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ORIGINAL ARTICLE

Molecular Identification of Lactic Acid Bacteria with Probiotic Potential from Local Curd

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KEYWORDS

Lactic acid bacteria;
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ABSTRACT: Researchers believe that lactic acid bacteria isolated from native sources that have probiotic potential can be beneficial for improving health. The aim of this study was molecular identification of the best lactic acid with probiotic potential from curd (Kashk). 10 samples of traditional Kashk were gathered and isolation of lactic acid bacteria was done. Identification of lactic acid bacteria using common biochemical tests was performed. The antagonistic activity of isolated bacteria was investigated by the modified double-layer method. The anti-adhesion effect of isolated lactic acid bacteria was evaluated by the 96-well microtiter plate method. Molecular identification of the best probiotic lactic acid bacterium was done by ribotyping method and the phylogeny tree was drawn. From Kashk samples, 9 lactic acid bacteria were isolated. 7 isolates were able to grow at all pH levels tested, while isolates K4 and K8 did not grow at pH equivalent to gastric acid. In terms of bile salt tolerance, all isolates showed the ability to tolerate bile salts, and isolates K2, K3, and K7 had the highest tolerance. The results of the double-layer and anti-attachment method showed that isolate K2 was the most effective against the tested pathogens *S. aureus, E. faecalis, E. coli, and P. aeruginosa*. According to the phylogeny tree, the most effective probiotic lactic acid bacterium (K2) was known as *Lactobacillus plantarum*. In this research, it was found that *Lactobacillus plantarum* with probiotic potential can have antagonistic and anti-attachment effects against pathogenic bacteria and is effective for the treatment and prevention of bacterial diseases and ameliorate human health.

INTRODUCTION

Curd (Kashk) is a dairy product made by coagulating milk with the help of microbial flora such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. These bacteria convert lactose, the primary sugar in milk, into lactic acid, which causes the milk proteins to coagulate and form curd. The microbial flora in curd not only gives it its characteristic tangy flavor but also provides several health benefits. They aid in digestion, boost the immune system, and improve gut health by maintaining a healthy balance of bacteria in the gut [1].

Lactic acid bacteria (LAB) are a group of gram-positive, non-spore-forming, and catalase-negative bacteria that produce lactic acid as an end product of carbohydrate fermentation. These bacteria are widely distributed in nature and are found in various habitats such as plants, animals, and fermented foods. LAB is used extensively in the food industry for the production of fermented foods such as yogurt, cheese, curd, sourdough bread, and pickles [2]. LAB produces metabolites such as acetic acid, ethanol, and carbon dioxide, depending on the type of carbohydrate and the environmental conditions [3].

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One of the most important characteristics of LAB is their tolerance to low pH and high salt concentrations. This allows them to survive and grow in acidic and salty environments, such as fermented foods and the human gastrointestinal tract. These bacteria are also able to tolerate bile salts and other antimicrobial compounds, which makes them suitable for use as probiotics [4]. *L. plantarum* has been shown to have probiotic properties, promoting gut health and potentially aiding in the prevention of certain diseases. Studies have also suggested that *L. plantarum* may have anti-inflammatory and antioxidant effects. Overall, this bacterium is a versatile and beneficial bacterium with numerous potential applications in the food and health industries [5].

Probiotics are live microorganisms that, when consumed in adequate amounts, confer health benefits to the host. They have been shown to have a wide range of health benefits, including improving digestive health, boosting the immune system, and reducing the risk of certain diseases [6]. Probiotics can help to restore the balance of bacteria in the gut, which can be disrupted by factors such as antibiotics, stress, and poor diet. Probiotics can also help to improve the absorption of nutrients from food, which can be particularly beneficial for people with malabsorption disorders [7]. In addition to their effects on digestive health, they have been shown to have immune-boosting properties. Probiotics can help to stimulate the production of antibodies and other immune cells, which can help to protect against infections and other diseases. Probiotics have also been shown to have anti-inflammatory effects, which can reduce the risk of chronic diseases such as heart disease, diabetes, and cancer [8]. Probiotic *lactobacilli* can control the colonization of pathogenic agents, which use different mechanisms to inhibit pathogenic agents, including direct competition for binding sites in epithelial cells and competition with pathogenic bacteria for substances. *Lactobacillus* species, having the property of cell aggregation (co-aggregation), aggregate with pathogenic microbes and by exerting their anti-adhesion effects, prevent infectious bacteria from reaching and binding to the target cell in the host, and ultimately prevent the onset of disease and its spread [9, 10]. The aim of this study was the molecular identification of lactic acid bacteria isolated from traditional Kashk with probiotic potential.

