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MICROBIAL DIVERSITY OF SIX COMMERCIALLY AVAILABLE KEFIR GRAINS

– Research paper –

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Abstract: Natural kefir grains are rich in beneficial bacteria, and analysis of their microbial diversity is a necessary condition for developing and applying kefir grains. In this study, six commercially available natural kefir grains were used as raw materials to explore their microbial diversity by metagenomics. The results showed that there were 14794 genes in 6 kinds of natural kefir grains, and the number of unique genes of X1, X2, X3, X4, X5, X6 were 111, 11, 0, 1899, 552, 1, respectively. From the relative abundance table of boundary, phylum, class, order, family, genus and species, the microbial diversity at each level was analyzed.The two dominant genera at the genus level are *Lactobacillus* and *Lactococcus*, and the dominant species at the species level are *Lactococcus lactis* and *Lactococcus kefiranofaciens*, *Lactococcus crispatus*, and *Lactococcus helveticus*, etc. Species distribution and species diversity of each sample were analyzed by species heat map, principal component analysis and non-metric multidimensional calibration methods. The results showed that the microbial diversity of natural kefir grains from 6 different sources were different. The research can provide reference for the development and application of natural kefir grains in the field of dairy products.

Key words: kefir grains; metagenomics; microbial diversity; species annotation

INTRODUCTION

Kefir, which originated in the Caucasus region of Russia, was fermented by adding kefir particles to milk (Lopitz-Otsoa et al., 2006; Karina et al., 2010). Kefir is rich in many vitamins, such as vitamin B1, B2, B5, vitamin C, etc. It is also a good source of minerals like calcium, magnesium, phosphorus (Miguel et al., 2011), and contains a wider variety of amino acids than milk (Otles et al., 2003). It has a dense, creamy texture, contains a lot of aromatic substances (Simova et al., 2002), a small amount of ethanol and carbon dioxide with low calories (Sabir et al., 2010). It is popular worldwide. Studies show that long-term references to Kefir have many benefits, such as lowering blood pressure (Gobbi et al., 2019; Silva-Cutini et al., 2019), fighting cancer, inhibiting inflammation (Liang et al., 2017; Chen et al., 2020), lowering cholesterol (Vujičić I et al., 1992; Dandy et al., 2019), relieving lactose intolerance (Alm, Livia et al., 1982; Steven et al., 2003), antioxidants (A Güven et al., 2010), and promoting sleep (Handan et al., 2019), etc.

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Kefir grains originated in the Caucasus Mountains of Central Asia about 1000 years ago (Nielsen et al., 2014). Herdsmen stored cow's milk, goat's milk or mare's milk in skins and fermented them for several times to form kefir grains (Li et al., 2016), which is essentially as same as "Tibetan Spirit Mushroom" (aka "Snow Lotus Mushroom") in Tibet, China (Gao, 2013). The appearance of kefir grains is white to pale yellow particles, generally between 0.5-3 cm in size, irregular spherical or cauliflower-shaped.
In general, kefir grains are mainly composed of lactic acid bacteria (LAB), yeast and a small amount of acetic acid bacteria (AAB). These microorganisms co-exist in a natural matrix of glucose, galactose and protein. According to reports, the lactic acid bacteria in kefir grains contents about 10^8-10^9 CFU/ml, the yeast contents about 10^5-10^6 CFU/ml, and the acetic acid bacteria contents about 10^5-10^6 CFU/ml (Garrote et al., 2001). The LAB in kefir grains are mainly in charge of converting the lactose in milk into lactic acid, thus lowering the pH level. Other microbial components, including ethanol and carbon dioxide producing yeast, non-lactose fermenting yeast and AAB, are also involved in the process (Magalhes et al., 2010; Rattray et al., 2011). After fermentation, the biomass of kefir grains increases by about 5%-7%.

During fermentation, the proportion of microorganisms in the grains differs from that in the final product. This difference is related to fermentation process conditions, such as fermentation time, temperature, degree of stirring, milk type, inoculum size and microbial distribution, etc (Rattray et al., 2011).

Traditional microbiological identification methods are used to consider the bacterial diversity of kefir grains (Simova et al., 2002; Rea et al., 2010), while sometimes this method does not have enough insight to identify closely related species or new species. As a complement to traditional methods, PCR-DGGE techniques in Molecular Biology methods is ideal for analyzing populations of complex microbes (Jianzhong et al., 2009; Magalhães et al., 2010). However, the technique cannot detect microbial species with a content of less than 1%. With the development of molecular biology techniques, especially NGS, macrogenomics has become an effective tool for the analysis of microbial communities of samples. The aim of the study is to analyze and detect the microbial diversity of six natural kefir grains by macrogenomics, and to provide a basis for subsequent studies on natural kefir microbial diversity.

MATERIALS AND METHODS

Preservation and activation of Kefir grains

Kefir grains X1 (Wuxi, Jiangsu), X2 (Dezhou, Shandong), X3 (Shanghai), X4 (Taiyuan, Shanxi), X5 (Fushun, Liaoning), and X6 (Dalian, Liaoning) were all preserved by the Shaanxi University of Science and Technology Laboratory 1C-419.

The whole goat milk powder was prepared into reconstituted goat milk according to the ratio of 1:8 with water, pasteurized, added with kefir grains, and fermented at 25°C for 22h. After fermentation, the filtered kefir grains were rinsed with sterile saline. This method was repeated to activate the grains.

DNA extraction and sequencing results preprocessing

The DNA of the samples was extracted using the standard procedure of the purchased kit (TIANamp Bacteria DNA Kit) (Zhao, 2018); then the purity of the obtained DNA was measured by ultraviolet spectrophotometer, and the appropriate DNA was selected as the template for PCR amplification. The amplification procedure was: denaturation at 94°C for 6 min, reaction at 58°C for one minute, and reaction at 72°C for 2 min, for 30 cycles. The final product was detected by agarose gel electrophoresis (1%). Referring to the method of Sun Yujiao (Sun, 2018), readfs was used to preprocess the data obtained by the sequencing platform.

Metagenome assembly

The MEGAHIT software (v1.0.4-beta) is used to assemble Clean Date data.

Prediction and abundance analysis of genes

Referring to the method of Zheng (2018), use MetageneMark to predict the ORF of the Scaftigs of each sample; use Bowtie2 (Bowtie2.2.4) to compare Clean Date of apiece sample with the initial Genetic Catalog to obtain the number of genes used for analysis; Calculate the abundance information of genes in each sample. The formula is as follows:

$$G_k = \frac{r_k}{L_K} \times \frac{1}{\sum_{i=1}^{n} \frac{r_i}{L_I}} \quad (1)$$

In the formula, r is the number of reads in the alignment, and L is the gene length.

Common Function Database Notes

Unigeges were aligned with functional databases using DIAMOND software. Starting from the comparison results, the relative abundance of different functional levels was counted; the number of genes in each sample at different taxonomic levels was obtained from the functional annotation results and gene abundances.

RESULTS AND DISCUSSION

DNA test results

The results of DNA detection of the six kefir grains are shown in Table 1 and Figure 1. The molecular weights of the six kefir grain samples were all about 7000bp, and the content of the target substances in X3 and X6 was the highest, which was consistent with the total amount of samples in Table 1.

Correlation analysis between samples based on the number of genes

Correlation coefficient heatmap

In Figure 2, different colors represent the level of the correlation thermal correlation coefficient; the ellipse skewed to the left represents a positive correlation coefficient, and the right skewed is negative; the darker the color of the ellipse, the flatter the curvature of the ellipse, the larger the absolute value of the correlation coefficient. The correlation coefficients among the 6 kefir grains are

all negative and the absolute values of the correlation coefficients is different between each samples, indicating that the diversity among the 6 kefir grains is different.

Petal illustration

The petal diagram is generally used to display the diversity of genes when the number of samples exceeds 5. In order to investigate the distribution of the number of genes among the samples, a petal diagram was drawn, and the results are shown in Figure 3; the number of genes shared by the six kefir species is 14,794. The unique gene numbers of X1, X2, X3, X4, X5, X6 were 111, 11, 0, 1899, 552, 1, respectively.

