

LACTIC ACID BACTERIA AND YEASTS FROM MILK KEFIR GRAINS: ISOLATION, CHARACTERIZATION, SCREENING, AND IDENTIFICATION

– Research paper –

Yunli XU¹, Guowei SHU¹, Yu LIU^{2,*}, Lin MA¹, Yichao LI², He CHEN¹, Chao SHAN²

¹ School of Food and Biological Engineering, Shaanxi University of Science and Technology, Xi'an, 710021, China

² Department of Research and Development, Shaanxi Heshi Dairy Co. Ltd., Baoji, 721200, China

Abstract: Lactic acid bacteria and yeast are abundant in kefir grains. Six natural kefir grains were used in this research to isolate and purify 64 strains of yeast and 108 strains of lactic acid bacteria. A total of three lactic acid bacteria and one yeast strain (*Lactococcus lactis* 2C6, *Lactobacillus helveticus* 6171, *Lactobacillus plantarum* 4M2 and *Saccharomyces cerevisiae* 6Y6) were examined for their ability to protein hydrolysis, water holding capacity, alcohol production, acid and bile salt tolerance and identified by 16S rDNA. The proteolytic ability, alcohol generation, and acid and bile salt tolerance of 4M2 and 6Y6 were excellent. Lyophilization was used to create bacterial powder, which laid the foundation for the subsequent development of direct-vat-set (DVS) kefir starter.

Keywords: kefir grains, lactic acid bacteria, yeast, 16S rDNA, proteolytic ability

INTRODUCTION

Around a thousand years ago, the Caucasus Mountains in Central Asia were where kefir seeds first appeared (Nielsen B et al. 2014). Sheep or horse milk was originally kept in skin sacs by herdsmen, who then fermented the milk multiple times to create kefir grains (Li et al. 2016).

Macroscopically, kefir grains are a unique natural microbial symbiosis with elastic, viscous particles, ranging in color from white to light yellow, presenting irregular spherical or cauliflower shapes, and variable in size, generally between 0.5 and 3 cm. A natural matrix made up of glucose, galactose, and proteins in the kefir grains supports the coexistence of lactic acid bacteria (LAB), yeast, and a negligible quantity of acetate bacteria (AAB).

Kefir grains are added to cow, goat and sheep milk and fermented into a low-calorie fermented dairy product containing small amounts of ethanol and carbon dioxide, known as kefir, also known as sour milk wine (Sabir et al. 2010). Vitamins A, K, B1, B2, B5, vitamin C, and antioxidants are all present in kefir. Kefir is an excellent source of calcium, magnesium, and phosphorus when it comes to mineral content. In addition, the fermentation process of kefir produces a variety of bioactive compounds.

Kefir has functions of lowering blood pressure, lowering cholesterol, anti-cancer, and anti-inflammatory, etc. It has gained global recognition as a healthy probiotic drink, but there are limitations in industrial production because the fermentation of kefir needs to rely on kefir grains. Therefore, it is important to develop industrially produced and functional kefir fermenters. In recent years, many scholars have devoted themselves to the development of pure ferments similar to kefir grains to replace natural kefir grains for industrial production. To prepare a DVS kefir starter, it is necessary to first isolate the strains of natural kefir grains, obtain the dominant strains by screening, and obtain the powder by centrifugal freeze-drying after pure culture of the strains separately, and then by compounding.

In this paper, we used selection medium to isolate and purify lactic acid bacteria and yeast from six kinds of natural kefir grains, perform primary and re-screening on the strains obtained from the grains, finally get the dominant strains, perform strain identification, and prepare the strains into bacterial powder by freeze-drying after pure culture, respectively, to subsequently prepare DVS kefir starter by compounding. Through this method, the purpose of making kefir product for industrial production can be achieved, thus allowing companies to produce kefir in a better and lower-cost way and expand their revenue.

Received: 08.04.2023

Accepted in revised form: 30.05.2023

* Corresponding author.

E-Mail address: liuyuhsh@gmail.com

MATERIAL AND METHODS

Materials

Six kefir grains were provided by the School of Food & Biological Engineering, Shaanxi University of Science & Technology (Dong et al. 2022).

Morphological observation of kefir grains

The activated kefir grains were filtered out and the fermented milk adhering to the surface was rinsed off with sterile saline for macroscopic observation first and then processed for observation using scanning electron microscopy (Gao 2013).

Separation and purification of lactic acid bacteria and yeast

Following the national standard, 5 mL of fermented kefir milk was injected into 45 mL of sterile physiological saline, and one of the activated kefir grains was aseptically ground and mixed into sterile physiological saline to make a dilution solution. Take 1 mL of the dilution solution into 9 mL of sterile saline and shake well to make the distribution of the bacteria evenly. Repeat the above steps until the dilution reaches 10^{-6} ~ 10^{-9} . Take 0.1 mL of the dilution and spread it on MRS agar medium, MC agar medium, M17 agar medium, Elliker agar medium, PDA agar medium, and YPD agar medium. They were incubated at 37 °C and 28 °C, respectively, and after several purifications, individual colonies were picked from each medium and transferred into the broth medium.

Yeast aroma production test

The yeast was sown in an ester-producing substrate and allowed to grow for 48 h at 28 °C. The strain with the bolder taste was chosen for the following experiment (Zhang 2016).

Fermented goat milk pH determination

The pH value of fermented milk was measured at room temperature using a PHS-3C pH meter. Three parallel tests were set up for each group of samples.

Water-holding capacity

The water-holding capacity was calculated using a modified version of a previously described technique (Yao et al. 2017). 20 g of yogurt (Y) were spun at 4000 g for 20 min at 4 °C in a refrigerator. After pouring off the supernatant, the precipitation (W) was measured. The following algorithm was used to determine water holding capacity:

$$\text{Water holding capacity (\%)} = \left(\frac{W}{Y} \right) \times 100\% \quad (1)$$

where W is the mass of the precipitate and Y is the mass of the yogurt that has been studied.

Protein hydrolysis degree

The Protein hydrolysis degree was determined according to a method previously described with

modifications (Adler-Nissen 1986).

Yeast alcohol production test

The activated bacterial suspension was diluted to a suitable gradient, 0.1 mL was applied to YPD medium or PDA medium, and incubated at 28 °C for 48 h. TTC medium was poured to cover the initial colonies on plates with colony counts between 100 and 300, and incubation was continued at 28 °C. At 1, 3, and 6 hours, the medium's color shift was noticed and noted, respectively. (Zhang et al. 2011).

Acid resistance

With a few minor changes, the Zhang et al. (2011) technique was used. Lactic acid bacteria and yeasts were incubated for 4 h at 37 °C and 28 °C, respectively, in a liquid medium with a pH of 3. The resulting broth was then suitably diluted and tested for the viable bacterial count.

