Screening and biological characteristics of excellent lactic acid bacteria from Kefir

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Abstract: Four excellent lactic acid bacteria were screened from Kefir. After morphological observation, physical and chemical detection, and 16SrDNA sequence comparison, three strains were preliminarily identified as Lactobacillus Kefir, and one strain was Lactobacillus acidophilus. Two strains were selected to study their biological characteristics. The results showed that the lactic acid bacteria isolated from Kefir had good probiotic properties, with a survival rate of more than 42% after artificial gastric juice digestion for 3 hours and more than 46% after artificial intestinal juice digestion for 6 hours; It has inhibitory effects on intestinal pathogenic bacteria such as Staphylococcus aureus and Escherichia coli; Does not produce hemolysis; There are different levels of resistance to antibiotics.

1. Introduction

Kefir is a fermented beverage with renowned probiotics that coexist in symbiotic association with other microorganisms in kefir grains.These gelatinous grains are a symbiotic culture of bacteria and yeast embedded in a polysaccharide matrix. Lactic acid bacteria, yeast and acetic acid bacteria are the primary microbial members of the sugary kefir grain.Amongst other contributions, species of lactic acid bacteria produce the exopolysaccharide matrix from which the kefir grain is formed, while yeast assists the bacteria by a nitrogen source that can be assimilated.This beverage consumption is associated with a wide array of nutraceutical benefits, including anti-inflammatory, anti-oxidative, anti-cancer,antimicrobial,antidiabetic,antihypertensive,andant i-hypercholesterolemic effects.

The microbial composition of kefir grains comprised 65 to 80 % of Lactic acid bacterias and the remaining portion was completed by yeasts.Lactic acid bacteria are gram positive bacteria that can ferment carbohydrates to produce organic acids such as lactic acid, and are widely found in fermented dairy products, pickles, silage, and other environments. Lactic acid bacteria are not only conducive to food fermentation, but also can improve the nutritional value of food and play a better regulatory role in the physiological functions of the human body .

In order to fully understand the functional characteristics of lactic acid bacteria in Kefir, lactic acid bacteria with excellent characteristics such as acid production and bacteriostasis were isolated from Kefir. Through morphological observation, biochemical property determination, and 16S rDNA strain identification, the bacteriostasis and gastrointestinal resistance of Kefir lactic acid bacteria were studied, and the safety of lactic acid bacteria in Kefir was evaluated from both hemolysis and drug resistance, To provide safe and high-quality lactic acid strains for the better development of Kefir milk drinks.

2. Materials and Methods

2.1 Materials and reagents

MC culture medium, blood agar plate, HKI013 EasylD lactic acid bacteria biochemical identification kit, antimicrobial susceptibility paper, Guangdong Huankai Company; MRS broth culture medium, Hangzhou Bess Company; Bacterial Genomic DNA Extraction Kit, Beijing Tiangen Company; 16S upstream and downstream primers, Shanghai Shenggong; Artificial gastric juice, artificial intestinal juice;, Hangzhou Microbiology Company. The rest are domestic analytical or chemical reagents.

2.2 Instruments and equipment

Model 722 Ultraviolet Visible Spectrophotometer, Shanghai Yidian; MyCycler PCR Amplifier, Bio-Rad, USA; SW-CJ-1D ultra-clean workbench, Shanghai Hujing; Electric heating thermostatic incubator, Shanghai Shangyi.

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2.3 Method

2.3.1Screening of excellent lactic acid bacteria

2.3.1.1 Isolation and purification of lactic acid bacteria

Dip in Kefir fermentation broth and scribe on an MRS plate. Incubate at 37 ℃ for 48 hours. Use a sterilized toothpick to pick up single colonies and transfer them to MRS liquid culture medium for cultivation. The strains with negative contact enzyme reaction and positive Gram stain microscopic examination were preliminarily identified as lactic acid bacteria.

2.3.1.2 Preliminary screening

Inoculate the purified single bacterial colony in MRS liquid culture medium, incubate at 37 ℃ for 48 hours, measure the OD value at a wavelength of 600 nm, and measure the pH value of the fermentation broth of each strain. Select strains with higher OD values and lower pH values for subsequent experiments.

2.3.1.3 Double screening

Fully activated bacterial solution was added to MRS liquid culture medium at 3% inoculation amount, and incubated at 37 ℃ for 48 hours under constant temperature oscillation. The acidity was measured according to the method in GB/T 5413.34-2010 [5].