Table 1. DNA test results of the six Kefir grain samples

Sample	Library type	Qubit Concentration (ng/μL)	Volume (μL)	Total amount (μg)
X1	Meta library	4.28	76.00	0.49
X2	Meta library	5.16	76.00	0.39
X3	Meta library	21.80	76.00	1.66
X4	Meta library	2.24	76.00	0.18
X5	Meta library	5.02	76.00	0.38
X6	Meta library	10.50	76.00	0.80

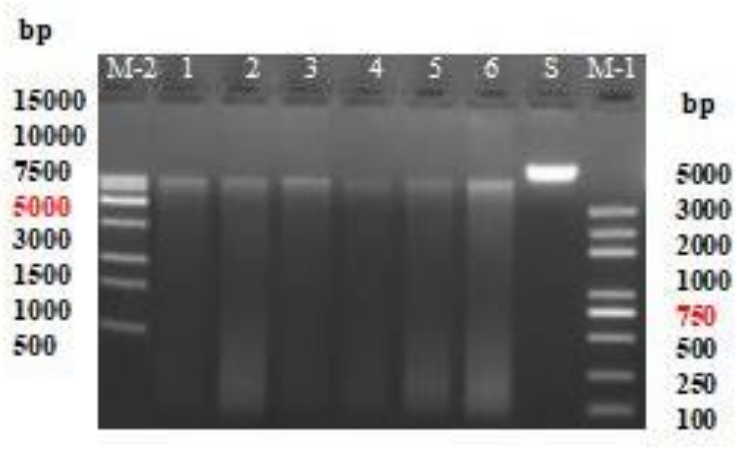


Figure 1. Agarose gel electrophoresis of six kefir grain samples

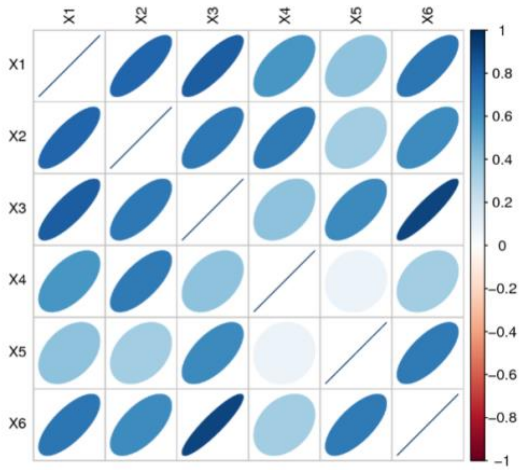


Figure 2. Heat map of correlation coefficient between six kinds of Kefir grains

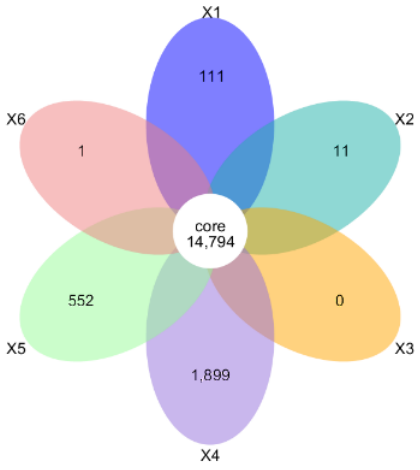


Figure 3. Petal map of the number of genes in six Kefir grains

Species annotation

According to the relative abundance tables of different taxonomic levels, the top 10 species with the largest relative abundance in each sample were selected. The relative abundance column charts of the species annotation results corresponding to each sample at different taxonomic levels were drawn.

Relative abundance of species

Figure 4 shows the species diversity analysis at the phylum level. At the phylum level, the top 10 microorganisms in 6 samples are *Firmicutes*, *Proteobacteria*, *Ascomycota*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Chlamydiae*, *Spirochaetes*, *Basidiomycota*, *Candidatus Woesebacteria*.Among them, *Firmicutes* has an absolute advantage. The proportions of *Firmicutes* in samples X1, X2, X3, X4, X5, and X6 are 86.17%, 93.68%, 89.78%, 92.26%, 83.81%, and 89.04%, respectively.

Figure 5 shows the species diversity analysis at the class level. At the class level, 10 classes were annotated from 6 kefir samples, of which *Bacilli* dominated each sample, accounting for X1, X2, X3, X4, X5, and X6 85.59%, 93.6%, 89.64%, 92.15%, 83.7%, 88.91%, respectively. Secondly, the abundance of *Alphaproteobacteria* in X5 is the largest, which is 7.26%. *Saccharomycetes* also occupies a certain proportion in each sample, and the abundance in X1 is the largest, which is 2.14%. In addition, species with relatively low abundance were also detected, such as *Clostridia*, *Actinobacteria*, *Gammaproteobcteria*, *Betaproteobacteria*, *Ktedonobacteria*, *Epsilonproteobacteria*, *Sphingobacteriia*, etc.

Figure 6 shows community composition based on the order level. The graph shows the absolute dominance of *Lactobacillales* in the six samples, with 84.54%, 93.36%, 88.68%, 91.83%, 83.36% and 88.96% in X1, X2, X3, X4, X5 and X6 respectively. *Rhodospirillales* accounted for the largest proportion of X5 at 7.19%. The abundance of *Bacillales* in each sample was 0.3%, 0.07%, 0.1%, 0.08%, 0.04%, 0.09%. The *Saccharomycetales* were more abundant in samples X1 and X4 with 2.14% and 2.13% respectively. *Caudovirales*, *Micrococcales*, *Neisseriales*, and *Ktedonobacterales* were all annotated, but the abundance was low.

Figure 7 is based on community composition at the family level. The microbial species annotated in the six samples were similar. The abundances of *Lactobacillaceae* in X1, X2, X3, X4, X5 and X6 were 27.93%, 79%, 10.44%, 68.06%, 58.82% and 18.63%, respectively. The abundances of *Streptococcaceae* in the above samples were 53.79%, 13.32%, 73.46%, 20.04%, 22.77% and 65.1%. The abundances of *Saccharomycetaceae* were 2.11%, 0.81%, 0.01%, 2.11%, and 0.04%. The abundances of *Bacillaceae* were 0.11%, 0.05%, 0.04%, 0.04%, 0.23% and 0.04%. The abundances of *Leuconostocaceae* were 0.13%, 0.1%, 0.09%, 0.85%, 0.06% and 0.08%. The abundances of *Enterococcaceae* were 0.39%, 0.08%, 0.46%, 0.12%, 0.15%, and 0.42%. In addition, 7.2% of *Acetobacteraceae* were annotated in X5.