MATERIALS AND METHODS

Sampling and culture conditions

10 samples of kashk were gathered from some traditional dairy shops in the northeast of Tehran and under standard protocol (cool box -4°C) transported to the laboratory. To enrich the lactic acid bacteria, 2 grams of powdered curd samples were dissolved in 10 ml of 2% sodium citrate solution and then dissolved in 100 ml of MRS broth medium supplemented with nystatin (0.1g) and incubated for 48 hours at 37°C under anaerobic condition. This process was repeated once. All culture mediums used in this research were prepared by Merck, Germany

Isolation of lactic acid bacteria from Kashk

To isolate lactic acid bacteria, serial dilution method was done in MRS broth. First serial delusion (10^{-1} to 10^{-10}) of each curd sample was prepared. Empty plate was poured with one milliliter of each dilution, mixed with MRS agar and incubated at 37 °C for 48 hours under anaerobic condition. To purified lactic acid bacteria, single colonies that had different color, shape and consistency during the isolation stage were selected and strict method was done. This process was repeated twice. Isolated bacteria were stored in Glycerol stock at -20°C [11].

Biochemical tests to identify lactic acid bacteria

Initial identification of lactic acid bacteria was bone by common biochemical tests such as gram staining, catalase (using 3% H2O2) and Oxidase (using oxidase disc) tests, motility in Sulfide Indole Motility *medium* (SIM), fermentation of carbohydrates (Glucose, lactose, galactose, dextrin, arabinose, terehalose, sucrose, maltose, sorbitol, mannitol, and xylose) in phenol red broth, pH tolerance (2.5 and 4.5) and growth in 15°C and 45°C in MRS broth under anaerobic condition [12].

Probiotic potential of lactic acid bacteria

To investigate the probiotic potential of lactic acid bacteria isolated from Kashk some probiotic standard tests were performed which as described below:

Resistance to bile salts

First, lactic acid isolates were cultured for 24 hours in MRS broth medium in an anaerobic condition at 37°C. For each lactic isolate, one tube containing 9 ml of MRS broth medium containing 0.3% bile salts and one tube containing 9 ml of MRS broth culture medium without bile salts were prepared as a control. One milliliter of culture medium containing each isolate was added to them and every one hour for 8 hours, their optical absorption at 600 nm was read by a spectrophotometer and a curve was drawn [13].

L-arginine amino acid hydrolysis

100 µl of fresh culture of each isolate was inoculated in MRS broth medium containing 0.3% L-arginine and incubated for 24 hours at 37°C in an anaerobic condition. Then 100 µl of this culture medium was added to filter paper containing Nessler's reagent and its color change was examined. *S. aureus* bacteria (ATCC25923) was also used as a positive control [14].

Hemolytic activity

Isolated lactic acid bacteria were cultured on blood agar and incubated for 48 hours at 37°C and in an anaerobic condition. The plates were examined for the presence or absence of hemolysis around the bacterial colonies. *S. aureus* (ATCC 25923) was used as a positive control [15].

Antagonistic activity

About 20 µl of each isolate was inoculated in the center of the plate containing MRS culture medium and allowed to absorb the moisture of the environment completely. The plates were incubated at 37°C for 48 hours under anaerobic conditions. The Moller Hinton agar culture (MHA) medium was poured as the second layer on the medium containing each isolate and McFarland standard suspension of pathogenic bacteria *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* (prepared from Islamic Azad University, Damghan branch) were cultured on the second medium and the plates were incubated for 24 hours at 37°C. The culture medium without pathogenic bacteria was considered as a control. The diameter of the halo of non-growth of pathogenic bacteria in the presence of each lactic isolate was measured using a ruler and reported in millimeters [16].