Bile salt tolerance

The approach used by Delgados et al. (2007) and Guo et al. (2009) underwent a minor change. The activated bacterial broth was inoculated with 3% (V/V) in a liquid medium with different concentrations of bovine bile salts and the time required for the absorbance (OD₆₂₀) to rise by 0.3 units was determined to determine the growth lag of the strain in different concentrations of bovine bile salts, which was calculated as follows:

$$\text{Lag period (h)} = \text{experimental group (h}_2\text{)} - \text{control group (h}_1\text{)} \quad (2)$$

where: h₂ is the time consumed for the rise of 0.3 units of OD₆₂₀ value in the experimental group; h₁ is the time consumed by 0.3 units of OD₆₂₀ value rise in the blank group.

Strain identification

Molecular biology was used to identify the variants. 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, 72 °C for 10 min, and 4 °C for the PCR reaction conditions. Bacterial primers were: rD1-REV/27F-FOR; yeast primers were: ITS4-FOR/ITS5-REV. phylogenetic trees were established by MEGA software.

Preparation of lyophilized powder

The lactic acid bacteria and yeasts that were obtained through screening were cultured in MRS broth medium and YPD broth culture based on 37 °C and 28 °C for 24 h and 48 h, respectively. The organisms were then collected by centrifugation and vacuum freeze-dried while adding the protective agent from the pre-screening in the laboratory. (Shi 2019) (freeze-drying conditions: -35 °C pre-freezing for 3 h, then programmed warming, every 3 h for one stage, vacuum level 15 Pa).

Statistical analysis

Three times the trial was conducted. Standard variation and the average were computed. The standard deviation was added to the mean to express

each number. The SPSS.17 program was used for statistical analysis of the data, and OringinPro 8 was used for figure creation.

RESULTS AND DISCUSSION

Morphological characterization of kefir

As shown in Figure 1, it is the kefir grains after activation. From a macroscopic point of view, kefir grains are elastic particles, kefir grains X1 and X6 are more regular large round spheres, kefir grains X2 and X3 are small round spheres, kefir grains X4 and X5 are more like irregular cabbage shapes; all kefir grains have wet surfaces and are milky white. Figure 2 depicts the results of scanning electron microscope observations of the kefir grains' microstructure. All kefir grains contained lactic acid bacteria and yeast that were attached to the substrate and combined to form the kefir grains. However, the composition, distribution, and proportion of the bacterial phases varied among the various sources of kefir grains. According to the available reports and the majority of the long and short rods for lactic

acid bacteria and acetate bacteria, which is consistent with the findings of Guzel-Seydim et al. (2010), microscopic images revealed that all kefir grains contained much more yeasts than lactic acid bacteria and acetate bacteria, and that most yeasts were attached to the surface of kefir grains. According to the available reports and the majority of the long and short rods for lactic acid bacteria and acetate bacteria, which is consistent with the findings of Guzel-Seydim et al. (2010) who observed kefir grains, microscopic images showed that all kefir grains contained much more yeasts than lactic acid bacteria and acetate bacteria. Additionally, most yeasts were attached to the surface of kefir grains. In the proximity of the electron micrographs, lactococci were also seen.

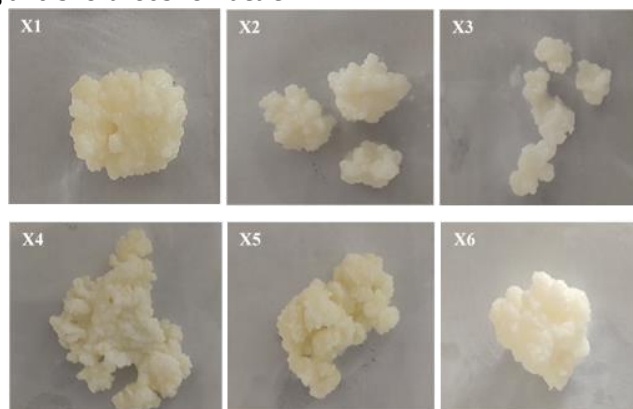


Figure 1 Morphological characteristics of different kefir grains

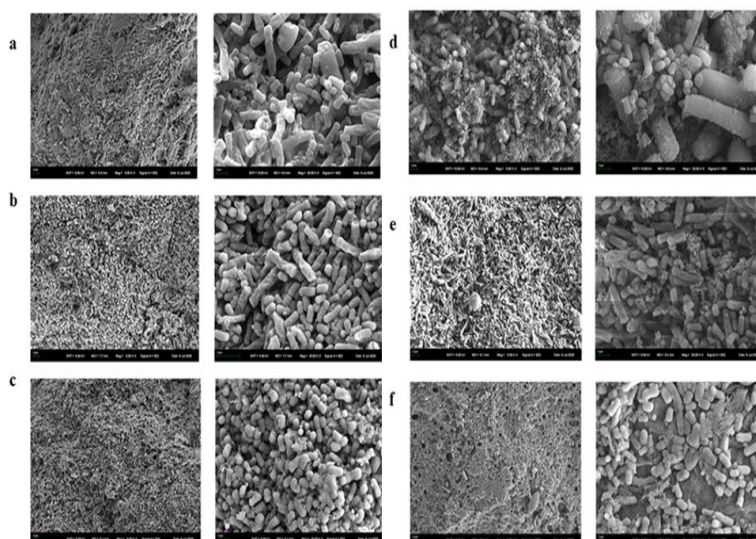


Figure 2. The scanning electron microscopy images of six kinds of natural kefir grains (left:5 kX; Right:20.00 kX; a:X1, b:X2, c:X3, d:X4, e:X5, f:X6)

Analysis of yeast primary screening results

After the isolation and purification of PDA and YPD solid media, a total of 64 yeast strains were obtained. In the fermentation process of kefir grains, yeasts serve as the main source of alcohol flavor, so it is especially important to screen out yeasts with better aroma production ability. According to the aroma production test, a total of 19 strains of yeast did not produce or produced a small amount of fermentation aroma during the fermentation process, namely 2P3, 2P4, 3P3, 4P1, 4P5, 5P2, 5P3, 6P1, 6P4, 1Y2, 1Y3, 1Y5, 4Y2, 4Y4, 5Y3, 5Y5, 6Y2, 6Y4, 6Y5. The next screening test was conducted for yeasts other than the above 19 strains.

Measurements were made of the fermented goat milk's pH and protein hydrolysis after *Saccharomyces cerevisiae* was incubated in 12.5% sterilized recovered goat milk at 28 °C for 24 hours with a 5% inoculum. The pH and protein hydrolysis degree were used as indicators for screening, and the test results are shown in Table 1.

The pH of yeast is generally high, where 1Y1, 2Y4, 2Y5, 4P4, 5Y2, 6P5, 6Y1, and 6Y6 can reach the pH of normal fermented milk, but the pH of yeast is used as a reference indicator since yeast is not involved in lactic acid fermentation and is responsible for the production of aroma species during the fermentation process.

