Kefir as a Probiotic Dairy Beverage: Determination Lactic Acid Bacteria and Yeast

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***Abstract*—Kefir is a fermented milk beverage with a slightly acidic taste. In this study, lactic acid bacteria and yeast were isolated from consumed kefir samples. The isolates which had antibacterial activity were identified using biochemical tests, API CHL, and riboprinter system. Lactic acid bacteria isolates were identified as *Lactobacillus brevis, Lactobacillus plantarum, Lactobacillus paracasei spp paracasei, Lactococcus lactis spp lactis, Leuconostoc mesenteroides*. Yeast was isolated from the kefir yeast as *Kluyveromyces marxianus, Kluyveromyces wickerhamii, Saccharomyces cerevisiae, Pichia angusta, Pichia guilliermondii, Candida glaebosa.* Lactic acid bacteria were tested for antimicrobial activity against food-borne bacterial pathogens according to the agar spot test and well diffusion assay. Lactic acid, hydrogen peroxide and proteolytic activity of the lactic acid bacteria were all identified. The amount of lactic acid was range 1.12–8.68 mg/mL. *Lactobacillus paracasei spp paracasei* KM-5 produced maximum hydrogen peroxide (0.69µg/mL). *L. plantarum* KM4-mr1 produced the highest (0.59mg/mL) proteolytic activity.**

***Index Terms*—kefir, antimicrobial, hydrogen peroxide, lactic acid, proteolytic activity, yeast, biofilm**

I. Introduction

Kefir is a fermented milk beverage with a weakly acidic taste and slightly alcoholic which has always been traditionally consumed in the Turkey. Kefir is prepared by inoculating cows, sheep's or goat's milk with the Kefir grains [1]. The milk is incubated at the room temperature for a day or two. During this time, lactose is fermented. The resulting beverage is a sour, slightly alcoholic drink. The consistency is similar to thin yoghurt.

Kefir grains consist of lactic acid bacteria, acetic acid bacteria such as *Lactobacillus* species, *Lactobacillus acidophilus, Leuconostoc, Acetobacter* species and *Streptococcus* species, yeasts as *Saccharomyces* and *Torula* and other microorganisms [2]. These bacteria and yeasts are under the control of pathogenic bacteria. These microorganisms are agglutinated with a water-soluble polysaccharide (kefiran).

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There are many health benefits of kefir. Kefir contains minerals, essential amino acids and beneficial bacteria and yeast that are beneficial for the human health. Kefir also has plenty of minerals as calcium and magnesium. These mineral are needed for a healthy nervous system. Phosphorus is the second plenty mineral in kefir. Phosphorus helps utilize carbohydrates, fats, and proteins for cell growth, maintenance and energy. Kefir contains Vitamin B1, B12, and Vitamin K.

Kefir shows antimicrobial activity against the some bacteria and fungi. Cell-free extracts of kefir inhibit *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Clostridium tyributyricum* and *Listeria monocytogenes*. The antimicrobial activity of kefir occurs due to lactic acid, diacetyl, acetaldehyde, hydrogen peroxide, carbon dioxide, bacteriocins and/or bacteriocins-like substrate produced by LAB.

Strains belonging to the genera *Lactobacillus*, *Streptococcus*, *Bacteroides*, *Escherichia*, *Bifidobacterium* and *Clostridium* were colonized normal gastrointestinal tract. *Lactobacillus* probiotic strains were shown positive effects on the host health and to inhibitory activity against the pathogenic bacteria. These bacteria were resistant to acid and bile. LAB adhered to the intestinal epithelial cells Finlay and Falkow [3] Jacobsen *et al.* [4].

In this study, lactic acid bacteria and yeasts were isolated from consumed kefir and these isolates were identified. The antimicrobial activity of lactic acid bacteria was determined against the food borne pathogens. In addition, produced lactic acids, hydrogen peroxide, proteolytic activities were studied as well. And biofilm formation of the lactic acid bacteria was also evaluated.

II. Material and Methods

A. Bacterial Strains, Media and Cultivation Conditions

Test bacteria were obtained from the USDA Agriculture Research Service, IL. USA and The American Type Culture Collection and our laboratory culture. The strains cultivation media, and incubation temperatures used in this study are described in Table I. Cultures were maintained at –80℃ in 20% glycerol. Cultures were inoculated to broth medium at the 1% level

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and propagated at their corresponding incubation temperature as indicated in Table I.