Anti-attachment activity

Anti-attachment effect of isolated lactic acid bacteria was done by 96-well microtiter plate method. First supernatant of lactic acid bacteria was prepared by centrifuge of overnight culture of each isolate at10000 rpm and 4°C. Then 80 µl of mentioned pathogenic bacterial suspension and 80 µl of supernatant of each isolate were added to wells. In control wells, only pathogenic bacteria and MHB culture medium were poured, and the microtiter plate was incubated at 37°C for two nights. The contents of the wells were removed and washed with phosphate buffer (2 times). Fixing the sample in the wells was done with 96% ethanol for 10 minutes. Then, the wells were stained with 2% crystal violet for 5 minutes, and washed with city water and dried in air. 100 µl of 33% acetic acid was added to the wells and the optical absorbance of the microtiter plate was measured at 490 nm and the anti-attachment effect was calculated from the following formula [17].

$$\text{Anti} - \text{attachment} = [\text{OD (control well)} - \text{OD (test well)} / \text{OD (Control well)}] \times 100$$

Molecular identification

DNA Extraction and PCR

DNA extraction of the best lactic acid bacterium with the most probiotic effect was done using the DNA extraction kit (Zhinogene). The extracted DNA quality was assessed using agarose gel electrophoresis. 4 µl of the extracted DNA was mixed with 1 microliter of loading dye and loaded into the wells and for 40 minutes at 95 volts was connected to an electrical source. Gel was stained with ethidium bromide, the band quality was observed using a transilluminator device (Biorad, UAS). For PCR method the 16S rRNA gene primer (F-5′AGAGGTTCCTGAGCTCAG3′, R-5′ ACAGCTTCCTTGTTACGATT3′) sequence was designed using the oligo7 software and blast in the NCBI

website. Taq DNA Polymerase Master Mix kit (Amplicon) was used for PCR reaction. The temperature program of PCR reaction was included the stages of initial denaturation (3 minutes at 95°C, and 30 cycles), secondary denaturation (60 second at 95°C), annealing (30 second at 52°C), extension 30 second at 72°C) and final extension (3 minutes at 72°C). To identify of the PCR band, 1µl of PCR product was mixed with 3 µl loading dye and electrophorese was done on 2% agarose gel. To observe the band, an electric source of 95 volts was used for 45 minutes. Molecular weight marker of 10 bp was used.

Phylogeny tree

The PCR product was sent to Pishgam Company for sequencing. The sequence determined on the NCBI website was compared with the sequences recorded in the gene bank and the closest bacteria to the desired strain were determined based on the similarity of the 16s rRNA sequence. These sequences with a high similarity percentage were placed into MEGA4 software and the Neighbor Joining method was used to draw the phylogenetic tree.

Statistical analysis

The experiments were done independently and with three repetitions and the data were calculated as mean ± standard deviation (SD). Data analysis was done using one-way analysis of variance (ANOVA) and SPSS version 20.0 statistical software. $p \leq 0.05$ was used to determine the significance of differences. The graphs were drawn with Excel software.

RESULTS

Biochemical characteristics of isolated lactic acid bacteria

14 bacteria were isolated from the 10 Kashk samples collected, and 5 samples were excluded from the study due to contamination and catalase positivity. The 9 isolated bacteria were coded by K letter (K1-K9). All isolates were catalase and oxidase negative. K1 and K5 were gram positive cocci but others were bacilli form. Morphological observation showed that the colonies of all isolates were white, except for isolates K6 and K9, which cream in color with a more prominent appearance (Figure 1). All isolates were non-motile except K5, which was motile.

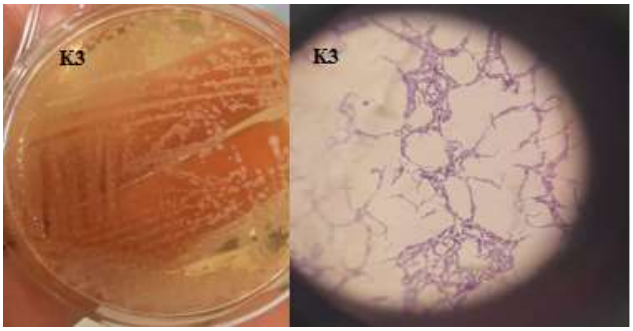

Figure 1. The colony (left) and gram staining (right) K3.