In the process of yeast fermentation, proteins in goat milk are hydrolyzed for human digestion and absorption, where 6Y6 had the highest protein hydrolysis of $(53.78 \pm 0.22) \%$, and Shi (2019) used the protein hydrolysis degree as one of the indicators in the screening of dominant strains, where the highest protein hydrolysis degree in yeast was about 64%, which was higher compared to the protein hydrolysis degree in this study.

Primary screening and performance analysis of lactic acid bacteria

A total of 108 lactic acid bacterial strains were isolated from six different types of kefir fermented milk after isolation and purification. The bacterial broth was then added to sterilized recovered goat milk at a rate of 5% inoculum, and the results are shown in Table 2.

One of the criteria used to assess the grade of fermented milk is pH. After dairy fermentation, a pH of about 4.5 was found to be ideal in earlier experiments. Among the 108 strains of lactic acid bacteria screened, strains 1M2, 1M4, 1M6, 1C6, 1E5, 1E6, 2M3, 2C5, 3M2, 3M4, 3C1, 3C3, 3E6, and SM2 had pH values above 6.0, indicating that these strains were extremely weak in fermenting goat milk.

Table 1. Determination results of pH and proteolysis degree of fermented milk

Number	Strain number	Protein hydrolysis degree (%)	pH	Number	Strain number	Protein hydrolysis degree (%)	pH
1	1P1	15.11±0.34	5.81±0.01	23	3Y1	31.30±0.43	5.03±0.02
2	1P2	23.76±0.43	5.78±0.02	24	3Y2	33.45±0.56	4.85±0.01
3	1P3	26.88±0.63	4.88±0.01	25	3Y3	44.99±0.31	4.66±0.02
4	1P4	23.65±0.62	5.81±0.03	26	3Y4	34.75±0.53	5.42±0.01
5	1P5	24.07±0.48	4.72±0.02	27	3Y5	27.80±0.64	5.64±0.01
6	1P6	27.41±0.73	5.58±0.02	28	3Y6	24.41±0.28	5.57±0.01
7	1P7	22.19±0.57	4.82±0.01	29	4P2	27.01±0.46	4.88±0.01
8	1P8	24.95±0.53	5.70±0.01	30	4P3	26.02±0.63	5.04±0.02
9	2P1	23.68±0.63	5.77±0.01	31	4P4	30.24±0.53	4.66±0.02
10	2P2	23.95±0.46	4.74±0.02	32	5P1	23.37±0.42	5.15±0.02
11	3P1	23.07±0.63	5.70±0.01	33	5P4	25.68±0.22	5.84±0.01
12	3P2	24.34±0.53	5.72±0.01	34	6P2	14.63±0.15	5.06±0.02
13	3P5	23.95±0.42	5.78±0.02	35	6P3	22.74±0.21	4.89±0.03
14	3P6	23.68±0.46	5.80±0.01	36	6P5	22.74±0.73	4.15±0.01
15	1Y1	44.25±0.18	4.55±0.02	37	4Y1	24.05±0.56	5.80±0.02
16	1Y4	23.67±0.53	5.79±0.03	38	4Y3	23.05±0.67	5.03±0.01
17	2Y1	18.53±0.53	5.99±0.02	39	5Y1	26.02±0.33	5.12±0.01
18	2Y2	21.19±0.56	5.10±0.01	40	5Y2	38.24±0.38	4.33±0.02
19	2Y3	24.91±0.67	5.36±0.01	41	5Y4	33.40±0.47	4.81±0.02
20	2Y4	42.62±0.43	4.16±0.01	42	6Y1	40.63±0.33	4.26±0.01
21	2Y5	50.30±0.37	4.17±0.02	43	6Y3	31.00±0.43	5.03±0.02
22	2Y6	27.23±0.54	6.09±0.01	44	6Y6	53.78±0.22	4.24±0.01

Table 2. Determination results of pH, water retention and proteolysis degree of fermented milk

Number	Strain number	water-holding capacity (%)	Protein hydrolysis degree (%)	pH
1	1M1	15.35±0.10	49.19±0.34	4.93±0.02
2	1M2	15.66±0.14	50.20±0.43	6.08±0.01
3	1M3	23.27±0.12	36.47±0.63	4.11±0.01
4	1M4	16.80±0.15	33.97±0.24	6.15±0.03
5	1M5	15.49±0.17	45.22±0.48	5.06±0.02
6	1M6	16.74±0.07	43.95±0.47	6.04±0.01
7	1C1	29.60±0.03	58.65±0.20	4.46±0.02
8	1C2	20.02±0.02	53.91±0.53	4.29±0.01
9	1C3	23.41±0.12	38.17±0.38	5.63±0.03
10	1C4	30.93±0.09	57.16±0.46	4.35±0.02
11	1C5	23.35±0.07	43.28±0.39	5.16±0.02
12	1C6	23.46±0.2	38.53±0.53	6.07±0.01
13	1E1	32.27±0.03	51.80±0.56	4.05±0.03
14	1E2	22.35±0.02	42.09±0.67	4.99±0.02
15	1E3	23.12±0.12	55.92±0.33	5.09±0.02
16	1E4	23.15±0.09	57.19±0.38	5.25±0.02
17	1E5	17.47±0.07	42.09±0.47	6.10±0.02
18	1E6	16.03±0.20	48.11±0.33	6.06±0.01
19	1E7	10.57±0.13	39.22±0.43	3.03±0.01
20	2M1	24.05±0.13	48.26±0.42	5.80±0.02
21	2M2	29.58±0.04	69.00±0.46	4.42±0.01
22	2M3	20.32±0.13	37.19±0.18	6.08±0.02
23	2M4	15.04±0.05	51.23±0.53	5.15±0.02
24	2M5	24.94±0.14	45.83±0.53	4.05±0.01
25	2C1	14.40±0.13	44.28±0.56	5.93±0.02
26	2C2	33.56±0.15	62.29±0.67	4.33±0.01
27	2C3	21.46±0.13	47.16±0.43	4.34±0.02
28	2C4	14.65±0.01	53.45±0.37	5.66±0.03
29	2C5	14.60±0.07	55.02±0.54	6.02±0.02
30	2C6	31.40±0.11	67.72±0.43	4.44±0.01
31	2E1	24.05±0.04	55.95±0.53	4.98±0.03
32	2E2	9.58±0.13	51.07±0.53	5.86±0.01
33	2E3	20.32±0.05	48.91±0.56	3.6±0.02
34	2E4	15.04±0.14	44.43±0.67	5.84±0.02
35	2E5	24.94±0.13	50.98±0.43	5.73±0.01
36	3M1	15.06±0.08	39.02±0.56	5.84±0.01
37	3M2	25.01±0.16	56.66±0.31	6.01±0.02
38	3M3	25.30±0.05	37.63±0.53	5.05±0.02
39	3M4	21.17±0.13	46.97±0.64	6.09±0.01
40	3M5	31.84±0.06	59.19±0.28	4.44±0.02
41	3M6	22.10±0.12	56.67±0.46	4.86±0.01
42	3M7	25.26±0.09	50.61±0.63	5.45±0.01
43	3C1	12.07±0.13	57.18±0.53	6.02±0.01
44	3C2	29.20±0.11	31.44±0.42	4.32±0.02
45	3C3	18.65±0.10	43.18±0.22	6.03±0.02
46	3C4	10.33±0.13	37.99±0.15	5.07±0.01
47	3C5	31.37±0.05	50.74±0.21	4.34±0.01
48	3C6	25.99±0.10	46.28±0.73	5.90±0.02
49	3E1	25.76±0.15	39.97±0.37	5.84±0.02
50	3E2	23.02±0.13	48.11±0.54	3.02±0.01
51	3E3	35.52±0.18	62.43±0.43	4.53±0.01
52	3E4	24.00±0.07	60.10±0.56	4.97±0.01
53	3E5	24.57±0.11	58.77±0.73	5.33±0.02
54	3E6	21.79±0.04	56.91±0.56	6.08±0.02
55	3E7	20.00±0.13	48.18±0.67	3.59±0.01
56	C1	22.29±0.04	49.78±0.33	4.67±0.01