2.3.2Screening and identification of lactic acid bacteria

2.3.2.1 Observation of morphological characteristics

Observe the culture characteristics of lactic acid bacteria on MRS plate, such as the color, size, texture, and edge of a single colony.The Gram stain microscope is magnified 1000 times to examine the color, morphology, and arrangement of bacteria.

2.3.2.2 Biochemical identification

The lactic acid bacteria biochemical identification kit was used to conduct biochemical tests such as aescin hydrolysis test, xylose fermentation test, cellobiose fermentation test, arabinose fermentation test, maltose fermentation test, galactose fermentation test, mannose fermentation test, melibiose, raffinose fermentation test, cane sugar fermentation test, trehalose fermentation test, and determine whether the test is positive or negative based on color changes.

2.3.2.3 Amplification and sequence analysis of 16S rDNA

The genomic DNA of lactic acid bacteria was extracted using a bacterial genomic DNA extraction kit. The conserved sequence of the 16S rDNA of the bacteria was amplified using the 16S universal primers 27f and 1492r. The purified PCR product was sent to Shanghai Shenggong for sequencing. The sequencing results were compared with similar sequences in the NCBI database.

2.3.3Screening of functional characteristics of lactic acid bacteria

2.3.3.1 Study on the Tolerance of Lactic Acid Bacteria to Artificial Gastrointestinal Fluid

The lactic acid bacteria were resuspended in artificial gastric juice and artificial intestinal juice for 3 and 6 hours, respectively, and the bacteria were collected. The viable bacteria were counted according to the National Standard for Food Safety - Microbiological Examination of Food - Determination of Total Colony Count (GB47892-2022). The tolerance was calculated based on survival rate [4] The formula is as follows:

Survival rate/%=lgN1/lgN0 × 100%

In the formula, N1 - viable bacterial count after tolerance treatment (CFU/mL); N0 - viable bacterial count before tolerance treatment (CFU/mL).

2.3.3.2 Bacteriostatic study

The Oxford Cup method was used to determine the antibacterial activity of lactic acid bacteria against common pathogenic bacteria. The supernatant was centrifuged from the bacterial solutions of Escherichia coli and Staphylococcus aureus. The supernatant was treated in three ways: adjusting the pH to 6.0 with 1 mol/L NaOH, treating with catalase and trypsin, and then filtering for sterilization. Add the prepared pathogenic bacteria plate to an Oxford cup, place the plate under 37 ℃ for 48 hours, and measure the diameter of the bacteriostatic ring (mm) .

2.3.3.3 Safety evaluation

1) Hemolysis

Select the activated lactic acid bacteria and draw a line on a blood agar plate. Incubate at 37 ℃ for 48 hours to observe whether there are hemolytic circles. At the same time, use Staphylococcus aureus as a positive control for comparison .

2) Drug resistance testing

Incubate the lactic acid bacteria liquid to 105~106 CFU/mL, evenly coat it on the MRS agar plate, paste vancomycin paper, erythromycin paper, chloramphenicol paper, tetracycline paper, gentamicin, amoxicillin paper, penicillin G paper, and streptomycin paper on the MRS agar plate, incubate at 37 oC for 48 hours, observe and measure the diameter of the bacteriostatic ring [8].

All trials were conducted three parallel trials, and the experimental data were statistically analyzed using Origin 8.5 and Excel 2016. Single factor analysis of variance was performed on the data of each group. $P<0.05$ indicates a significant difference between the data.

3. Results and Analysis

3.1 Screening of excellent lactic acid bacteria

Using Kefir as raw material, a total of 34 strains of lactic acid bacteria, named KFR-1, KFR-2, KFR-3, were obtained through isolation and purification, microscopic examination, and contact enzyme experiments. The strains with fermentation broth OD600 values above 1.0 and pH values below 5 include 11 strains with strong acid production capacity, including KFR-2, KFR-5, KFR-7, KFR-11, KFR-15, KFR-16, KFR-20, KFR-23, KFR-27, KFR-29, and KFR-31. The acidity of the fermentation broth of the initially screened strains is shown in Figure 1. The total acid content of the fermentation broth of lactic acid bacteria KFR-11, KFR-23, KFR-27, and KFR-31 is significantly higher than that of other strains ($P<0.05$), among which the total acid content of strains KFR-11 and KFR-27 is the highest and the difference is not significant ($P>0.05$), being 91 o T and 98 o T, respectively. KFR-11, KFR-23, KFR-27, and KFR-31 were selected as experimental subjects for the next step of the experiment.

