belong to both cow milk and goat milk, while the other areas are the specific OTUs of cow's milk vs goat's milk (204 vs. 38 OTUs).

At the family level, the most dominant family in cow milk was *Lactobacillaceae* (95.38% RA), while in goat milk (96.57% RA) was *Lactobacillus*. The study presented

Lactobacillaceae as the dominant family (Yegin et al. 2022). In the cow milk sample, we detected *L. agilis (Ligilactobacillus agilis), L. delbrueckii, L. kefir(Lentilactobacillus kefiri), Lactobacillus mucosae, Lactobacillus murinus, Lactobacillus plantarum,* and *Lactobacillus kefiranofaciens*. These were slightly different from those in goat milk that included *Lactobacillus delbrueckii, Lactobacillus fermentum, Lactobacillus kefir, Lactobacillus salivarius,* and *Lactobacillus kefiranofaciens.*

The results of this study indicated that *L. kefiranofaciens* was the most dominant species in the *Lactobacillus* genus observed in both cow milk kefir and goat milk kefir. These results were similar to bacterial composition analyzed in kefir products from sheep's milk, goat's milk, and cow's milk in China. In the study, the microbiota showed clear, uniform compositions, and the microbial communities were dominated by *L. kefiranofaciens, Acetobacter*, and *Lactococcus lactis* (Nalbantoglu et al. 2014). *L. kefiranofaciens*, primarily producing kefiran and forming grains (Dertli and Çon 2017), generally dominates the kefir microbiota. However, *L. kefiranofaciens* identified through the culture-based methods was neglected, mainly due to the difficulty of isolating the microorganism because it has more stringent anaerobic properties (Bourrie et al. 2016). As the main exopolysaccharide, kefiran improves rheological characteristics and can be used as a nutraceutical due to its various biological activities (Prado et al. 2015).

Yeast composition of kefir

We obtained 91,806 and 151,978 high-quality total tags from cow's milk kefir and goat's milk kefir, respectively. After chimera removal, 91,646 tags of cow's milk kefir remained, and 151,796 tags of goat's milk kefir were used for OTU analysis. Before bacterial analysis, data were filtered using median abundance value and interquartile range for low variance data filtering. Fungi composition was dominated by phylum *Acomycota* (99% RA) in cow milk, whereas goat milk contained 2% RA of *Acomycota and* 97% RA of the unassigned genus. The diversity of fungal communities at the genus level in cow's milk kefir was dominated by *Kazachstania* and *Kluyveromyces* (50.42% RA vs 49.47% RA) and *Aspergillus* and *Botryotrichum.* Meanwhile, goat's milk kefir contained 97.69% RA of the unassigned genus, but the rest percentage consisted of nine different families, namely *Kazachstania, Kluyyeromyces, Podospora, Conocybe, Clavulina, Aspergillus, Cladosporium, Botryotichum,* and *Conlarium.*

Several alpha diversity indices were performed to assess bacterial diversity between cow's milk and goat's milk kefir (observed, Shannon, Simpson, and Chao1). Goat's milk kefir has higher observed and Chao1 diversity indexes, while the higher value of Shannon and Simpson indexes indicates cow's milk kefir. These conditions indicated that although cow's milk kefir has a lower yeast composition than goat's milk kefir, the yeast distribution in cow's milk was more diverse than goat's milk kefir (Table 1).

Figure 3 shows that goat milk produces a broader distribution of yeast OTUs than cow milk, as illustrated by the circular area in the Venn diagram. The value of the overlapping area represents the general OTUs of both samples (10 OTUs), while general OTUs show less yeast diversity in cow milk than in goat milk (123 vs 32 OTUs).