Continued Table 2

Number	Strain number	water-holding capacity (%)	Protein hydrolysis degree (%)	pH
57	C2	28.79±0.08	40.22±0.38	5.17±0.02
58	C3	24.57±0.11	60.81±0.43	5.84±0.02
59	C4	28.65±0.10	46.67±0.46	5.03±0.02
60	SM1	23.09±0.05	50.36±0.47	4.56±0.03
61	SM2	26.76±0.04	33.92±0.33	6.02±0.02
62	MC1	23.09±0.13	46.87±0.46	4.59±0.02
63	MC2	25.10±0.01	52.18±0.26	4.69±0.01
64	4M1	26.02±0.12	48.01±0.42	5.21±0.02
65	4M2	30.24±0.06	71.92±0.46	4.35±0.01
66	4M3	23.37±0.07	51.09±0.18	4.09±0.02
67	4M4	25.68±0.20	60.01±0.53	5.59±0.01
68	4M5	14.63±0.11	58.17±0.53	5.10±0.02
69	4E1	17.66±0.01	48.16±0.56	5.10±0.02
70	4E2	27.12±0.06	38.19±0.73	5.01±0.02
71	4E3	23.32±0.12	44.99±0.56	4.93±0.01
72	4E4	25.76±0.13	52.91±0.67	3.62±0.01
73	4E5	23.02±0.07	39.09±0.33	4.57±0.02
74	4E6	19.45±0.03	46.94±0.38	5.77±0.03
75	4171	29.44±0.05	63.22±0.53	4.44±0.01
76	4172	21.09±0.15	38.17±0.42	5.01±0.01
77	4173	25.32±0.10	49.26±0.22	3.31±0.02
78	4174	22.73±0.08	51.86±0.15	4.88±0.01
79	5M1	22.74±0.14	46.02±0.56	5.41±0.03
80	5M2	22.74±0.13	43.28±0.57	5.22±0.01
81	5M3	24.05±0.05	47.18±0.53	3.7±0.02
82	5M4	23.05±0.14	39.93±0.33	4.85±0.02
83	5M5	17.28±0.13	58.28±0.46	5.22±0.01
84	5E1	31.00±0.13	62.18±0.53	3.58±0.02
85	5E2	21.57±0.12	39.28±0.42	5.16±0.01
86	5E3	21.79±0.15	43.16±0.28	5.22±0.02
87	5E4	22.16±0.11	47.77±0.18	4.76±0.01
88	5E5	25.30±0.07	49.97±0.53	3.52±0.01
89	5E6	21.17±0.07	60.17±0.53	3.95±0.02
90	5171	17.40±0.13	54.83±0.21	4.10±0.02
91	5172	31.15±0.08	67.59±0.40	4.41±0.01
92	5173	25.30±0.06	52.15±0.43	5.20±0.01
93	5174	20.17±0.05	49.26±0.56	3.89±0.02
94	5175	23.84±0.13	40.10±0.73	5.44±0.03
95	6M1	21.26±0.15	67.10±0.63	4.77±0.02
96	6M2	24.11±0.13	33.27±0.53	4.26±0.01
97	6M3	14.30±0.15	44.48±0.42	3.40±0.01
98	6M4	22.72±0.13	51.34±0.43	4.57±0.01
99	6E1	23.84±0.11	53.10±0.56	4.09±0.03
100	6E2	22.10±0.04	57.39±0.57	4.68±0.02
101	6E3	23.26±0.09	36.68±0.28	5.64±0.01
102	6E4	12.07±0.04	46.11±0.46	5.55±0.02
103	6E5	19.20±0.08	43.90±0.63	3.78±0.02
104	6171	32.27±0.07	68.16±0.56	4.10±0.02
105	6172	17.28±0.11	35.35±0.67	4.99±0.01
106	6173	21.26±0.09	49.92±0.33	5.19±0.02
107	6174	24.11±0.10	56.18±0.38	5.03±0.02
108	6175	28.97±0.15	35.35±0.19	4.59±0.02

The lower water holding capacity indicates the lower viscosity of the fermented milk and the poorer taste and texture of the fermented milk. The water holding capacity of fermented milk in this experiment ranged from 9.58% to 35.52%, where the strain with the highest water holding capacity was 3E3 with 35.52% water holding capacity. It has been found that the production of extracellular polysaccharides can increase the water holding capacity of fermented milk (Bensmira et al. 2010) as extracellular polysaccharides have a high water binding capacity in some strains (Hess S J et al. 1997). In the later stages of fermentation, when the kefir grains are completely activated, the production of extracellular polysaccharides rises significantly along with the growth in microbial activity and population. As a result, the kefir will have a high water holding capacity.

The results of the study showed that microorganisms in kefir were able to hydrolyze proteins and peptides in milk. The highest protein hydrolysis was observed in strain 4M2 during the primary screening process with 71.92 ± 0.46 %. The hydrolyzed peptides are likely to be further hydrolyzed by extracellular kefir microbial proteases or absorbed by kefir microorganisms (Dallas et al. 2016).

Lactic acid bacteria and yeast re-screening and fermentation characteristics investigation

Alcohol production test

Take 0.1 mL of the diluted suspension and spread it on YPD medium or PDA medium, incubate at 28 °C for 48 h and then pour in TTC medium to cover the colony, continue to incubate, observe and record the color change of the medium at 1 h, 3 h, and 6 h, respectively. Kefir's distinctive taste is due in large part to the ethanol that yeast produces during the fermentation process. Therefore, the alcohol production ability of yeast was used as a screening index, as shown in Table 3, and the alcohol production performance of strains 2Y5, 3Y3, and 6Y6 was better.

Table 3. Results of ethanol production test by yeast

Time Strain	1h	3h	6h
1Y1	+	++	+++
2Y4	+	++	+++
2Y5	+	+++	+++++
3Y3	+	+++	+++++
5Y2	+	++	++++
6Y1	+	++	+++
6Y6	+	+++	+++++

Note: The + number represents the degree of color. The more + means the heavier the color.