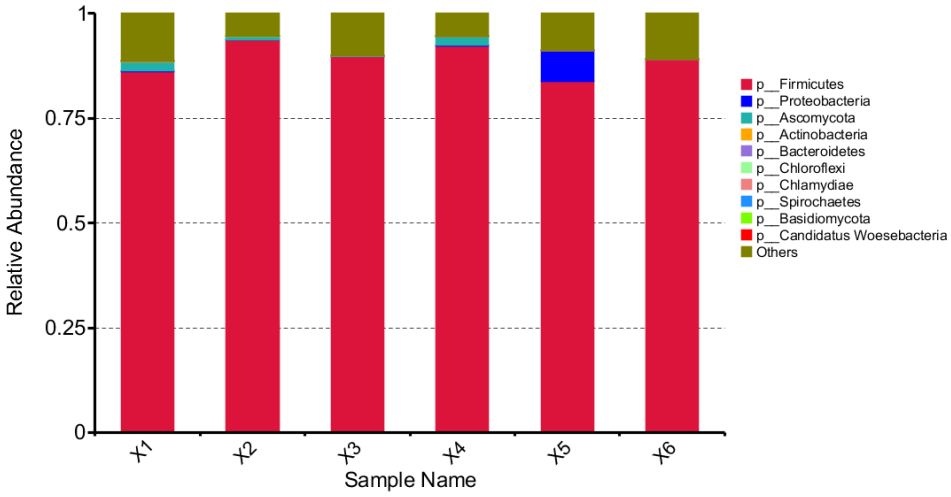


Figure 4. Species diversity analysis of six Kefir grains at the phylum level

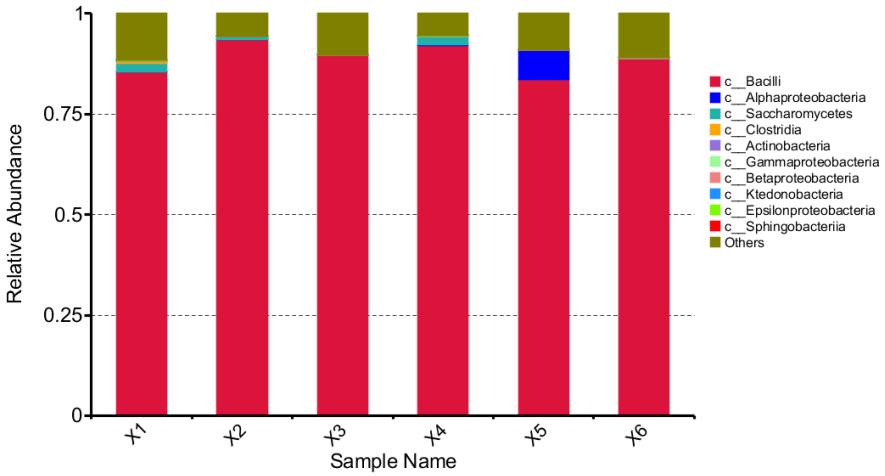


Figure 5. Species diversity analysis of six grains of Kefir at class level

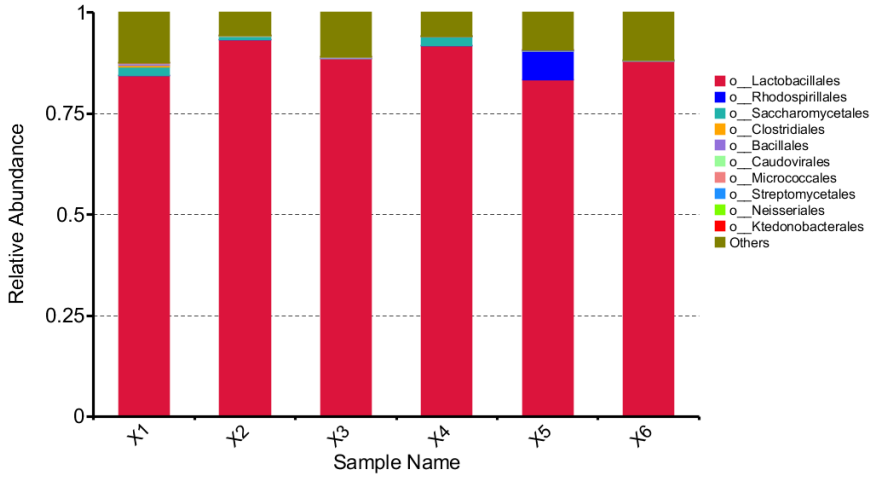


Figure 6. Species diversity analysis of six Kefir grains at order level

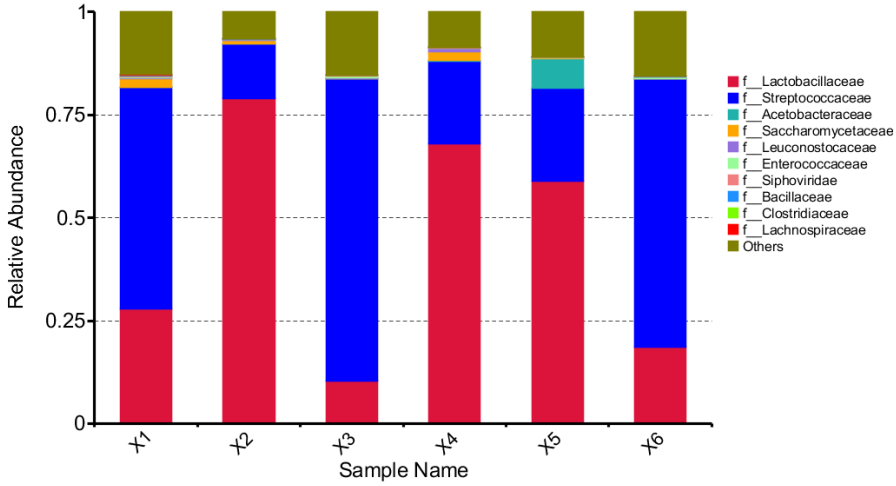


Figure 7. Species diversity analysis of six Kefir grains at the family level

Figure 8 shows the community composition based on the genus level. The genus-level microbial species contained in the six samples are similar, but there are certain differences in the number distribution. Among them, the first two genera that dominate at the genus level are *Lactobacillus* and *Lactococcus*. *Lactobacillus* accounted for 21.4% in X1, 78.38% in X2, 10.35% in X3, 67.55% in X4, 58.53% in X5 and 18.54% in X6. *Lactococcus* accounted for 51.96% in X1, 12.4% in X2, 72.38% in X3, 19.03% in X4, 22.17% in X5 and 63.88% in X6. *Kluyveromyces* only accounted for more than 1% in X1 and X4, and accounted for less than 1% in other samples. *Acetobacter* accounted for 6.77% in

X5, and the proportion in other samples was less than 1%. *Streptococcus* accounted for 1.54% in X1, and accounted for less than 1% in other samples. The content of *Weissella*, *Enterococcus*, *Bacillus*, *Leuconostoc* and other genera were all less than 1%. Figure 9 is a species-level annotation map showing the top ten species with the greatest relative abundance, which are *Lactococcus lactis*, *Lactobacillus kefiranofaciens*, and *Lactobacillus crispatus*, *Lactobacillus helveticus*, *Acetobacter orientalis*, *Kluyveromyces marxianus*, *Lactobacillus gasseri*, *Lactobacillus kefiri*, *Streptococcus suis*. The abundances of *Lactococcus lactis* in X1 to X6 samples were 49.24%, 11.78%, 68.18%, 18.11%, 20.92% and 60.16%, respectively. The proportion of *Lactobacillus kefiranofaciens* in the above samples was 8.96%, 52.14%, 6.29%, 44.87%, 25.13%, 6.32%. The proportion of *Lactobacillus crispatus* in the six samples was 0.61%, 1.55%, 0.31 %, 0.1%, 10.01%, 4.03%. The proportion of *Lactobacillus helveticus* was 0.63%, 1.88%, 0.36%, 1.94%, 6.68%, 2.5%. *Acetobacter orientalis* is the most abundant in X5, accounting for 5.59%. The abundance of *Kluyveromyces marxianus* in X1 and X4 is 1.77% and 1.79%, respectively, and the abundance in X6 is the smallest, and its content is less than 0.01 %. *Lactobacillus gasseri* had the highest abundance in X5, about 1.45%; *Lactobacillus kefiri* accounted for the highest proportion in X1, 1.24%, and was less than 1% in the rest of the samples.

In the microbial composition of kefir grains, LAB are the absolute dominant flora, but there are also some risk bacteria that have not been isolated yet. We speculate that due to its small proportion, processes such as growth and metabolism are inhibited by dominant bacteria and cannot produce harmful effects.

Yuan (2020) analyzed the bacterial composition of naturally fermented dairy products in Kyrgyzstan by PacBio SMRT sequencing technology, and found that the genus Lactobacillus was mainly composed of *Lactobacillus* and *Lactococcus*, and the dominant bacteria belonged to *Lactobacillus*.