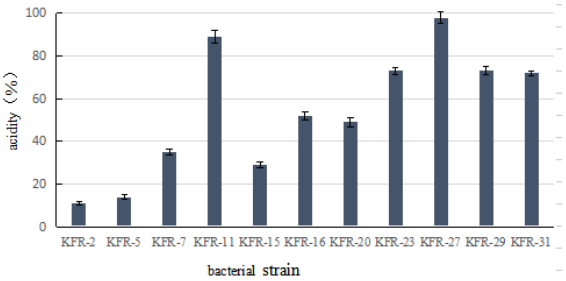

Figure 1 Acid production by lactic acid bacteria

3.2 Identification of lactic acid bacteria

3.2.1Observation of morphological characteristics

The colonies of selected KFR-11, KFR-23, and KFR-31 on MRS medium were small, white, round, and smooth and moist in surface; The colony of KFR-27 on MRS medium is small and its surface is smooth and moist. Four strains of lactic acid bacteria were Gram positive, and their bodies were all rod shaped, arranged in pairs or chains.

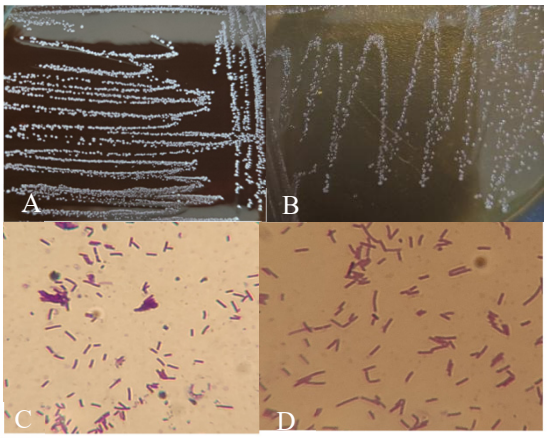

Figure 2 Lactic acid bacteria colony and microscopic examination
(A:KFR-11 Colony B:KFR-27 Colony
C:KFR-11 microscopic inspection
D:KFR-27 microscopic inspection)

3.2.2Biochemical identification

Using a biochemical identification kit for lactic acid bacteria, the ability of KFR-11, KFR-23, KFR-27, and KFR-31 to utilize carbon sources was tested. The results showed that the aescin hydrolysis test, cellobiose fermentation test, L-arabinose, maltose fermentation test, galactose fermentation test, mannose fermentation test, and fucose fermentation test were all positive; The xylose fermentation test, arabinose fermentation test, and raffinose fermentation test and sucrose fermentation test were all negative.

3.2.3Amplification and sequence analysis of 16S rDNA

Using the DNA of lactic acid bacteria as a template, a fragment of about 1.5 kb was obtained by PCR amplification. The 16S rDNA sequencing results were submitted to NCBI for BLAST comparative analysis. The strains KFR-11, KFR-23, and KFR-31 isolated in this study have 99% homology with Lactobacillus kefir, and KFR-27 have 99% homology with Lactobacillus acidophilus.

According to the colony characteristics, morphological characteristics, sugar fermentation tests, and 16S rDNA sequence analysis of lactic acid bacteria, KFR-11, KFR-23, and KFR-31 were identified as Lactobacillus kefir, and KFR-27 as Lactobacillus acidophilus. According to the acid production performance, KFR-11 and KFR-27 were selected as the research objects for biological characteristics research.

3.3 Research on biological characteristics

3.3.1Study on Tolerance of Artificial Gastrointestinal Fluid

Kefir is generally consumed without heating. Most acid intolerant microorganisms from food are killed by the acid

in the gastric juice. Fluid foods stay in the stomach for about 2-3 hours, and the pH of human gastric juice is usually around 3.0. Food enters the small intestine after being digested by gastric juice. Bile salts in the small intestine have antibacterial effects. Bile salt stress is an important factor affecting the probiotic function of lactic acid bacteria, which stay in the small intestine for about 2 to 6 hours. In this study, artificial gastric juice containing pepsin was selected, with a pH value of about 3.0 and a digestion time of 3 hours; The artificial intestinal fluid contains trypsin and bile salts, with a pH of about 8.0 and a digestion time of 6 hours. The tolerance of KFR-11 and KFR-27 to artificial gastric and intestinal fluids is shown in Table 1. After 3 hours of digestion in artificial gastric juice, the survival rates of the two strains were 42% ± 0.49 and 58% ± 0.28, respectively. There was a significant difference between the two strains ($P<0.05$), with the survival rate of KFR-27 being greater than 50%. The survival rate of both strains of bacteria after 6 hours of digestion in artificial intestinal fluid was close to 50%, and there was no significant difference between the two strains ($P>0.05$).