Acid resistance

The yeast and lactic acid bacteria obtained from the primary sieve were inserted into the broth medium with adjusted pH and incubated at 28 °C and 37 °C for 4 h to determine the number of viable bacteria, and the results are shown in Table 4.

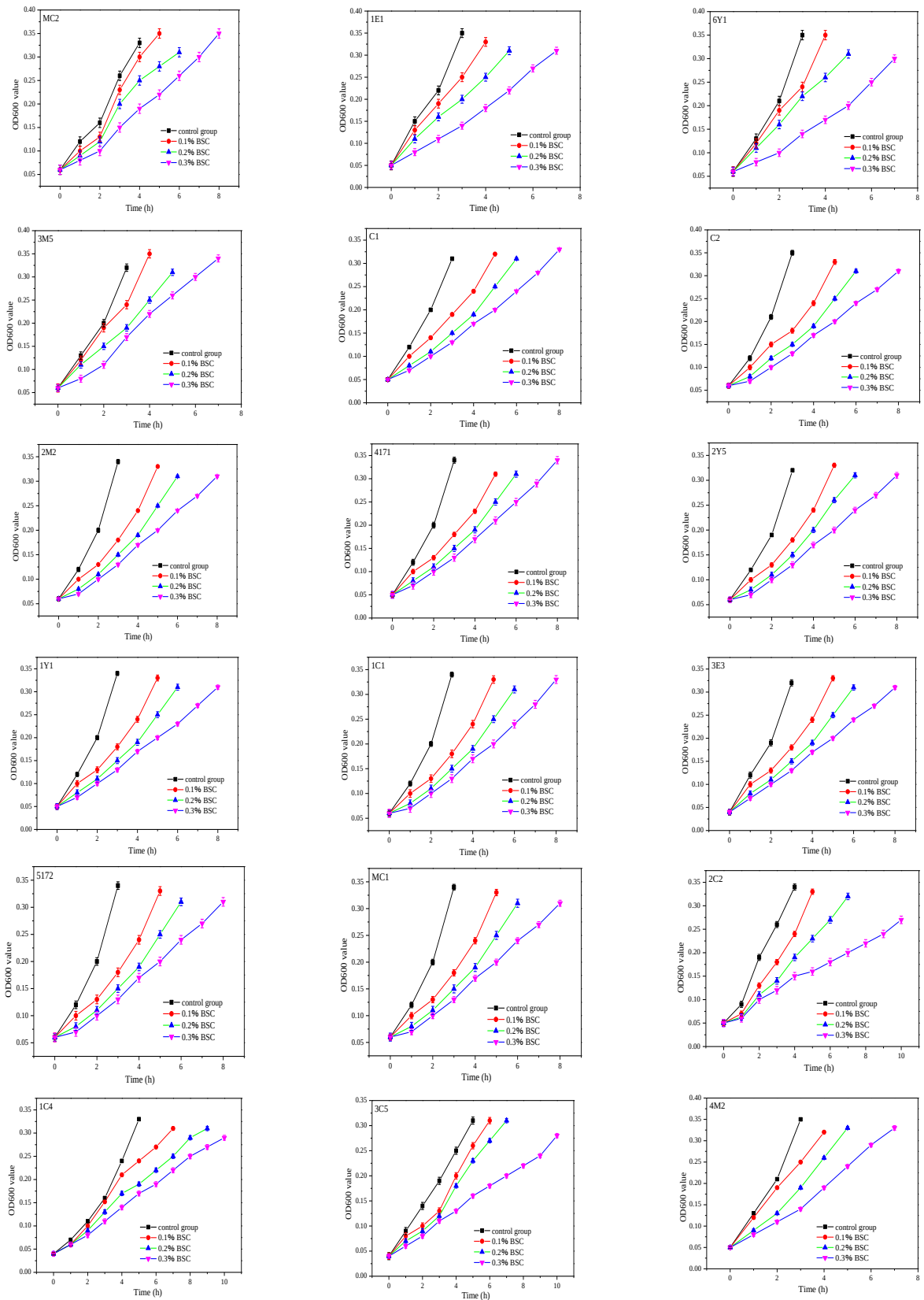
The survival of strains after 3 h of digestion in an environment with pH 3 was used as an indicator of acid tolerance of the strains. As shown in the above table, all strains have some tolerance to an acidic environment, and the acid tolerance of different strains has a significant difference ($p > 0.05$); among them, strains 2C6, 2Y5, 3E3, 4M2, 5E1, 6171, 6Y6 have relatively good tolerance to acid, and the number of viable bacteria can reach 10^8 orders of magnitude after 4 h digestion, strains 1Y1, 2Y4, 3C5, C2, 6Y1 had relatively poor tolerance to acid, and the viable bacteria count could only reach 10^5 orders of magnitude after 4 h digestion.

Bile salt tolerance

The growth rate of lactic acid bacteria and yeast is inhibited in the bile salt environment. The bile salt concentration inside the human small intestine is high, so bile salt-tolerant strains are screened for development of direct-vat-set subsequent preparation, and the results of bile salt tolerance of lactic acid bacteria and yeast are shown in Figure 3.

Table 4. Determination of viable bacteria number

Number	Strain number	Number of active bacteria (CFU/mL)	Number	Strain number	Number of active bacteria (CFU/mL)
1	1C1	$(8.73 \pm 0.21) \times 10^7$	14	C1	$(2.93 \pm 0.11) \times 10^6$
2	1C4	$(4.19 \pm 0.18) \times 10^6$	15	C2	$(1.57 \pm 0.32) \times 10^5$
3	2C6	$(3.59 \pm 0.12) \times 10^8$	16	MC1	$(3.33 \pm 0.23) \times 10^7$
4	1E1	$(8.61 \pm 0.22) \times 10^6$	17	MC2	$(3.41 \pm 0.19) \times 10^7$
5	1Y1	$(3.91 \pm 0.51) \times 10^5$	18	4M2	$(2.32 \pm 0.17) \times 10^8$
6	2M2	$(1.66 \pm 0.23) \times 10^7$	19	4171	$(2.08 \pm 0.27) \times 10^6$
7	2C2	$(2.93 \pm 0.14) \times 10^6$	20	5E1	$(1.21 \pm 0.31) \times 10^8$
8	2Y4	$(3.25 \pm 0.18) \times 10^5$	21	5172	$(4.93 \pm 0.37) \times 10^6$
9	2Y5	$(2.27 \pm 0.29) \times 10^8$	22	5Y2	$(5.25 \pm 0.44) \times 10^7$
10	3M5	$(2.62 \pm 0.23) \times 10^7$	23	6171	$(5.28 \pm 0.56) \times 10^8$
11	3C5	$(7.18 \pm 0.26) \times 10^5$	24	6175	$(3.91 \pm 0.43) \times 10^7$
12	3Y3	$(1.39 \pm 0.98) \times 10^7$	25	6Y1	$(7.70 \pm 0.42) \times 10^5$
13	3E3	$(2.82 \pm 0.15) \times 10^8$	26	6Y6	$(3.66 \pm 0.22) \times 10^8$