TABLE I. TEST STRAINS USED FOR THIS WORK

Microorganisms		Optimum Growth Temperature
C. albicans	Anadolu University Faculty of Science	30 ℃
C. glabrata	Anadolu University Faculty of Science	30 ℃
B. cereus	NRRL B–3711	30 ℃
B. subtilis	NRLL B–744	30 ℃
E. coli	NRRL B–3704	37 ℃
E. feacalis	ATCC 29212	37 ℃
L. monocytogenes	ATCC–7644	30 ℃
L. monocytogenes 1	Gazi University Faculty of Science	30 ℃
L. monocytogenes 2	Gazi University Faculty of Science	30 ℃
P. aeroginosa	ATCC 27853	30 ℃
S. aureus	ATCC 6538	30 ℃
L. plantarum	Anadolu University Faculty of Science	30 ℃
L. buchneri	Anadolu University Faculty of Science	30 ℃
L. bulgaricus	Anadolu University Faculty of Science	30 ℃
S. lactis	Anadolu University Faculty of Science	30 ℃

NRRL: Northern Regional Research Laboratory of the USDA, Peoria, Illinois, USA. ATCC: American Type Culture Collection, USA.

B. Isolation of Lactic Acid Bacteria from Kefir

Ten different kefir samples were isolated from lactic acid bacteria and yeast. Lactic acid bacteria was isolated using on Lactobacillus Agar acc. to De Man, Rogosa and Sharpe (MRS) agar and M17 agar. The plates were incubated at 30 ℃ for 48 h in an atmosphere of 10% CO_2. Growing colonies were subcultured from MRS and M17 agar plates. All of the pure isolates were tested for Gram reaction, catalase and oxidase activity [5]. Gram-positive, oxidase and catalase negative isolates were stored. Carbohydrate fermentation test of the isolated LAB strains were analysed using an API 50CHL identification kit (Bio-Merieux, France). Growth at 3.9pH, ammonia production from arginine, CO_2 production from glucose, growth at different temperatures (4, 15 and 45 ℃), growth at different NaCl concentrations (6.5, 7, 10%) [6], [7].

Ribotyping was made with an Automatics RiboPrinter Microbial Characterization System (Qualicon Inc., Wilmington, DE) and the EcoRI DNA preparation kit as described in the manufacturer's operations and analytical guides. The ribotype profiles of the isolates were compared with the reference DuPont identification database DUP2003.

C. Isolation and Identification of Yeasts from Kefir

Yeasts were isolated on YM agar and malt extract agar [8]. Plates were incubated for 3-5 days at 28 ℃. Colonies that exhibited different morphology were inoculated on YM agar plates. Identification was carried out according to Kreger van Rij [8] and Barnett *et al.* [9]. The yeasts were identified on the fermentation tests using Biolog identification systems.

D. Detection of Antagonistic Activity

Antagonistic activity screening was examined by the agar spot test and well diffusion assay, as described by Schillinger and Lücke [6] and Harris *et al.* [10].

Agar spot test were performed by spotting 10µl of an 18-24h lactic acid bacterial culture onto the surface of a MRS agar plate. The plates incubated for 48 h at 30 ℃ in an atmosphere of 10% CO_2. These plates were then overlaid with 8ml of soft agar (0, 75% agar) seeded with 8µl of a test bacteria culture (approximately 10^7 stationary-phase cells). After 24h incubation at 35 ℃, the plates were examined for zones of inhibition in the test bacteria.

Agar well diffusion assay was described according to Kıvanc *et al.* [11]. LAB cultivated optimum conditions in MRS broth for 24h. LAB cultures centrifuged at 2500 $\times g$ for 5 min. Cell free supernatants obtained and Cell free supernatants were adjusted pH 5.5 $\pm$ 0.2 and then 0.2 µm filter-sterilized. Cell-free neutralized supernatants of LAB isolates were screened for antagonistic activity by the agar well test method.

E. pH and Lactic Acid Determination

The pH of the samples was measured by using pH meter (Corning pH/ion analyser 350). Acid production was determined in sterilized skim milk. Lactic acid bacteria was inoculated in the rate of 2ml/100ml. Measurements of titratable acidity, expressed as grams lactic acid/mL, were made at 42h [12]. These tests were performed three times.