The growth results at 15 and 45°C showed that the isolated lactic acid bacteria were different in terms of growth temperature and only K2, K3 and K7 isolates were able to grow at both 15 and 45°C. Based on the results obtained from the investigation of growth in three pH of 2.5, 4.5 and 6.5, 7 isolates had the ability to grow in all three pH, and the microbial population after their dilution was about 10^6 CFU ml^{-1}, but the K4 and K8 did not have the ability to grow at pH equivalent to gastric acid and were excluded from further experiments. Carbohydrate fermentation test showed that K7 and K9 had the ability to ferment all tested sugars, but K3 was able to ferment all sugars except xylose. K4 had a very weak fermentation power and only fermented glucose, lactose and sucrose. In Table 1 Growth temperature, pH tolerance and Carbohydrate fermentation of isolated lactic acid bacteria was shown.

Table 1. Growth temperature, pH tolerance and carbohydrate fermentation.

Isolates / Tests	K1	K2	K3	K4	K5	K6	K7	K8	K9
Growth at 15°C	-	+	+	+	-	-	+	+	-
Growth at 45°C	+	+	+	-	+	+	+	-	+
pH 2.5	+	+	+	-	+	+	+	-	+
pH 4.5	+	+	+	-	+	+	+	-	+
pH 6.5	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	-	+	+	+
Galactose	+	+	+	-	+	+	+	-	+
Dextrin	+	+	+	-	-	-	+	+	+
Arabinose	-	+	+	-	-	-	+	-	+
Trehalose	+	+	+	-	+	+	+	-	+
Maltose	-	+	+	-	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	-	+	+	+	-	+
Mannitol	+	+	+	-	+	+	+	+	+
Xylose	+	+	-	-	+	-	+	-	+

Probiotic potential

Bile salt resistant

Based on the results, isolates K2, K3 and K7 had the highest ability to tolerate bile salts, respectively. It is worth mentioning that all 7 isolates were able to tolerate bile salts. Statistically, a significant difference of 0.05 was observed between the isolates and the control, (Figure 2).