Table 1. Microbial alpha diversity indices for cow milk and goat milk

Sample	Community	OTUs	Shannon	Simpson	Chao1	ACE	Coverage
Cow's milk	Yeast	42	1.573	0.630	42.000	42.345	1.000
	Bacteria	246	0.858	0.194	249.615	250.757	1.000
Goat's milk	Yeast	134	0.369	0.073	158.500	158.00	1.000
	Bacteria	82	0.984	0.300	79.000	79.411	1.000

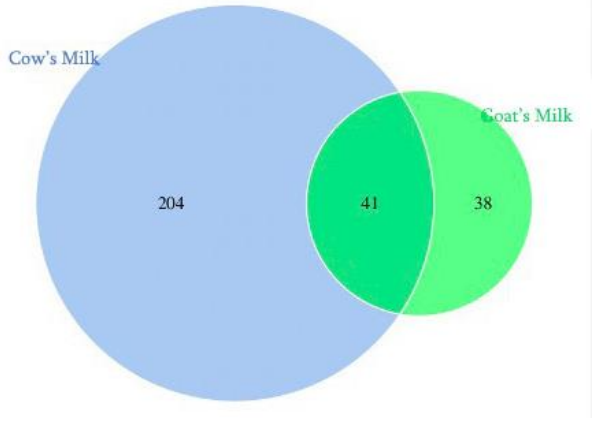

Figure 2. Venn diagram showing specific and common OTUs of bacterial community

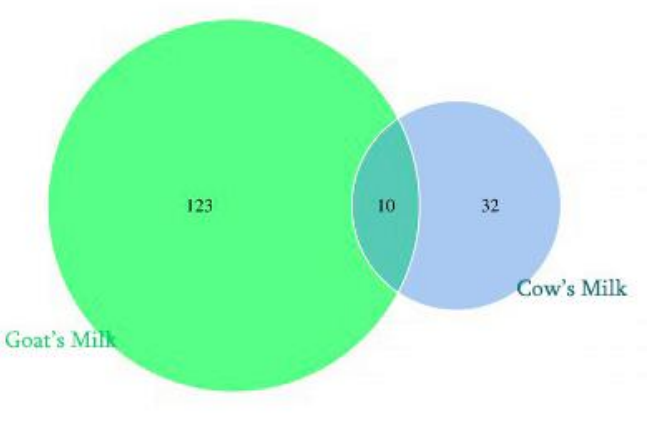

Figure 3. Venn diagram showing specific and common OTUs of yeast community in kefir grains

Interestingly, *Saccharomycetaceae* was the most dominant yeast family in the cow's milk sample (99.88% RA) but only a small amount in the goat milk sample (2.01% RA). Some studies show that *Saccharomycetaceae* is generally found in kefir (Gao et al. 2012) but not in kefir grain (Bengoa et al. 2019). *Saccharomyces* genus is a common probiotic as an antibiotic to treat diarrhea. Study outcomes demonstrate that *Saccharomyces* exhibits multiple functions, including immunomodulator, antioxidant, gastrointestinal modulation, production of β-glucan, protein, fiber, vitamin B, and folic acids derived from the cell membrane (Fakruddin et al. 2017; Hong et al. 2019a; Hong et al. 2019b).

Kazachstania (50.42% RA) and Kluyveromyces (49.47% RA) are the most dominant genus in cow milk. *Kazachstania* detected in this study consisted of *Kazachstaniaunispora* (29.44% RA) and *Kazachstaniahumatica* (20.98% RA). Tang et al. (2020) reported the abundance and high adaptability of *Kazachstaniaunispora* in the environment of milk products because it is the most common species. Other studies stated that *Kazachstaniaunispora* is frequently identified in kefir (Magalhães et al. 2011; Gao et al. 2012).

*Kluyveromyces*is is the dominant genus in this study, and other studies report that the components of kefir grains are *Kluyveromyces, Saccharomyces, candida,* and *Torulaspora* (Marsh et al. 2013). *Kluyveromyces*is was detected in 17 kefir grains and 18 milk (Marsh et al. 2013). This study only detected the *Kluyveromyces marxianus* genus. While Ireland's kefir grain is known to have *Kluyveromyces* spp as its dominant genus (Marsh et al. 2013), Argentina's kefir grain consists of *Lactobacillus kefiranofaciens, Lactobacillus kefir, Lactobacillus para kefir, Lactococcus lactis, Kluveromyces marxianus, Saccharomyces unisporus,* and *Saccharomyces cerevisiae* (Londero et al. 2012). Other kefir grains are reported to have a higher abundance of *Saccharomyces* spp., *K. lactis, Kazakhstania* spp., and *Candida* spp. (Leite et al. 2013; Marsh et al. 2013). *Kluyveromycesmarxianus* are more likely to present in Belgian kefir (Korsak et al. 2015).