F. Proteolytic Activity

Protease activities were assessed in sterilized skim milk (SSM; pH 6.50) (Oxoid) at 37 ℃ for lactobacilli and 30 ℃ for lactococci. LAB were grown in MRS broth, centrifuged at 4000 $\times g$ for 10min, washed twice with sterile distilled water, and re-suspended. The proteolytic activities of cultures were spectrophotometrically determined according to the Hull as modified by Citti *et al.* [13]. The results were calculated from a calibration curve obtained from dilution of tirosin in distilled water and were determined as µg/ml tirosin. Proteolytic activity was determined in triplicate.

G. Hydrogen Peroxide Detection

Hydrogen peroxide (H_2O_2) was determined spectrophotometrically by the method of Patrick and Wagner [14]. H_2O_2 was quantified by using a H_2O_2 standard curve, performed with concentrations, ranging from 1µg/ml to 10µg/ml.

H. In Vitro Biofilm Assay

Biofilm assay on microplates: Biofilm formation on polystyrene was quantitatively determined using the method developed by Zhang *et al.* [15].

Congo red agar methods: Slime production by the isolates was determined by Congo red agar (CRA) methods as described by Freeman *et al.* [16].

I. Resistance to Bile and pH 2

The tests were performed in microwell plates. A 200-µl volume each of MRS (pH 2) and MRS (0.3% oxgall), each inoculated with a LAB strains (10^6 cfu/ml), was tested. Growth rates were determined 24h of incubation at 37 ℃. Survival was tested at 37 ℃ after 4h and plating of 50µl onto MRS agar plates.

III. Result and Discussion

All yeast isolated from kefir were identified as *Kluyveromyces marxianus*, *Kluyveromyces wickerhamii*, *Saccharomyces cerevisiae*, *Pichia angusta*, *Pichia guilliermondii*, *Candida glaebosa*. *Kluyveromyces marxianus* was identified in the isolates from all the kefir samples, followed by *Kluyveromyces wickerhamii*. *Pichia angusta*, *Pichia guilliermondii*, *Candida glaebosa* were not described in previous reports [17], [18]. *K. marxianus var. lactis* isolated from kefir grains was distinguished by the feature of occurring with lactose - negative yeasts, which was also confirmed by our results [18]. *K. marxianus* var. *lactis* provided characteristic yeasty flavour and aroma of kefir [19].

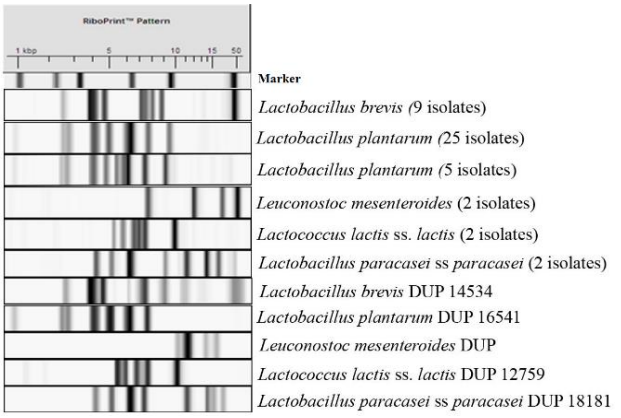

Figure 1. Riboprinter pattern of some isolates and some standard lactic acid bacteria