Table1 Three strains of lactic acid bacteria resistant to gastrointestinal tract

	gastric juice 3 h	intestinal juice 6 h
KFR-11	42%±0.49	48%±0.42
KFR-27	58%±0.28	51%±0.56

3.3.2Bacteriostatic study

The fermentation supernatant of both strains of lactic acid bacteria can inhibit Escherichia coli and Staphylococcus aureus, but not Pseudomonas aeruginosa. From Table 2, it can be seen that neutralization treatment and catalase treatment eliminate the impact of organic acids and hydrogen peroxide on the bacteriostatic effect of the indicator bacteria. Using trypsin to treat the fermentation broth of the two strains, the bacteriostatic effect was significantly reduced ($P<0.05$), and it was determined that the bacteriostatic substance contained bacteriocins with protein properties. The antibacterial effect of lactic acid bacteria fermentation supernatant on pathogens has potential application value. Lactic acid bacteria can maintain gastrointestinal balance by inhibiting the growth of pathogens, thereby improving human health.

Table 2 Bacteriostatic activity of lactic acid bacteria

Indicator bacteria	Supernatant treatment method	Bacteriostatic diameter	
		KFR-11	KFR-27
E.coli	untreated	13.25±0.123（＋＋）	14.25±0.094（＋＋）
	Neutralization treatment	13.12±0.098（＋＋）	14.32±0.087（＋＋）
	Catalase treatment	9.08±0.08（＋）	11.08±0.032（＋）
	Trypsin treatment	-	-
S. aureus	untreated	11.05±0.076（＋）	12.05±0.073（＋＋）
	Neutralization treatment	11.03±0.059（＋）	12.03±0.042（＋＋）
	Catalase treatment	9.23±0.056（＋）	10.23±0.050（＋）
	Trypsin treatment	-	-

Note: If the diameter is<9 mm and there is no antibacterial activity, it is marked as "-"; 9 mm ≤ diameter<12 mm, with antibacterial activity, marked as "+"; Diameter ≥ 12mm, strong antibacterial activity, marked as "++".

3.3.3Safety evaluation results

As an edible probiotic strain, the strain isolated from Kefir was tested for potential adverse effects on human health. Analysis of antibiotic resistance and hemolytic activity of lactic acid bacteria is an important feature of their safe use.

3.3.3.1 Hemolysis

The agar around Staphylococcus aureus showed a green ring, while the agar around KFR-11 and KFR-27 did not show a green transparent ring. The strains with transparent hemolytic circles are β- Hemolytic or completely hemolytic, the green circle is α- Hemolytic or partially hemolytic. Only non hemolytic lactic acid bacteria can be used as potential probiotics in fermented foods. In this experiment, no hemolytic phenomenon was found in the screening of lactic acid bacteria.

3.3.3.2 Resistance

With the overuse of antibiotics, the problem of microbial resistance is becoming increasingly serious. Fermented products containing lactic acid bacteria may enter the human gut through the food chain, and drug resistance genes are transmitted to pathogenic bacteria in the body through molecular binding and other actions, posing a serious threat to human health. The sensitivity of the two strains of lactic acid bacteria tested to eight common antibiotics is divided into three levels according to the standards specified by the American Society for Clinical and Experimental Standards (CLSI): sensitive (S), moderately sensitive (I), and resistant (R). From Table 3, it can be seen that the two strains of lactic acid bacteria

have no resistance to most antibiotics, only gentamicin, while most lactic acid bacteria have natural resistance to aminoglycoside antibiotics. KFR-11 and KFR-27 are moderately resistant to vancomycin, while KFR-27 is moderately resistant to tetracycline. Xu Nv [a] et al. evaluated the antibiotic resistance of 97 strains of lactic acid bacteria isolated from traditional fermented foods and analyzed their resistance genes. All the isolated strains were highly resistant to vancomycin, gentamicin, and tetracycline, with a resistance rate of over 50%. Drug resistance of lactic acid bacteria may become an important food safety hazard. As an edible strain, it is necessary to strengthen the detection of drug resistance of lactic acid bacteria.