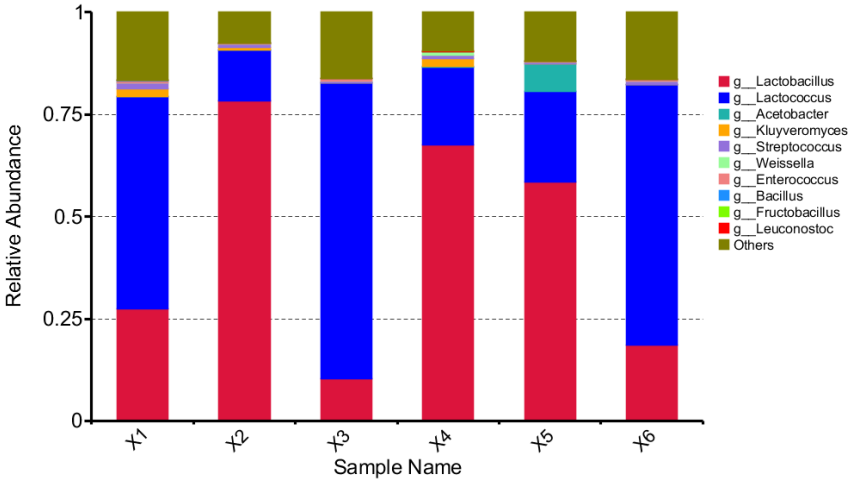


Figure 8. Species diversity analysis of six Kefir grains at the genus level

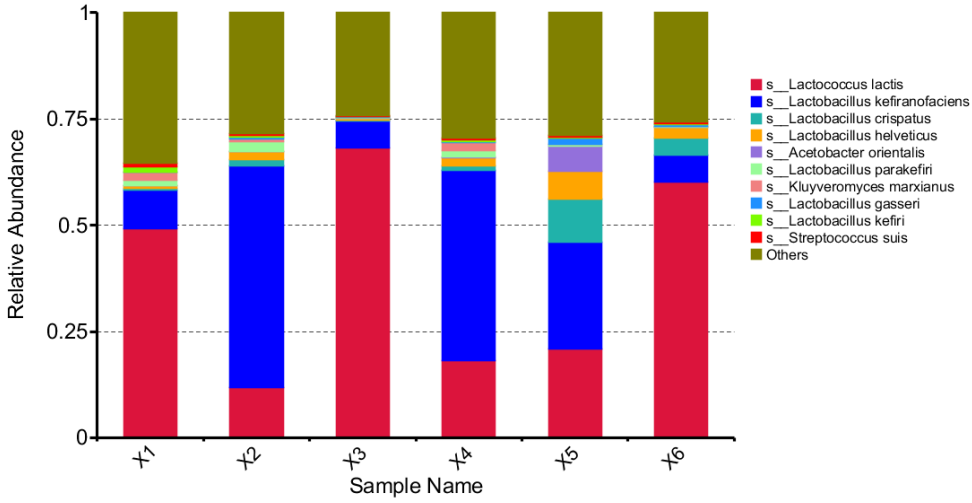


Figure 9. Species diversity analysis of six grains of kefir at the species level

The diversity of LAB in different types of naturally fermented dairy products was analyzed based on the species level, and it was found that the LAB in different fermented milk (Kyrgyzstan kefir, yogurt, cheese) samples consisted of *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, *Lactobacillus kefiri*, etc. In domestic (42.11%) and foreign (84.62%) studies on kefir grains, *Lactobacillus helveticus* is the most richest species, which can avoid bitterness and produce a nutty taste during the dairy fermentation (Simova et al., 2002). Garrote et al. (2001) isolated a wide variety of *Lactobacilli* from kefir and kefir grains, including *Lactobacillus kefi*ri, *Lactobacillus kefiranofaciens*, *Lactobacillus kefirgranum* and *Lactobacillus parakefiri*, which constitute the main population.

Gene relative abundance clustering analysis

Figure 10 is the genus-level relative abundance clustering heat map, the sample information is presented on the horizontal axis, the genus name is showed on the vertical axis, each row represents a genus. The species clustering tree is on the left. The value in regard to the inter-specific heat map is the Z value after normalizing
relative abundance of species in each row. It can be seen from the figure that there are 20 genera in sample X1 showing red, indicating that the content of these genera is relatively large. Except for the less dominant genera in the remaining 5 samples, the abundance of other genera were very low.

Figure 11 is a heat map of species-level relative abundance clustering, with sample information in the horizontal direction and strain names in the vertical direction, with each row representing a species.

The dominant strains of different samples are different, and the content of the same strain in different samples is also different (Figure 11). Garofalo et al. (2015) concluded that over 98% of the bacteria in kefir grains are *Lactobacillus*. Among the three kefir grains, other genera had relative abundances below 1%, including *Pseudomonas*, *Bacillus*, and *Curvibacter*, etc.

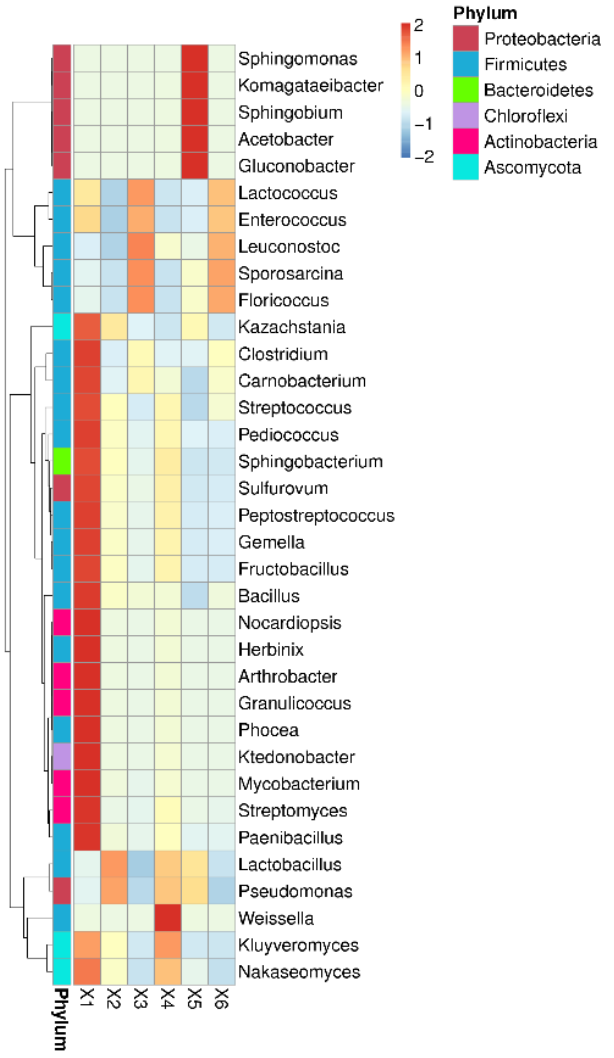


Figure 10. Clustering Heat Map of Abundance of Six Kefir grains at the Generic Level

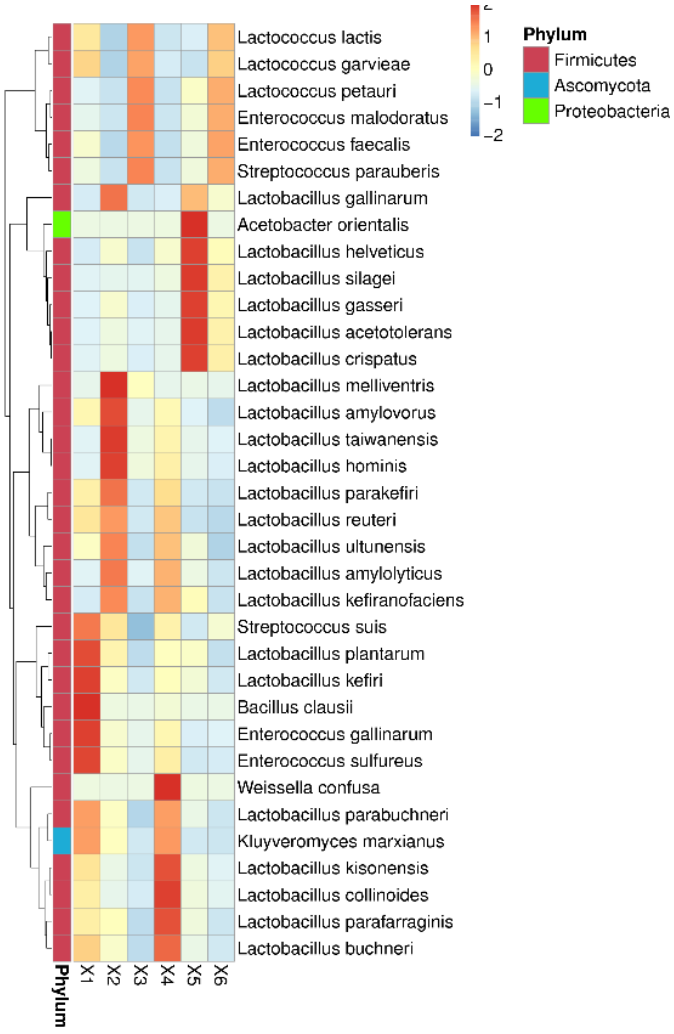


Figure 11. Clustering Heat Map of Abundance of Six Kefir grains at Species Level

Dimensionality reduction analysis based on species abundance

Principal component analysis

Principal Component Analysis (PCA) is a dimensionality reduction analysis based on a linear model, which can reflect the differences of multidimensional data in a two-dimensional coordinate graph, thereby reflecting the simple laws behind complex data.