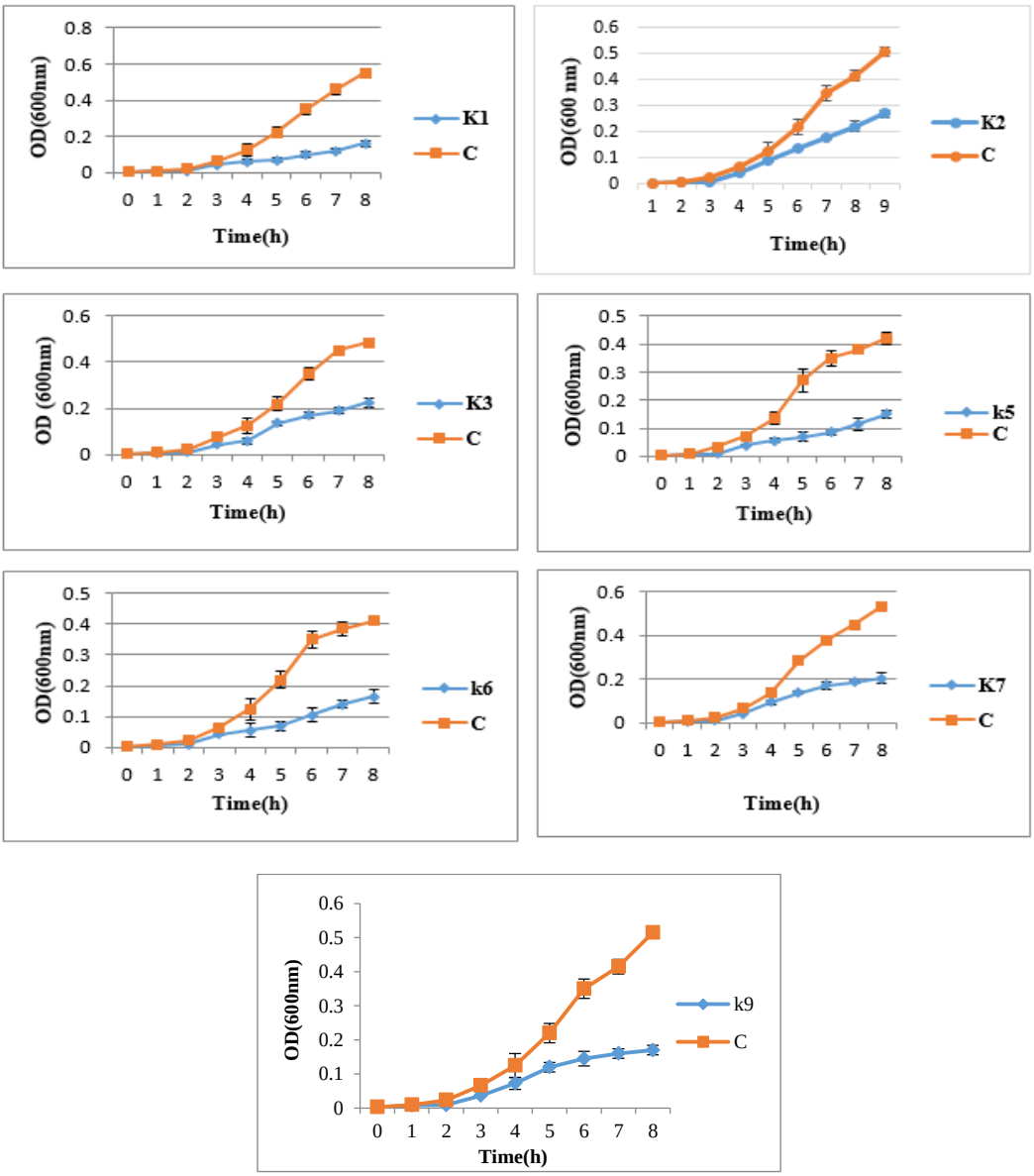

Figure 2. The effect of bile salts on the growth of k1, K2, K3, K5, K6, K7 and K9

Hemolytic activity and hydrolysis of L- arginine

The results showed that none of the isolates had the ability to lyse red blood cells, which is one of the important characteristics of probiotics.

Based on the obtained results, Only K1 had the ability to hydrolyze this L- arginine, but other isolates were not able to hydrolyze this amino acid. Figure 3 shows the results of L-arginine hydrolysis of K1 and K2 in comparison with the positive control of *Staphylococcus aureus*.

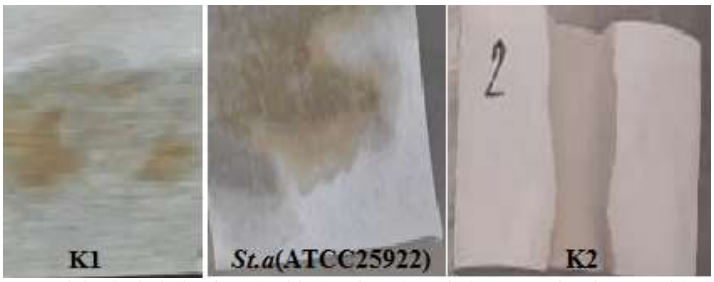

Figure 3. Arginine hydrolysis of K1 (positive) and K2 (negative) compared to the control (Positive).

Antagonistic activity

The highest antagonistic effect against all four pathogens was related to k2 with the average diameter of the non-growth halo of *Staphylococcus aureus* (32 mm), *Enterococcus faecalis* (26 mm), *Escherichia coli* (26 mm) and *Pseudomonas aeruginosa* (24 mm) and the lowest effect was related to the isolate was K5. Most isolates had the highest and lowest inhibitory effect against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, Figure 4.