Table3 Three strains of lactic acid bacteria resistance test results

titer/U·mg^{-1}	KFR-11	KFR-27
vancomycin	15.9/I	16.8/I
erythromycin	20.9/**S**	21.2/S
chloramphenicol	21.6/S	22.3/S
tetracycline	23.5/S	16.9/I
gentamicin	12.1/R	10.2/R
Amoxicillin	25.2/S	26.3/S
Penicillin G	24.8/S	21.1/S
streptomycin	22.8/S	21.1/S

4. CONCLUSIONS

Some lactic acid bacteria in Kefir are naturally occurring or artificially added. Both Lactobacillus Swiss and Lactobacillus Kefir belong to the genus Lactobacillus, which can ferment to produce a variety of antibacterial substances, inhibiting and killing spoilage bacteria and pathogenic bacteria in fermented foods. Many lactobacilli can produce volatile substances that give fermented foods a special flavor. If lactic acid bacteria want to play a role in improving the structure of intestinal flora and inhibiting intestinal pathogenic bacteria, it is required to overcome the impact of physical and chemical factors in the stomach and intestines, and reach the stomach and intestines in a viable state. KFR-11 and KFR-27 have a certain survival rate after being treated with artificial gastric and intestinal fluids. According to the safety evaluation of the 2 strains of lactic acid bacteria, hemolysis will not occur. In this study, it was found that 2 strains of bacteria had no antibiotic resistance except for gentamicin.

The two excellent lactic acid bacteria selected from Kefir in this experiment can withstand the stomach and intestinal environment, inhibit the growth of common pathogenic bacteria such as Escherichia coli and Staphylococcus aureus, and provide a reference basis for the development and utilization of probiotics. Currently, the lactic acid bacteria used in fermented foods are basically wild type, which are generally consumed directly without secondary disinfection, and may carry out resistance gene transmission with normal intestinal flora or pathogenic bacteria. Before lactic acid bacteria are used as edible production strains, it is necessary to detect the drug resistance of the strains and prevent the spread of drug resistant genes.

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References

1. Azizi N, Kumar M, Yeap S, Abdullah J, Khalid M, Omar A, Osman M, Mortadza S, Alitheen N. (2021)Kefir and Its Biological Activities. Foods. 10:1210. DOI：10.3390/foods10061210
2. Zhihua G, Cong W, Hongling C.(2021) Screening, identification and functional characteristics of nitrite-degrading lactic acid bacteria from black tea bacteria . Chinese Journal of Food, 21 : 309-316. DOI:10.16429/j.1009-7848.2021.08.033.
3. Daiwen L,Xiuzhu D.(1999)Classification, Identification, and Experimental Methods of Lactic Acid Bacteria. Beijing: China Light Industry Press, 1999:6-7
4. Lynch K, Wilkinson S, Daenen L, Arendt E.(2021) An update on water kefir: Microbiology, composition and production. Int J Food Microbiol. 345:109128. DOI:10.1016/j.ijfoodmicro.2021.109128.
5. Ministry of Health of the People's Republic of China. GB/T 5413.34-2010 National Food Safety Standard: Determination of Acidity in Milk and Dairy Products [S]. Beijing: China Standards Press, 2010
6. Ojha A, Shah N, Mishra V. (2021)Conjugal Transfer of Antibiotic Resistances in Lactobacillus spp.[J]. Current microbiology, 78 : 1-11.DOI : 10.1007/s00284-021-02554-1
7. Rosa, D., Dias, M., Grześkowiak, Ł, Reis, S., Conceição, L., & Peluzio, M. (2017). Milk kefir: Nutritional, microbiological and health benefits. Nutrition Research Reviews, 30:82-96.
8. SHEKH S, DAVE J, VYAS B. (2016)Characterization of Lactobacillus plantarum strains for functionality, safety and γ-amino butyric acid production. LWT-Food Science and Technolgy, 74: 234-241. DOI:10.1017/S0954422416000275
9. Mengjiao W, Shaoying L,Shufen L.(2014)Safety evaluation of enterococcal lactic acid bacteria in horse milk and its products.Food Science, 35: 204-208 DOI：10.7506/spkx1002-6630-201417039
10. Haiyan X,Siduo Z,Mingzhe Z.(2019)Screening and functional characteristics of probiotic lactic acid bacteria from fermented chili. Food Science,40: 93-99 DOI：10.7506/spkx1002-6630-20180315-199
11. Nv X,Yaru L,Chaoyu W.(2020) Evaluation of antibiotic resistance and analysis of resistance genes of lactic acid bacteria in traditional fermented foods.

Chinese Journal of Food, 20: 160-171. DOI:10.16429/j.1009-7848.2020.07.020.

12. Bo Y, Ji B,Yamei J.(2022) Screening of excellent lactic acid bacteria starter from donkey milk in Xinjiang and its probiotic characteristics. Food Science, 3: 224-232 DOI:10.7506/spkx1002-6630-20201209-104
13. Fang Z,Yanqin L,Binchun L.(2016)Screening of probiotic lactobacillus in a simulated human gastrointestinal environment.Microbiology Bulletin, 43: 1396-1403 DOI:10.13344/j.microbiol.china.150898