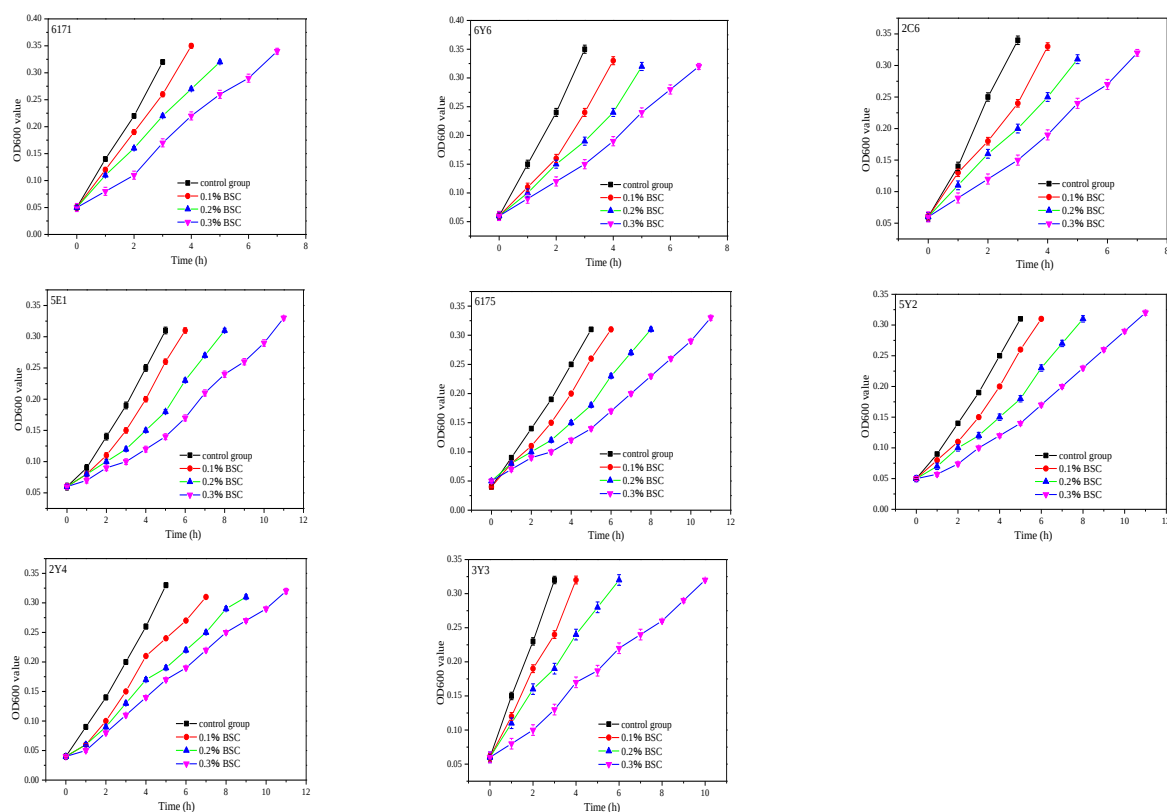


Figure 3. Bile salt tolerance of each strain (BSC: bile salt concentration)

As shown in Figure 3, strains 1E1, 2C6, 3M5, 4M2, 6171, 6Y1, 6Y6, and MC2 showed better bile salt tolerance, with growth lag times of 1 h, 2 h, and 4 h in environments with bile salt content of 0.1%, 0.2%, and 0.3%, strains 1Y1, 1C1, 3E3, C1, C2, MC1, 4171, and 5172 with 2 h, 3 h, and 5 h, strains 6175, 5E1 and 5Y2 had lag times of 1 h, 3 h and 6 h afterward, strain 2Y4 had lag times of 2 h, 4 h, and 7 h, strain 3Y3 had lag times of 1 h, 3 h, and 7 h, and strains 1C4, 2C2 and 3C5 had lag times of 2 h, 4 h; 1 h, 3 h; and 1 h, 3 h in bile salt concentrations of 0.1% and 0.2%, the OD values failed to reach 0.3 after 10 hours of growth in the environments of and respectively, showing that the bile salt

concentration of 0.3% had a strong inhibitory impact on these three strains. Gao et al. (2020) determined the acid tolerance and bile salt tolerance of strains isolated from traditional fermented milk in Xinjiang, and found that *Lactiplantibacillus plantarum* 4-1 had strong acid tolerance and *Lactiplantibacillus plantarum* 3-1 had strong bile salt tolerance.

Strain identification

The strains 6171, 4M2, 2C6, and 6Y6 were identified and phylogenetic trees were constructed as shown in Figures 4, 5, 6, and 7.