Figure 12 shows the results of principal component analysis at the genus level and Figure 13 shows the results of principal component analysis at the species level.. The abscissa and ordinate are the first principal component (PC1) and the second principal component (PC2), respectively. The contribution of PC1 and PC2 to the sample is 45.86% and 26.16%, and the sum of the two is 72.02%, indicating that 72.02% of the information of the sample can be reflected, and the analysis results are relatively accurate. The closer the distance between the two samples in the figure, the more similar the genus-level composition of the two samples. For example, the distance between samples X2 and X4 is relatively close, indicating that the compositions of samples X2 and X4 are more similar.

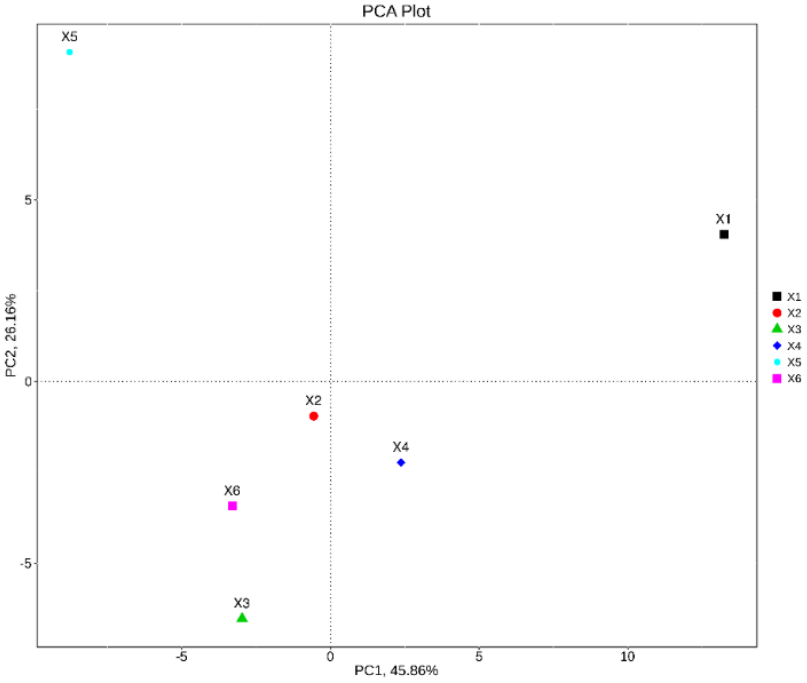


Figure 12. Genus-level PCA plots for six kefir grains

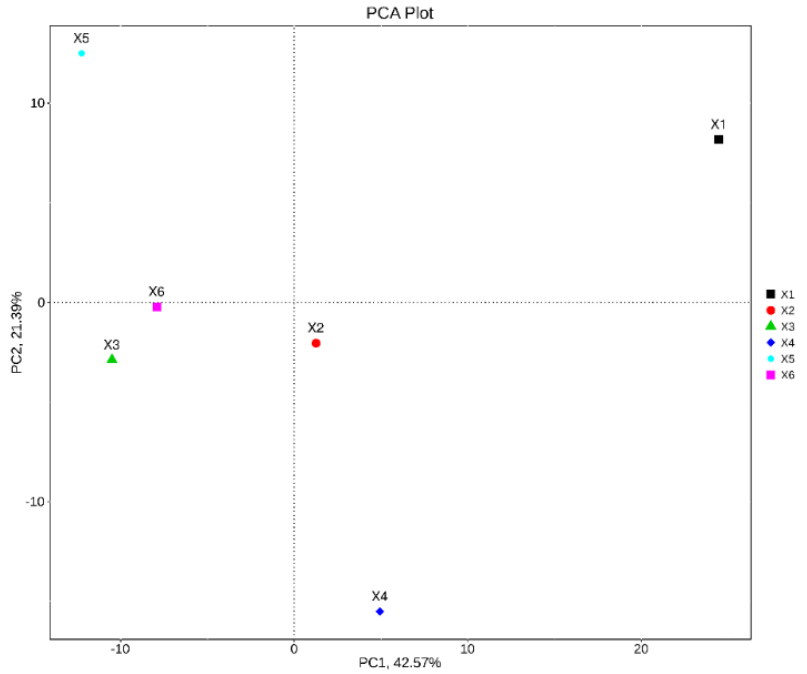


Figure 13. Species-level PCA plots for six kefir grain samples

The contribution of PC1 to the sample is 42.5%, the contribution of PC2 to the sample is 21.39%, and the sum of the two is 63.89%, indicating that it can reflect 63.89% of the information of the sample. The close distance between samples X3 and X6 indicated that the similarity between them is relatively high. Studies have shown (Wang et al.; 2021) that the branching distance between kefir grains is small, indicating that the microbiota between them is highly similar.

Analysis of metric-free multidimensional calibration results

The non-metric multidimensional calibration method (NMDS) is to make up for the shortcomings of the linear model and better reflect the nonlinear structure of the data. Figures 14 and 15 show the NMDS results of each sample at the genus level and species level, respectively.