Lactic acid bacteria isolated from kefir were gram positive, catalase negative and non-motile. Lactic acid bacteria were identified according to the physiological and morphological tests used for identification. According to the carbohydrate fermentation reactions, physiological and morphological tests, and isolates are members of the species *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactococcus lactis*, *Lactococcus pentosus*, *Leuconostoc mesenteroides*. Similarities and differences were observed when these results were compared with those obtained by Santos *et al.* [20] using kefir grains. They reported *L. kefir* which was predominant, *L.brevis*, *L. delbruekii*, *L. acidophilus*, *L. kefiranofaciens*, *L. paracasei*, *Lactobacillu plantarum*. While our samples consumed kefir, researcher isolated them from kefir grain. Yüksekdağ *et al.* [21] *L. lactis*, *L. acidophilus*, *L. helveticus*, *L. bulgaricus*, *L. brevis*, *L. plantarum* and *L. casei* were isolated from the Turkish kefir samples. *L. brevis*, *L. plantarum*, *L. lactis* ss *lactis*, *Lactobacillus paracasei* ss *paracasei* were detected with RiboPrinter® system. As seen Fig. 1, most of the isolates belonged to member of *Lactobacillus plantarum* (K2-1, K2-3, K2-4, K2-11, K2-13, K2-14, K2-15, K2-16, K2-17, K2-18, K2-22, K2-23, K2-24, K2-5, K2-6, K2-7, K2-9, K2-8, K2-12, KM1-mr6, KM1-mr22, KM1-mr23, KM3-mr3, KM4-mr1, KM4-mr3). The smallest groups were *L. mesenteroides* (KM1-mr20, KM1-mr2), *Lactococcus lactis* spp *lactis* (KM1-m3, KM2-m7), *Lactobacillus paracasei* spp *paracasei* (K2-2, K2-10) represented with two members (Fig. 1).

TABLE II. Antagonistic Activity of Neutralizedsupernatant against Tested Bacteria by the Well Diffusion Test (mm)

Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14
L. plantarum														
K2-1, K2-3, K2-4, K2-11, K2-13, K2-14, K2-15, K2-16, K2-17, K2-18, K2-22, K2-23, K2-24	-	-	-	-	+	-	+	-	-	-	+	+	-	-
K2-5, K2-6	-	-	-	+	+	+	+	-	-	-	+	+	-	-
K2-7, K2-9	-	-	-	-	+	+	+	-	-	-	+	+	-	-
K2-8	-	-	-	-	+	-	+	-	-	-	+	+	-	-
K2-12	-	-	-	+	+	-	+	-	-	-	+	+	-	-
KM1-mr6	-	+	-	-	-	-	-	-	-	+	-	-	-	-
KM1-mr22	-	+	-	-	-	-	-	-	-	-	-	-	-	-
KM1-mr23	+	+	-	-	-	+	+	+	+	-	-	+	-	
KM3-mr3	+	+	-	-	-	+	+	-	-	-	-	-	-	-
KM4-mr1	+	-	-	-	-	-	-	-	+	+	-	-	+	-
KM4-mr3	-	+	+	-	+	-	+	+	-	+	+	+	+	+
L. paracasei sp. paracasei														
K2-2	-	-	-	-	+	-	+	-	-	-	+	+	-	-
K2-10	-	-	-	-	+	+	+	-	-	-	+	+	-	-
KM-5	-	-	-	-	+	+	+	-	-	-	+	+	-	-
L. brevis														
K2-19, K2-20, K2-21	-	-	-	-	+	-	+	-	-	-	+	+	-	+
KM1-m3	+	-	-	-	-	-	+	-	+	+	-	-	-	-
KM1-m4	+	-	-	-	-	-	-	+	-	-	-	-	-	-
KM2-m6	-	-	-	-	-	-	-	-	+	+	-	+	-	-
KM2-m8	-	-	-	-	-	-	-	+	+	+	-	-	-	-
KM1-mr1	-	-	-	-	-	-	-	-	-	+	-	-	-	-
MK-1	+	-	-	-	-	-	+	+	+	+	-	-	-	-
L. lactis spp. lactis														
KM1-m3	+	-	-	-	-	-	+	-	+	+	-	-	-	+
KM2-m7	+	-	+	-	-	-	+	+	-	+	-	+	+	-
L.pentosus														
KM3-m14	-	+	-	-	-	+	-	-	-	-	-	+	+	+
Leu. Mesenteriodes														
KM1-mr20	-	-	-	-	+	-	-	-	-	-	-	-	-	-
KM1-mr2	-	-	-	-	-	-	-	-	+	-	-	-	-	+

1; *B. cereus*,2; *B. subtilis*, 3; *E.coli*, 4; *C. albicans*, 5; *E. faecalis*, 6; *S. aureus*, 7; *L. monocytogenes* ATTC, 8; *L. monocytogenes1*, 9; *L. monocytogenes* 2, 10; *P. aeruginosa*, 11; *L. plantarum* , 12; *S. lactis*, 13; *L. bulgaricus*, 14; *L. buchneri*