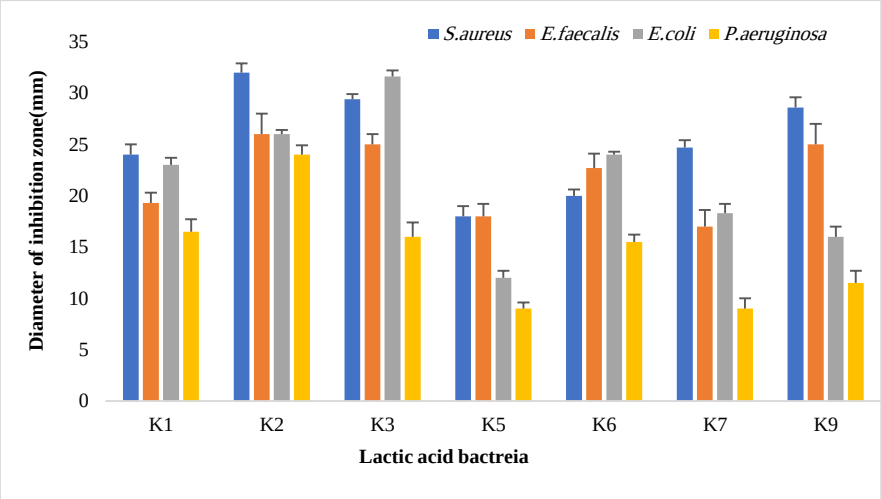

Figure 4. Antagonistic effect of isolated lactic acid against pathogens.

Anti-attachment activity

The results of anti-attachment effect of isolated lactic acid bacteria showed that the highest average anti-adhesion effect was obtained by K2 on *Staphylococcus aureus* (61%), *Enterococcus faecalis* (55%), *Escherichia coli* (58%) and *Pseudomonas aeruginosa* (47%), Figure5.

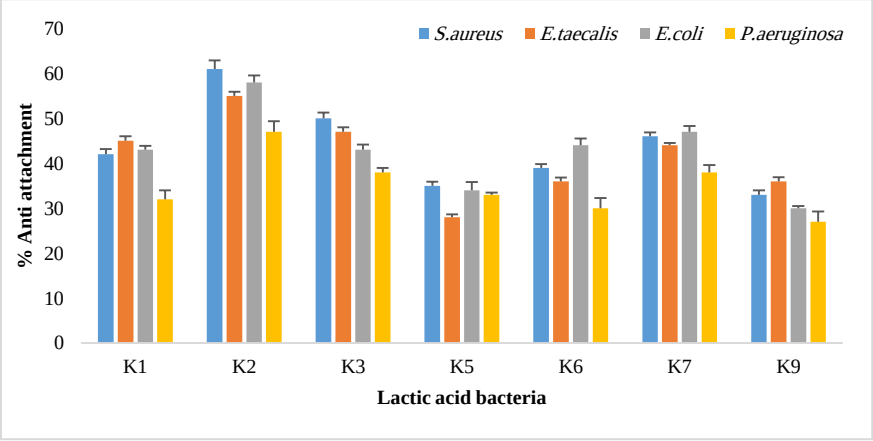

Figure 5. Anti-adhesion effect of isolated lactic acid bacteria against pathogens.

Molecular identification of most effective probiotic isolate

Using the ribotyping method, the best lactic isolate with the best probiotic potential, isolate K2, was investigated from a molecular point of view, (Figure 6).

The sequence obtained in the NCBI database was blasted and according to molecular identification and phylogeny tree, the K2 is *Lactiplantibacillus Plantarum*, Figure 7.

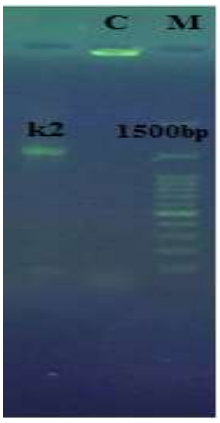

Figure 6. PCR product (16S rRNA gene) of K2.

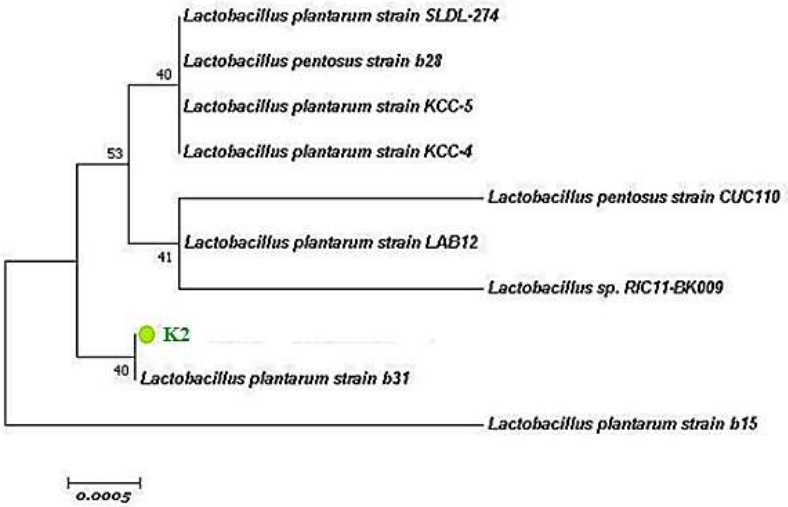

Figure 7. Phylogenetic tree of K2.