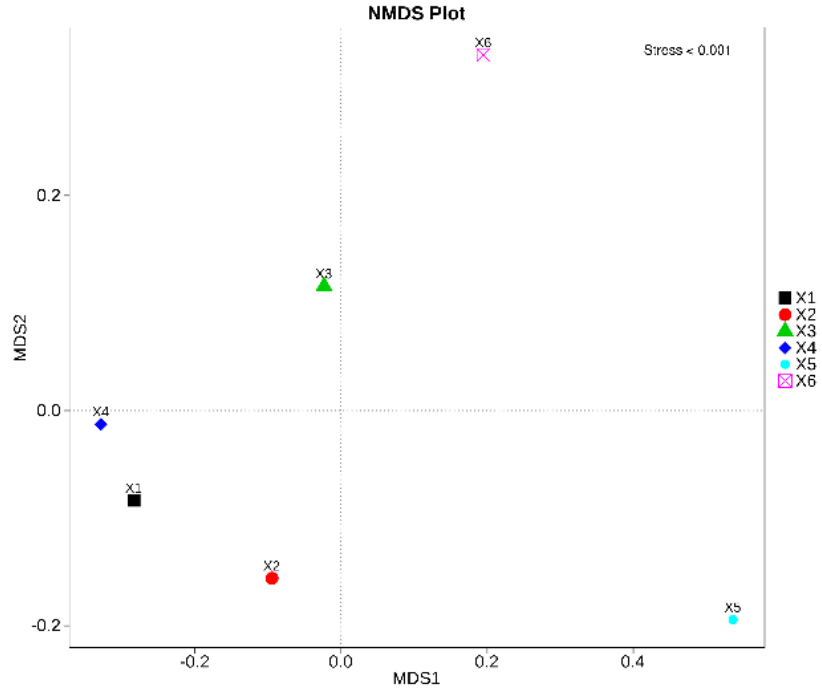


Figure 14. The genus-level NMDS results of six Kefir grain samples

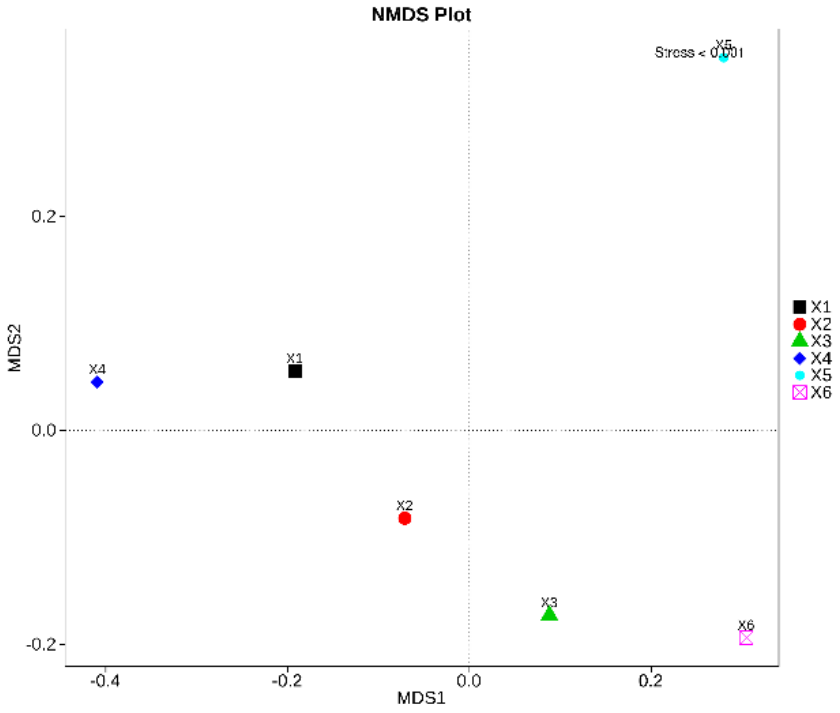


Figure 15. The species-level NMDS results of six Kefir grain samples

Figure 15 is a presentation of species NMDS results based on species level. Stress is less than 0.001, indicating that the analysis results are highly reliable. At the species level, the differences between X5 and the other five samples were relatively large, and the differences between X1 and X2, X2 and X3, and X3 and X6 were relatively small, which was consistent with the previous species diversity results. Figure 14 indicates the species NMDS results based on the genus level. Each point in the figure represents a sample, and the distance between the points indicates the degree of difference. When stress is less than 0.2, NMDS analysis has certain reliability. The stress in Figure 14 is less than 0.001, indicating that the analysis has some reliability. The differences between X1 and X4 at the genus level were relatively small, and the differences between X2 and X6 were relatively large, which was consistent with the previous species diversity analysis.

Cluster analysis of samples based upon species abundance

Clustering the samples to construct a sample clustering tree can study the similarity of these 6 samples. Figure 16 is a clustering tree for each sample at the genus level. The Kefir grains' relative abundance distribution at the gate level is shown to the right of the graph. The Bray-Curtis distance clustering tree structure is on the left. At the genus level, the six samples can be divided into two categories, the first category contains X1, X2 and X4, where X2 and X4 are more similar; the second category contains X3, X5 and X6, where X3 and X6 are more similar. It is also reflected in the principal component analysis above.

Figure 17 shows the clustering tree of 6 kinds of kefir grains at the species level. From the figure, we can see that the similarity at the species level and the genus level is consistent, X2 and X4 are more similar, X3 and X6 are more similar, but there are certain differences in the abundance of each bacterium.

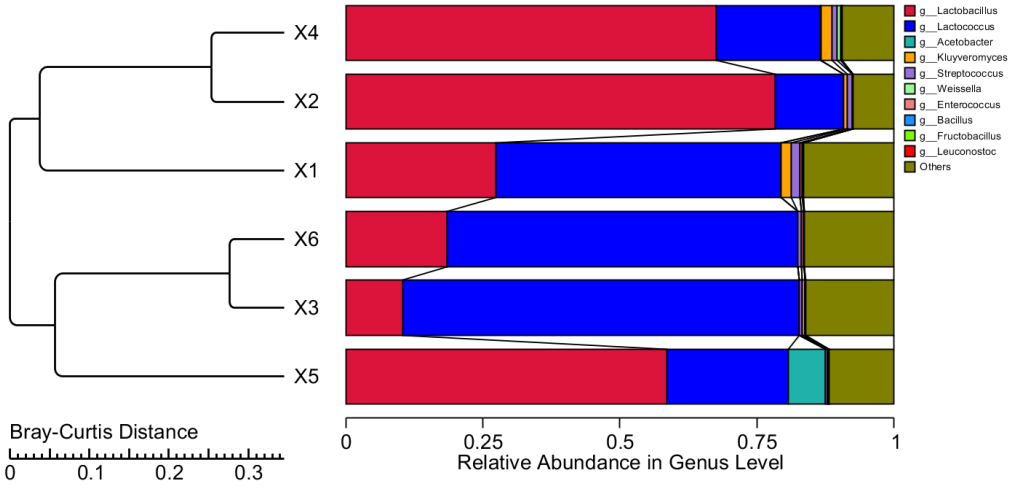


Figure 16. Clustering tree based on genus level of six Kefir grains

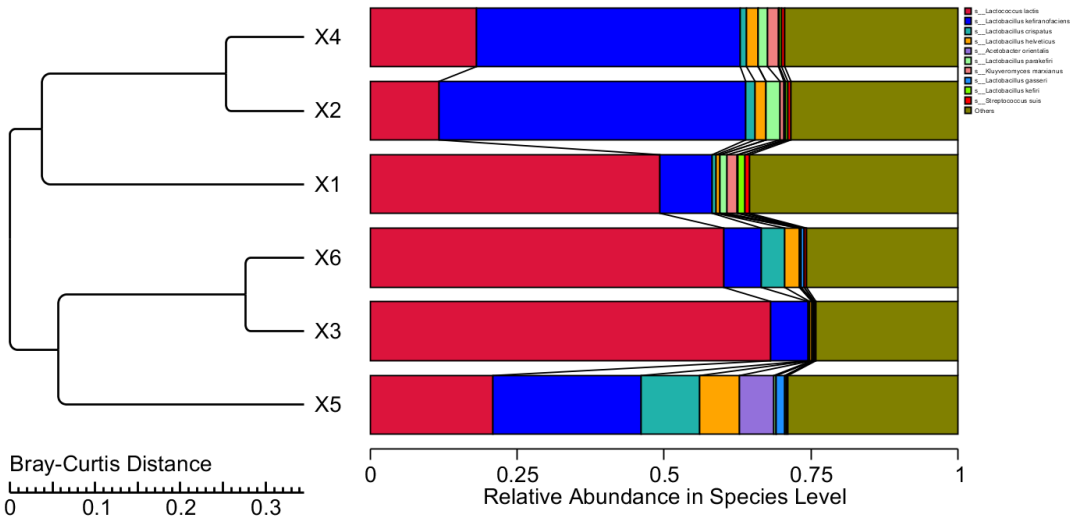


Figure 17. Clustering tree based on species level of six Kefir grains

Common Function Database Notes

KEGG analysis

Figure 18 shows the relative abundance of the six samples at level 1 in the KEGG database. Six types of biological metabolic pathways were annotated, namely cellular processes, environmental information processing, genetic infromation processing, human disease, metabolism and organic systems. Among them, metabolism accounted for the largest proportion of all the annotated functions, accounting for more than 10%, which provided the genetic basis for the growth and metabolism of each sample. Next is genetic information processing, accounting for about 5%, which may be related to the passage and proliferation of samples. The smallest proportion is the biological system, which is less than 1%.

Figure 19 is a relative abundance map at Level 2. At present, there are 57 sub-pathways at Level 2 in KEGG, and the top 10 sub-pathways are listed in this study. Fig.19 shows the proportion of each metabolic pathway at Level 2 in the 6 samples is different, followed by Carbohydrate metabolism environmental information processing, Membrane transport metabolism, Amino acid metabolism metabolism, Nucleotide metabolism genetic information processing, Translation metabolism, Energy Metabolism metabolism, Metabolism of cofactors and vitamins cellular processes, Cellular community-prokaryotes metabolism, Lipid genetic information processing, Replication and repair.