Aspergillus spp. was found in this study's cow milk sample and goat milk sample despite in a small amount, 0.006% RA vs. 0.038% RA, respectively. *Aspergillus* spp. is the most common yeast in kefir grain; even kefir grain in Nyingchi (China) shows a high variation compared to other regions (Liu et al. 2019). This species has been found in fermented food and, therefore, can be explored for the food industry (Bourdichon et al. 2012). Other studies reported the prevalence of *Aspergillus* spp. in fermented drinks, and it is responsible for producing some types of carbohydrates, such as amylase, amyloglucosidase, and maltase (Tamang et al. 2016a; Tamang et al. 2016b).

This study found that Firmicutes was the most dominant phylum in the samples of cow milk (96.57% RA) and goat milk (99.22% RA). Other studies reported Firmicutes being the most dominant phylum, followed by Actinobacteria and Proteobacteria (Leite et al. 2012; Biçer et al. 2021). This phylum consists of a cluster of gram-positive bacteria with low GC content, including LAB. Firmicutes can be found as the dominant microbiota in Ireland's kefir after analysis was conducted on the exterior and interior of the kefir (Kalamaki and Angelidis 2020). This study also detected Phylum Bacteroidetes, Actinobacteria, and Proteobacteria, although in a smaller amount in both cow milk (2.76% RA, 0.03% RA, and 0.24% RA) and goat milk (0.01% RA, 0.03% RA, and 0.09% RA). These three phyla are the smallest components in a study by Leite et al. (2012). Further, this study detected 2.76% RA of Bacteroidetes phylum in the cow milk sample but only 0.01% RA in the goat milk sample.

The present metagenomic study on kefir found that *L. kefiranofaciens* was one of the most abundant and dominant bacteria, while *Bifidobacterium* is not yet classified as dominant (Bengoa et al. 2019). This study did not detect any *Bifidobacterium; B. longum* is considered GRAS (Generally Recognized as Safe) and has been widely used as probiotic bacteria. Probiotic products are commonly used to improve the therapeutic properties of some drinks, and *Bifidobacteria* spp. is one of the bacteria with potential application as a bio-functional food in industry (Linares et al. 2017).

To date, kefir is mainly produced traditionally on a scale of small industry, which could be due partly to the diversity of bacteria and yeast in the kefir grains. The microbial composition of kefir grains is very complex and varies from one geographical area to another. Microbiota, maintenance, and storage conditions are the main factors for microbial diversity in kefir grain (Schwan et al. 2016). Our findings showed that the population of bacteria and yeast in both samples was affected by the type of milk for making kefir. Accordingly, kefir drinks for commercial purposes should be made using the same kefir grain to ensure the uniformity of the product yields (Schwan et al. 2016). On an industrial scale, kefir production using kefir grain needs proper handling in the maintenance and production stages to maintain bacterial diversity. This is because microbial diversity is responsible for each kefir product's physicochemical properties and biological activities (da Cruz Cabral et al. 2013).

This study evaluated bacterial and yeast communities in two kefir grains made of different types of milk - cow milk and goat milk - and subjected the evaluation to metagenomic analysis. Identification through sequencing would help the mapping of bacterial and yeast communities in kefir grains. It was based on the symbiosis of bacteria and yeast in kefir microbiota, which significantly affects the taste and texture, as the fundamental kefir quality. The food industry must investigate the microbial diversity and compositions of kefir grains to ensure the functional benefits they offer. Also, increasing the opportunities for down-streaming kefir grain products on an industrial scale is important.

In conclusion, metagenomic analysis of the microbial communities in kefir grains shows that the milk type affects the diversity of the population of both bacteria and yeast. The most prevalent lactic acid bacterium discovered in kefir grains cultivated in cow's and goat milk is *L. kefiranofaciens*. In the meantime, the two most common yeast genera are *Kazachstania* and *Kluyveromyces*. This study offers a variety of opportunities to further investigate

the potential of kefir as a functional beverage, a source of probiotics, and an agent that has antibacterial properties.

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