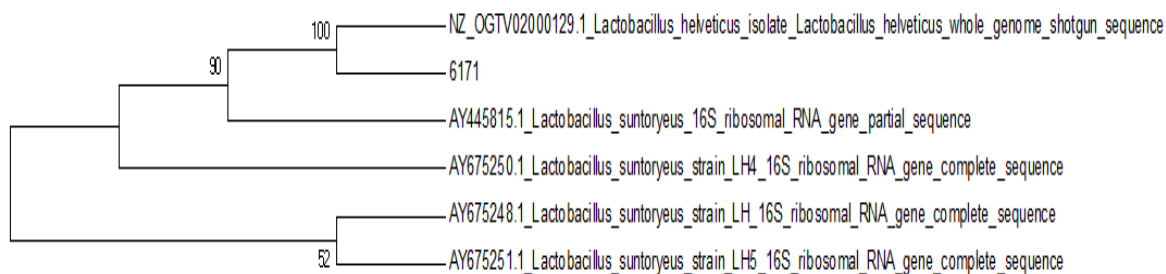


Figure 4. Phylogenetic tree of strain 6171

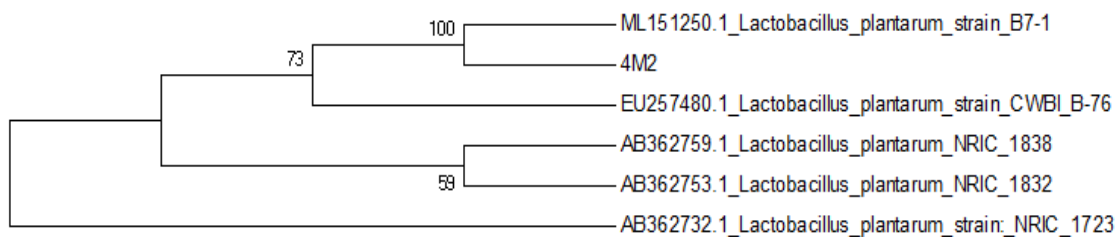


Figure 5. Phylogenetic tree of strain 4M2

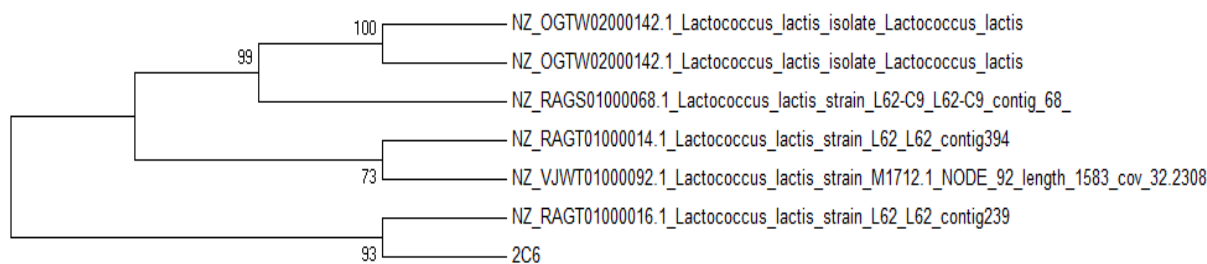


Figure 6. Phylogenetic tree of strain 2C6

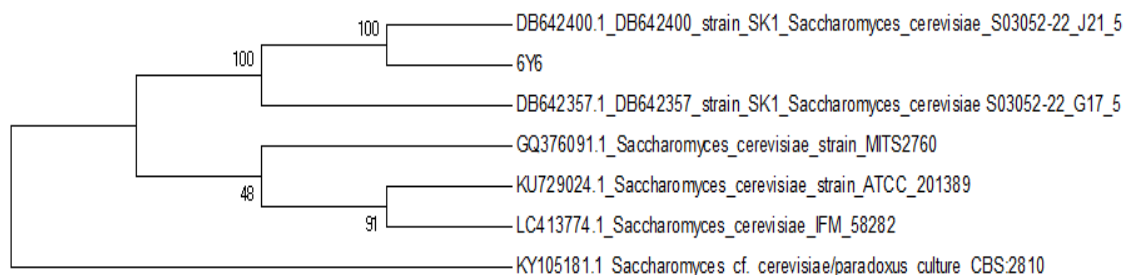


Figure7. Phylogenetic tree of strain 6Y6

Based on homology analysis, we obtained that 6171 is *Lactobacillus helveticus*, 4M2 is *Lactiplantibacillus plantarum*, 2C6 is *Lactococcus lactis* and 6Y6 is *Saccharomyces cerevisiae*. Gong (2018) isolated 22 yeast strains and 51 lactic acid bacteria from 10 kefir grains collected from

herders' families in Xinjiang, in which the yeasts were mainly *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*, and the lactic acid bacteria were mainly *Leuconostoc mesenteroides*, *Lactobacillus kefir* and *Lacticaseibacillus casei*.

DISCUSSIONS

Kefir not only has a unique flavor and aroma, but also has the function of improving immunity and regulating the intestinal tract, which is referred to as "the champagne of fermented dairy products". Kefir grains are a naturally occurring mixed bacterial fermentation system inhabited by a variety of microorganisms. According to reports, kefir grains contain 10^8 – 10^9 cfu/mL of lactic acid bacteria, 10^5 – 10^6 cfu/mL of yeast, and 10^5 – 10^6 cfu/mL of acetate. (Garrote et al. 2001). Lactose in milk is converted by lactic acid bacteria in kefir into lactic acid, which reduces the pH of the milk and preserves it. Other microorganisms participating in the process include *Acetobacter*, non-lactose

fermenting yeasts, and yeasts that produce ethanol and carbon dioxide (Magalhes et al. 2010). (Rattray et al. 2011).

Kefir grains have limited the large-scale and wide-scale production of kefir due to slow proliferation and unstable phase composition, among others. The symbiotic metabolic activity of numerous bacteria and yeasts results in the distinctive flavor and aroma of traditional kefir, and the use of fermenters containing lactic acid bacteria, acetic acid bacteria, and yeasts enables the production of a product with properties comparable to those of good traditional kefir (Assadi et al. 2000; Farnworth 2005). Therefore, a useful method to produce kefir for

business use is to use fermentor culture. The microorganisms in the kefir grains were isolated and purified, cultured individually, centrifuged to add a protective agent, freeze-dried to prepare the powder, and then compounded to prepare direct-vat-set (DVS) kefir starter for industrial production purposes.

Streptococcus thermophilus, *Lactobacillus bulgaricus*, *Lactobacillus swissensis*, *Lactococcus lactis*, and *Saccharomyces cerevisiae* were recombined in specific proportions to ferment cow's milk as a direct-vat-set (DVS) kefir starter after Beshkova et al. (2002) isolated and identified the strains in kefir grains. Gadaga et al. (2007) isolated four strains of lactic acid bacteria and nine strains of yeast from kefir grains and fermented buttermilk

using these bacteria in combination and found that the flavor and taste were better than naturally fermented milk. Zhong (2016) isolated and purified 10 strains of bacteria from natural kefir grain X, and used *Leuconostoc mesenteroides* xr-1, *L. kefir* xr-2, *L. kefir* xr-3 and *L. kefir* xr-4 in a 5:1:2:3 ratio to compound the fermented milk, and the results showed that the fermented milk made by the compounded fermenters had high nutritional value and similar flavor to kefir fermented milk fermented by kefir grains X. Shi (2019) isolated the dominant strain from natural kefir grains and compounded the direct-injection kefir fermenters by mixing test, and the kefir obtained after fermentation had less difference in taste from natural kefir.

CONCLUSION

A total of 64 yeast strains and 108 lactic acid bacteria strains were isolated from six natural kefir strains. A total of seven yeast strains were screened by the primary screening of lactic acid bacteria by the indexes of aroma production performance, pH, and proteolytic ability, and 19 bacteria strains were screened by the indexes of pH, proteolytic ability, and water holding capacity. The 19 strains of bacteria and seven strains of lactic acid bacteria were re-screened by acid tolerance, bile salt tolerance and alcohol production performance, and

finally, A total of three lactic acid bacteria and one yeast strain (*Lactococcus lactis* 2C6, *Lactobacillus helveticus* 6171, *Lactobacillus plantarum* 4M2 and *Saccharomyces cerevisiae* 6Y6) were screened and identified by 16S rDNA. 4M2 and 6Y6 showed good proteolytic ability, alcohol production and acid and bile salt tolerance. The four strains' freeze-dried powders had viable counts of 2.86×10^{11} cfu/g, 3.06×10^{11} cfu/g, 4.57×10^{11} cfu/g, and 9.84×10^{10} cfu/g, respectively, which will provide support for the subsequent development of kefir DVS starter.

ACKNOWLEDGMENTS

The work was partially supported by the Scientific Research Program Funded by Shaanxi Provincial Education Department (No.22JC022), science and technology project of Xi'an city (No.22NYF049), Technology Innovation Leading Program of Shaanxi [Program No.2023KXJ-122].