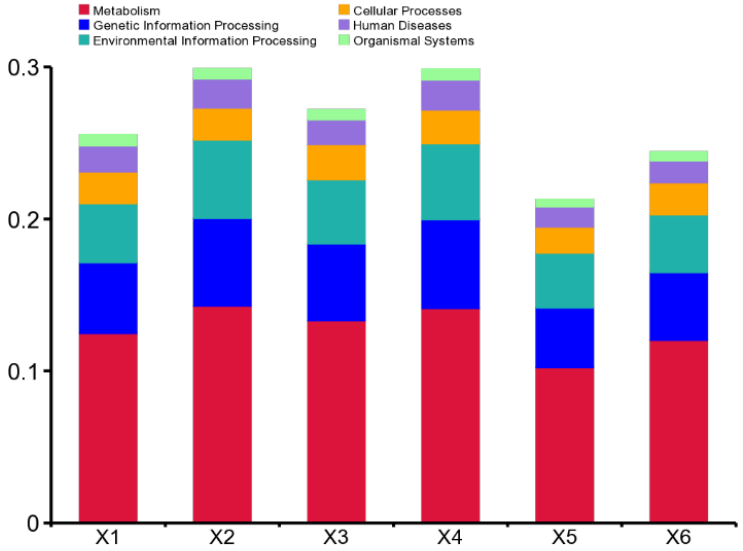


Figure 18. Functional notes of the six types of kefir grain relative abundance bar graph at level 1

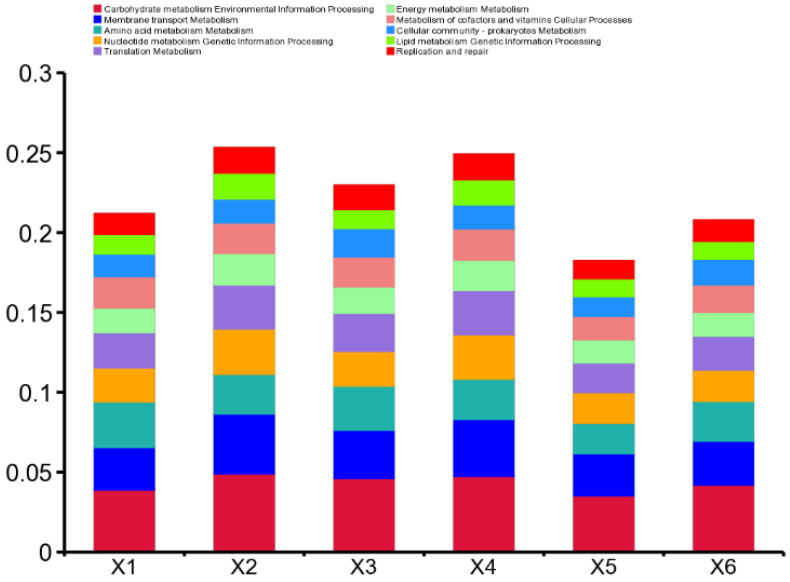


Figure 19. Functional notes of the six types of kefir grain relative abundance bar graph at level 2

EGGNOG function annotation

Figure 20 and Figure 21 show the functional

annotation results of the level 1 and level 2 levels of the eggnog database for the six kefir grain samples. Figure 20 shows the top 9 annotated results of the 6 samples in the eggnog database level 1 level. The functions of the six kefir grains have similar proportions. The functions with the largest proportion are: Replication, recombination and repair, and the other functions with a large proportion are Carbohydrate transport and metabolism; Amino acid transport and metabolism; Translation, ribosomal structure and biogenesis; Transcription; cell wall/membrane/envelope biogenesis; Inorganic ion transport and metabolism; nucleotide transport and metabolism and Defense mechanisms.

Figure 21 shows the top9 annotated results of 6 samples in eggNOG database level 2. Firstly, transposase is the most dominant factor in each sample, which may be affected by the recombination and repair functions in level 1. Secondly, transcription regulators account for a larger proportion, as well as retrotransposon proteins, (ABC) transport, virulence-associated proteins, phage portal proteins, and acetyltransferases.

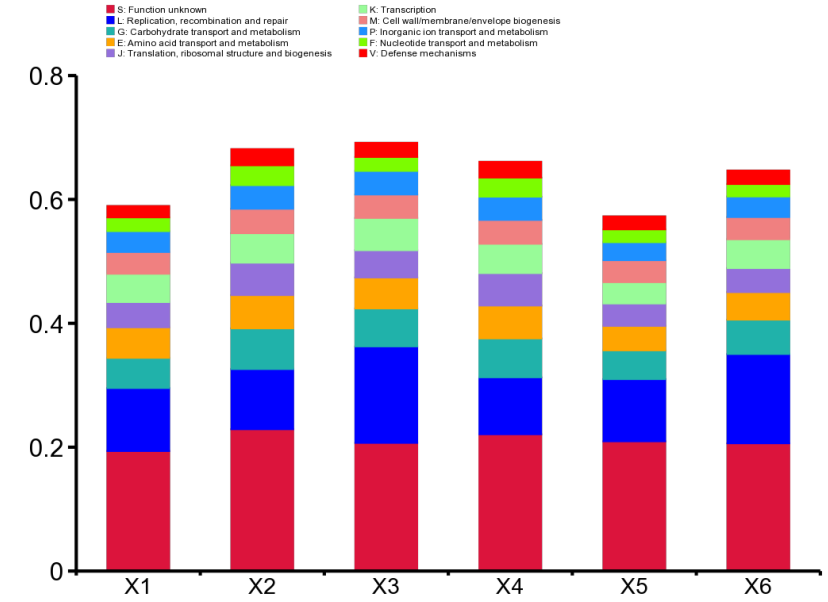


Figure 20. Histogram of the relative abundance of functional annotations of the six Kefir grains at level 1

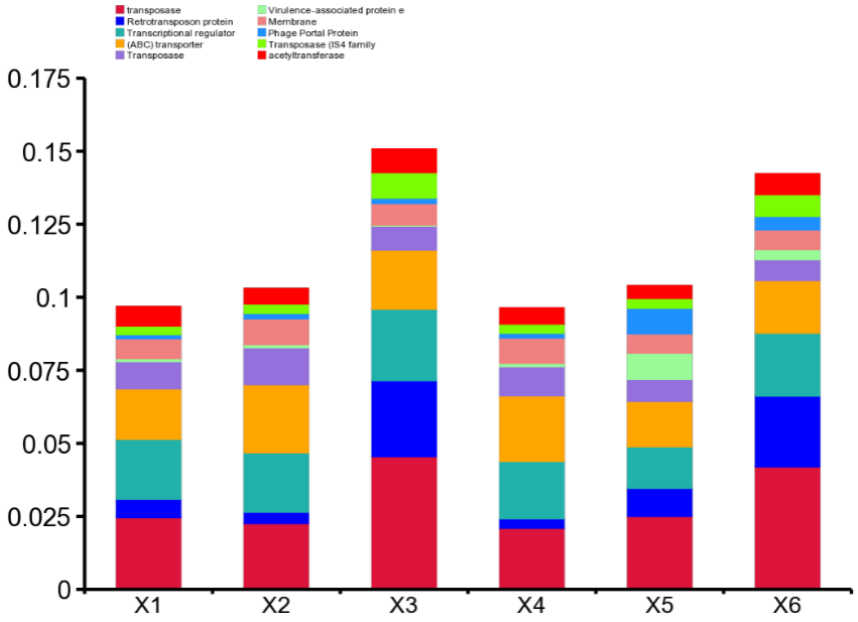


Figure 21. Histogram of the relative abundance of functional annotations of the six Kefir grains at level 2

CAZy database analysis

The CAZy database is a professional database for the study of carbohydrate enzymes, mainly including 6 functional categories: glycoside hydrolase, glycosyltransferase, polysaccharide lyase, carbohydrate esterase, auxiliary oxidoreductase and carbohydrate binding module. As shown in Figure 22, in the level 1 level of the CAZy database, 6 categories of functions are annotated in each sample,of which glycoside hydrolases accounted for the largest proportion, followed by glycosyltransferases, which may be related to the active growth and metabolism of the samples.

Figure 23 shows the functions of top10 annotated in the level 2 level of the CAZy database, mainly the next level of glycoside hydrolases and glycosyltransferases. The proportions of GH25, GH73 and GH1 are relatively high, and the proportion of GT23 in sample X5 is also relatively high.