In our study, *L. plantarum* was found to be the dominant species. Some species *L. kefir*, *L. kefiranofaciens*, *L. kefirgranum* and *L parakefir* [20]-[22] were not found in our study. These species may not consume kefir or their number might be very low in the

consumed kefir. These differences can be explained by the different geographical origins of the kefir grains [23]. In this study, different kefir samples showed similar lactobacilli composition. Recent studies have explained the role of lactobacilli in the prevention and treatment of gastrointestinal disorders [24], [25]. The inhibitor activity against microorganisms was variable. The most of lactic acid bacteria isolates (87%) showed antimicrobial activity against one or more test bacteria under the agar spot test (data not shown table). As can be seen in Table II under the well diffusion test, L. *plantarum* KM4-mr3 showed antimicrobial activity against *Escherichia coli*. The other strains did not inhibit the growth of *E. coli. Enterococcus feacalis* was inhibited by 80% of the *L. plantarum* isolates. *Stapylococcus aureus and Pseudomonas aeruginosa were* inhibited 24% and 12% of the lactic acid bacteria isolates, respectively. *Listeria monocytogenes* ATCC 7644, *L. monocytogenes* 1 and *L. monocytogenes* 2 were inhibited 88%, 8% and 8% of the lactic acid bacteria isolates, respectively. According to our results, it appears that the antibacterial activity of LAB observed can be attributed to secreted antimicrobial substances. The most of the strains (90%) showed no inhibition against *Candida albicans*. LAB strains did not inhibit the growth of *C. glabrata* (not shown in Table I).

Selected of 23 strains were tested for their lactic acid, hydrogen peroxide and proteolitic activity. All tested isolates produced acid products with pH between 3 ±0.5 and 5.6±0.2. The acid produced by lactic acid bacteria in skim milk is shown in Table II. Lactic acid is used food, pharmaceutical and manufacturing industries [26]. *L. plantarum* KM2-m3 strain produced the highest lactic acid amount (8, 68mg/ml). *L. plantarum* KM4-mr1 produced the lowest (2, 15mg/ml) lactic acid amount. Raukas and Katzekidov [27] reported that the maximum lactic acid concentration (46g/l) in *L. lactis* was obtained in fed batch culture. Yüksekdağ *et al.* [21] reported that the lactic acid concentration produced in *Lactobacillus* strains ranged between 1.7-11.4mg/mL.

Hydrogen peroxide production may beneficial for food preservation. Hydrogen peroxide inhibited pathogenic or spoilage bacteria [28]. *L. lactis spp. lactis* KM2-m7 strain produced the highest amount (0, 22 µg/ml) and *L. plantarum* KM1-mr6, KM1-mr9, KM3-m13 and KM2-m3 strains produced the lowest (0, 01µg/ml) amount of hydrogen peroxide (Table II). Yüksekdağ *et al.* [21] identified the amount of hydrogen peroxide generated by lactic acid bacteria to be in the range of 0.04-0.19 µg/mL. *L. plantarum* KM4-mr1 produced the highest proteolytic activity (0,59mg/ml) (Table III). Aroutcheva *et al.* [29] reported that there was no correlation between lactic acid, hydrogen peroxide production and bacteriocin activity. They were detected *Lactobacillus* strains produced hydrogen peroxide but did not show any inhibitory effect. Similar results were obtained in this study, *L. plantarum* KM2-m3 strain produced maximum lactic acid but did not produce hydrogen peroxide.

Biofilm formation of the strains isolated from kefir was studied using a microtitre plate assay and CRA. The results are shown in Table IV. The most of lactic acid bacteria isolates were the best biofilm producers in media with lactose as the carbon source. The adhesion of probiotic bacteria to gastrointestinal system was the primary and most significant step of colonisation. The production of exopolysaccharides was a key factor in the adherence of biofilm. Biofilm formation of the strains isolated from kefir was studied using a microtitre plate assay and Congo red agar. The results are not shown. The isolates showed greater biofilm formation with lactose. By CRA method, all isolates were slime producers developing almost black or very black colonies on CRA plate. Exopolysaccharides have been recognized to have an antitumor activity [30], [31]. Extracellular polysaccharides of LAB have been shown antitumor action.