REFERENCES

1. Adler-Nissen, J. (1986). *Enzymic hydrolysis of food proteins*. Amsterdam: Elsevier applied science publishers.
2. Assadi, M. M. Pourahmad, R. & Moazami, N. (2000). Use of isolated kefir starter cultures in kefir production. *World J. Microb. Biot.* 16(6), 541-543. DOI: 10.1023/A:1008939132685.
3. Bensmira, M. Nsabimana, C. & Jiang, B. (2010). Effects of fermentation conditions and homogenization pressure on the rheological properties of kefir. *LWT-Food Sci. Technol.* 43(8), 1180-1184. DOI:10.1016/j.lwt.2010.04.005
4. Beshkova, D. M. Simova, E. D. & Simov, Z. I. (2002). Pure cultures for making kefir. *Food Microbiol.* 19(5), 537-544. DOI:10.1006/fmic.2002.0499.
5. Delgado, S. O'Sullivan, E. & Fitzgerald, G. (2007). Subtractive Screening for Probiotic Properties of *Lactobacillus* Species from the Human Gastrointestinal Tract in the Search for New Probiotics. *J. Food Sci.* 72(8), M310-315. DOI:10.1111/j.1750-3841.2007.00479.x.
6. Dallas, D. C. Citerne, F. & Tian, T. (2016). Peptidomic analysis reveals proteolytic activity of kefir microorganisms on bovine milk proteins. *Food Chem.* 197(APR.15PT.A), 273-284. DOI:10.1016/j.foodchem.2015.10.116.
7. Dong, X. Shu, G., & Kang, J. (2022) Microbial diversity of six commercially available kefir grains. *Acta Universitatis Cibiniensis. Series E: Food Technol.* 26(2), 287-302. DOI:10.2478/auaft-2022-0023.
8. Farnworth, E. R. (2005). kefir – a complex probiotic. *Food Sci. Technol. Bull.* 2(1), 1-17. DOI:10.1616/1476-2137.13938.

9. Gao, J. (2013). *Investigation on Microflora Composition and Antitumor Activity of Tibetan kefir grains*. Published doctoral dissertation, Zhejiang University, Hangzhou City, Zhejiang Province, China.
10. Gao, Y., Li, B. & Li, S. (2020). Screening and probiotic properties of extracellular polysaccharide-producing lactic acid bacteria from traditional dairy products in Xinjiang. *China Brew.* 39(05), 28-34. DOI:10.11882/j.issn.0254-5071.2020.05.006.
11. Gong, X. (2018). *Isolation and Identification of High Quality Lactic Acid Bacteria and Yeast in Kefir Grains and the Preparation of Kefir Compound Fermentation Starter*. Published master's dissertation, Jiangsu University, Zhenjiang City, Jiangsu Province, China.
12. Guo, Z. Wang, J. & Yan, L. (2009). In vitro comparison of probiotic properties of *Lactobacillus casei* Zhang, a potential new probiotic, with selected probiotic strains. *LWT-Food Sci. Technol.* 42(10), 1640-1646. DOI:10.1016/j.lwt.2009.05.025.
13. Guzel-Seydim, Z. Wyffels, J. T. & Seydim, A. C. (2010). Turkish kefir and kefir grains: microbial enumeration and electron microscobic observation. *Int. J. Dairy Technol.* 58(1), 25-29. DOI:10.1111/j.1471-0307.2005.00177.x.
14. Garrote, G. L. Abraham, A. G. & De Antoni, G. L. (2001). Chemical and microbiological characterisation of kefir grains. *J. Dairy Res.* 68(4), 639-652. DOI:10.1017/S0022029901005210.
15. Gadaga, T. H. Viljoen, B. C. & Narvhus, J. A. (2007). Volatile organic compounds in naturally fermented milk and milk fermented using yeasts, *lactic acid bacteria* and their combinations as starter cultures. *Food Technol. Biotechnol.* 45(2), 195-200. DOI:10.1177/1082013207078066.
16. Hess, S. J. Roberts, R. F. & Ziegler, G. R. (1997). Rheological Properties of Nonfat Yogurt Stabilized Using *Lactobacillus delbrueckii* ssp. *bulgaricus* Producing Exopolysaccharide or Using Commercial Stabilizer Systems. *J. Dairy Sci.* 80(2), 252-263. DOI:10.3168/jds.S0022-0302(97)75933-2.
17. Li, H. Y. Qiao, C. Y. & Liu, Z. M. (2016). Study on the fermentation characteristics of kefir fermenters. *China Brew.* 35(02), 24-28. DOI:10.11882/j.issn.0254-5071.2016.02.006.
18. Magalhes, K. T. de M. & Pereira, G. V. (2010). Microbial communities and chemical changes during fermentation of sugary Brazilian kefir. *World J. Microbiol. Biotechnol.* 26(7), 1241-1250. DOI:10.1007/s11274-009-0294-x.
19. Nielsen, B. Gürakan, G. C. & Ünlü, G. (2014). kefir: A Multifaceted Fermented Dairy Product. *Probiotics Antimicrob. Proteins.* 6(3-4), 123-135. DOI:10.1007/s12602-014-9168-0.
20. Rattray, F. P. O'Connell, M. J. (2011). Fermented Milks kefir. *Encyclopedia of Dairy Sciences*. 518-524. DOI:10.1016/B978-0-12-374407-4.00188-6.
21. Sabir, F. Beyatli, Y. & Cokmus, C. (2010). Assessment of Potential Probiotic Properties of *Lactobacillus* spp. *Lactococcus* spp. and *Pediococcus* spp. Strains Isolated from kefir. *J. Food Sci.* 75(9), M568-M573. DOI:10.1111/j.1750-3841.2010.01855.x.
22. Shi, X. (2019). *Preparation and Application of Functional Direct-Vat-Set (DVS) Kefir Starter Cultures for Goat Milk*. Published master's dissertation, Shaanxi University of Science & Technology, Xi'an City, Shaanxi Province, China.
23. Yao, S. Xie, S. & Jiang, L. (2017). Effect of dandelion extract, sucrose and starter culture on the viscosity, water-holding capacity and pH of plain yogurt. *Mljekarstvo*, 67(4), 305-311. DOI:10.15567/mljekarstvo.2017.0408.
24. Zhang, Z. Zhang, T. & Li, D. (2011). In vitro studies on the probiotic properties of two strains of *Streptococcus thermophilus*. *China Dairy Ind.* 39(7), 8-11. DOI:10.3969/j.issn.1001-2230.2011.07.002.
25. Zhang, H. (2016). *Isolation and Identification of Strains in Kefir grains and Research on Compound Fermented Milk of Excellent Strains*. Published master's dissertation, Jiangsu University, Zhenjiang City, Jiangsu Province, China.
26. Zhong, X. (2016). *Identification and Application of Yeasts Isolated from Koumiss and Yak Milk Dreg Collected in Western China*. Published master's dissertation, Northwest Sci-Tech University of Agriculture and Forestry, Yangling, Shaanxi Province, China.