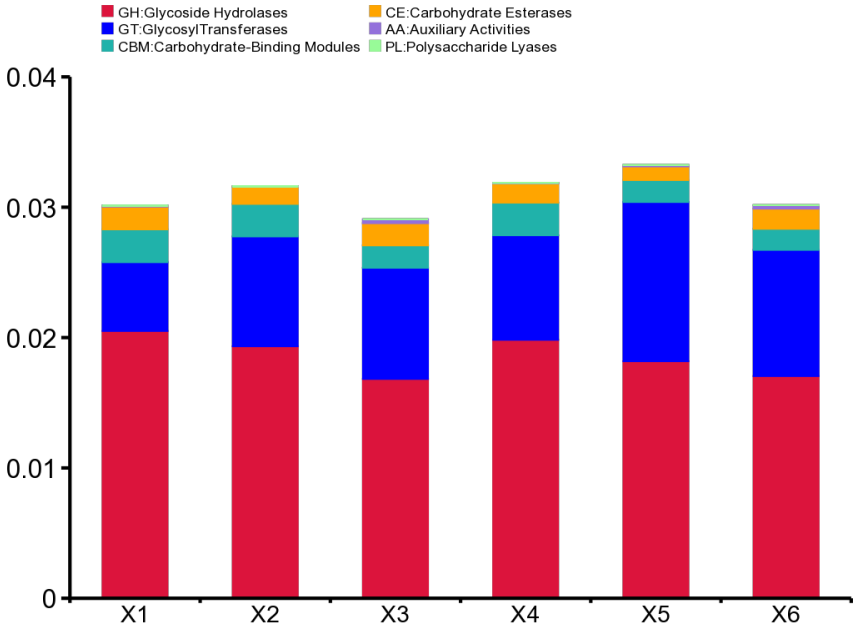


Figure 22. Bar chart of relative abundance at level 1 for functional notes of six types of kefir grains

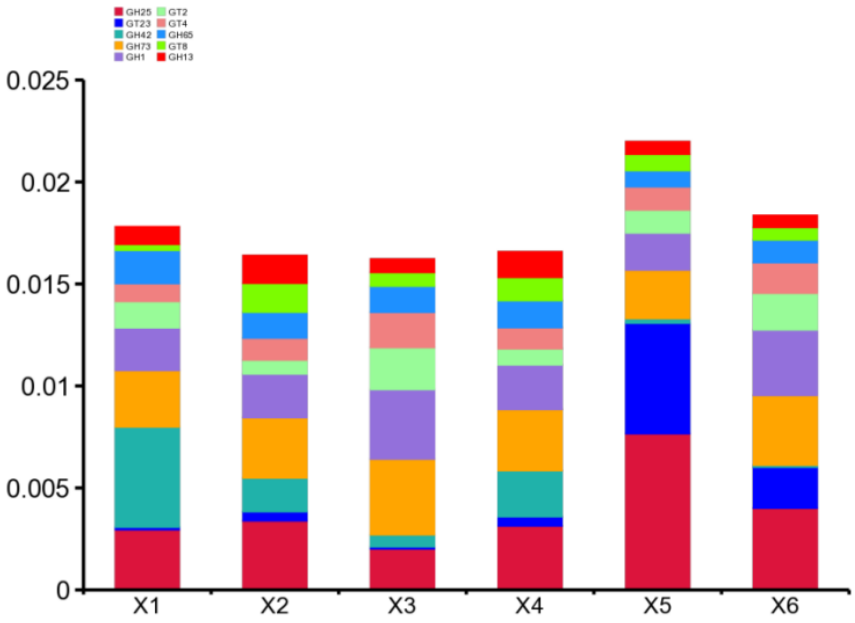


Figure 23. Bar chart of relative abundance at level 2 for functional notes of six types of kefir grains

The study analyzed the microbial diversity of 6 natural kefir and kefir grains through metagenomic detection, and analyzed the microbial diversity at each level from the relative abundance table at different levels. The results showed that the dominant species at the species level were *Lactococcus lactis*, *Lactobacillus kefiranofaciens*, *Lactobacillus crispatus*, *Lactobacillus helveticus*, *Lactobacillus kefiri* , etc. Gulitz et al. (2011) used auxotrophic medium for culturing microorganisms and molecular identification methods to isolate and identify kefir grains, and the content of *Lactobacillus* in the obtained strains was as high as 70%-80%, mainly including *Lactobacillus kefiranofaciens, Lactobacillus helveticus*, *Lactobacillus casei*, *Lactobacillus Nagelii* and so on. In the microbial composition of kefir grains, lactic acid bacteria are the absolute dominant flora. Nalbantoglu et al. (2014) performed whole genome shotgun pyrosequencing using two Turkish Kefir grains. They found the two grains showed high concordance in communities, where *Lactobacillus* was the most abundant genus with a mapped abundance of 99.42% and 99.79%. Garofalo et al. (2015) found the main bacterial species in milk kefir grains collected in some Italian regions was *Lactobacillus kefiranofaciens* while *Dekkera anomala* was the predominant yeast. Dertli et al. (2017) identified microbial flora of four kefir grains collected from different regions of Turkey with pyrosequencing approach. They found *Lactobacillus kefiranofaciens* presented in all grains at important levels. *Enterobacter*, *Acinetobacter*, *Enterococcus* and *Pseudomonas* spp. were observed in traditional kefir grains. The fungal microflora of kefir grains was dominated by yeast species and *Dipodascaceae* family was dominant and *Saccharomyces cerevisiae* presented in all grains. Although the species of kefir grains from different sources are different, the degree of diversity is similar.

Lactococcus is generally distributed on the surface of kefir grains, and produces acid quickly during fermentation, but its own intolerant acidity inhibits its growth in the later stage of fermentation (Jiang et al., 2009; Fan et al., 2014; Zhou et al., 2003). *Lactobacillus* mainly produces exopolysaccharides and other substances, which can be divided into homofermentative *Lactobacillus* and heterofermentative *Lactobacillus* (Liu et al., 2005). Yeasts are not only responsible for the production of carbon dioxide and ethanol, but also for pH regulation, aroma formation, and production of amino acids, vitamins, and biological compounds (E Simova et al., 2002; Özdestan et al., 2010). Wang et al. (2021) speculated that the different content of *Lactobacillus* genera in different kefir grains was highly associated with the proteolytic ability in goat milk kefir by Clustering analysis. And the contents of volatile compounds in goat milk kefir were also correlated to the bacteria and fungi in kefir grains (especially for *Lactobacillus* spp. and *Saccharomyces* spp.). Garrote et al. (2001) studied characteristics of the corresponding fermented milk, Chemical and microbiological composition of four Argentinean kefir grains from different sources.They found the four kefir grains have fermenting activity, while the grain weight growth was not significant. In the level 1 analysis of EGGNOG database, the two functions of Replication, recombination and repair and Carbohydrate transport and metabolism accounted for the largest proportion, which showed that kefir grains were undergoing vigorous growth and metabolism. In the level 1 analysis of the CAZY database, glycoside hydrolases accounted for the largest proportion of the six sample species, followed by glycosylransferases, which may be related to the active growth and metabolism of kefir grains, which is mutually confirmed with the conclusion of EGGNOG enrichment analysis.

CONCLUSION

The study performed inter-sample correlation analysis based on the amount of genes. The correlation heat coefficient map showed that six natural kefir grains vary in diversity, and the genes of kefir grains were diverse. There were 14794 common genes in samples, 111 unique genes of X1, 11 unique genes of X2, 1899 unique genes of X4, 552 unique genes of X5, and 1 unique gene of X6. The principal component analysis at the species level showed that X3 and X6 were highly similar, and X1 and X4 were significantly different. The NMDS results at the genus level showed that X2 and X6 were significantly different. There are differences in the microbial diversity of natural kefir grains from different sources, which may be related to the source of kefir grains, environment, culture conditions, researchers and other factors. There were different types of kefir grains from different sources, but the degree of diversity was similar. The dominant species at the species level are *Lactococcus lactis*, *Lactobacillus kefiranofaciens*, *Lactobacillus crispatus*, *Lactobacillus helveticus*, *Lactobacillus kefiri* ,etc. The level 1 analysis of EGGNOG database and the analysis of CAZY database both indicated that kefir granules were vigorously grown and metabolized. The research on the microbial diversity of natural kefir grains is conducive to the in-depth study of the particle

formation mechanism of kefir grains, the further study of the relationship between microorganisms and the flavor of kefir, and the optimization of the production process of kefir series products. The research can provide reference for the development and application of natural kefir grains in the dairy industry.

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