TABLE III. AMOUNT OF LACTIC ACID, PROTEOLYTIC ACTIVITY AND HYDROGEN PEROXIDE PRODUCED BY LACTIC ACID BACTERIA

Strains	Lactic Acid (mg/ml)	Proteolytic Activity (Tirosin mg/ml)	H_2O_2 (mg/ml)
L. brevis			
KM1-m3	5,30 ±0,02	0,30 ±0,12	0,10 ±0,01
KM1-m4	6,00 ±0,02	0,38 ±0,02	0,18 ±0,02
KM2-m6	6,23 ±0,01	0,21 ±0,01	0,10 ±0,02
KM2-m8	4,32 ±0,01	0,30 ±0,01	0,15 ±0,01
KM1-mr1	6,32 ±0,01	0,16 ±0,00	0,01 ±0,01
KM1-mr 3	7,25 ±0,02	0,31 ±0,01	0,06 ±0,01
MK-1	1,14±0,02	0,01±0,00	0,24±0,03
L. plantarum			
KM1-mr 6	2,45 ±0,01	0,06 ±0,01	0,01 ±0,01
KM1-mr22	4,19 ±0,01	0,54 ±0,01	0,14 ±0,01
KM1-mr23	5,81 ±0,01	0,49 ±0,01	0,17 ±0,01
KM4-mr1	2,15 ±0,01	0,59 ±0,01	0,14 ±0,01
KM4-mr 3	2,64 ±0,02	0,50 ±0,01	0,21 ±0,01
KM1-mr 9	6,41 ±0,01	0,37 ±0,01	0,01 ±0,00
KM1-mr10	3,19 ±0,01	0,09 ±0,01	0,16 ±0,01
KM1-mr17	4,12 ±0,01	0,10 ±0,02	0,13 ±0,01
KM3-m13	5,88 ±0,04	0,25 ±0,02	0,01 ±0,01
KM2-m3	8,68 ±0,02	0,38 ±0,01	0,01 ±0,01
L. lactis spp. lactis			
KM2-m7	5,35 ±0,04	0,49 ±0,01	0,22 ±0,04
L. pentosus			
KM3-m14	6,83 ±0,09	0,47 ±0,00	0,17 ±0,03
Leu. mesenteriodes			
KM1-mr 2	7,06 ±0,01	0,15 ±0,01	0,05 ±0,01
KM1-mr20	5,43 ±0,04	0,34 ±0,01	0,08 ±0,01
L. paracasei sp. paracasei			
KM-5	1,12±0,09	0,10±0,01	0,69±0,09

Among the tested, LAB were observed to have variable tolerances to 0.3% oxgall. The most of LAB showed resistance to bile. But, the growth was delayed in all the tested strains. All of the tested LAB were survival following 4th of incubation at pH 2, although none grew at pH 2. The results are not shown. Hudault *et al.* [24] explained the role of lactobacilli in the prevention and treatment of gastrointestinal disorders. These findings can

explain the production of antimicrobial substances. Natural probiotics to produce immunomodulatory molecules may help to further increase the benefit to the host.

TABLE IV. BIOFILM FORMATION OF LAB IN THE MICROTITER-PLATE TEST AND CRA METHOD

Species	No of strains tested	Methods	Number of strains showing No adherent (0)	Weak Adherent (+)	Moderate Adherent (++)	Strong Adherent (+++)
L. plantarum		MTP	4	5	10	6
	5	CRA	2	4	12	7
L. paracasei	2	MTP	1	1	0	0
sp. paracasei		CRA	0	2	0	0
L.brevis	9	MTP	0	0	5	4
		CRA	0	0	5	4
L. lactis spp. lactis	2	MTP	0	0	2	0
		CRA	0	0	2	0
L. pentosus	1	MTP	0	1	0	0
		CRA	0	0	1	0
Leu. mesenteriodes	2	MTP	0	0	0	2
		CRA	0	0	0	2

MTP; Microtiter plate CRA; Congo red agar

In conclusion, the results show that the *Lactobacillus* strains isolated from kefir have good probiotic features. It is essential that the probiotic candidates show antimicrobial activity to pathogen and capacity of adhesion. Also, these bacteria are used in the industry of fermented dairy products. In addition, kefir consumption as a probiotic product is of great importance in terms of health.

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