DISCUSSION

Today, probiotic lactic acid bacteria isolated from traditional fermented food products with therapeutic properties are being investigated for commercial use. Fermented food products have been studied in the pharmaceutical and food industries because of their probiotic properties. Therefore, this research focused on the isolation and monitoring of LAB with the best probiotic potential from traditional curd. Isolation and identification of probiotic microorganisms from natural sources, such as local curd, is an important process in developing functional foods [18]. In this study, several tests were performed to isolate and identify potential probiotic strains, with a specific focus on *Lactobacillus* spp.

Biochemical profiling and molecular techniques were performed to precisely identify the lactic acid bacteria. According to the study of Rai and Tamang (2021), 22 LAB were isolated from different fermented food products and screened for their probiotic potential. In the current study, 9 isolated lactic acid bacteria could ferment most of the sugars, except for K4, which was weaker than the others in fermenting sugars. Fermentation of sugars by lactic acid bacteria plays a crucial role in the identification and characterization of these microorganisms. Such tests provide valuable insights into the metabolic capabilities and substrate preferences of lactic acid bacteria, aiding in their classification and understanding of their functional properties. The fermentation of sugars, such as glucose, lactose, and fructose, by lactic acid bacteria leads to the production of various end products, including lactic acid, acetic acid, and ethanol. This diverse range of fermentation end products contributes to the unique flavor profiles, texture, and nutritional attributes of various fermented foods and beverages [19]. In the studies of Smith et al. (2022), investigating the

fermentation capability of lactic acid bacteria (LAB) isolated from different dairy products, focusing on their ability to ferment sugars has been shown [20]. Also, the diversity of sugar fermentation capabilities among lactic acid bacteria strains isolated from other dairy sources such as cheese and curd has been observed [19, 21].

Selection criteria for probiotic strains include resistance to bile toxicity as well as resistance to low pH. Based on the results obtained from the investigation of growth at different pH, seven isolates could grow at all three PHS, but K4 and K8 isolates could not grow at a pH equivalent to stomach acid. The results of the present research are consistent with the results of other researchers, and the tolerance of acid conditions by lactic acid bacteria can be used as a criterion for selecting probiotics [22, 23]. According to the results of this study, 7 isolates were able to tolerate bile salts and isolates K2, K3, and K7 had the highest ability to tolerate bile salts, respectively. The results of this research were consistent with the results of other researchers [24, 25]. Several mechanisms have been proposed to explain the tolerance of bile salts by LAB. In the first step, the cell membrane of LAB contains complex lipids that can interact with bile salts and prevent their destructive effects. Second, LAB has efflux pumps that actively remove bile salts from the cytoplasm, reducing their intracellular concentration. Finally, some LAB can produce enzymes that modify bile salts, such as bile salt hydrolases, which can efficiently metabolize bile salts into less toxic forms [26, 27].

The evaluation of L-arginine amino acid hydrolysis and growth performance at different temperatures is a crucial aspect in the identification of lactic acid bacteria with probiotic potential [28]. In the presented study, the ability to hydrolyze L-arginine was assessed, and strain K1 exhibited positive results, indicating its capacity to break down this amino acid. On the other hand, the remaining strains showed no hydrolytic activity towards L-arginine. This finding signifies the diversity within lactic acid bacteria strains and highlights the importance of this specific enzymatic activity in probiotic functionality. Moreover, temperature tolerance is another significant characteristic of lactic acid bacteria with probiotic attributes. In the study, the growth performance of the isolated strains was examined at both 15 and 45 degrees Celsius. Surprisingly, only strains K2, K3, and K7 were able to thrive and multiply at both temperature extremes. This observation suggests that these strains possess a broader range of temperature adaptation, which is advantageous for their survival and functionality in the host environment. In this present investigation, the hemolytic ability of lactic isolates on blood agar medium revealed that none of the isolates could lyse red blood cells and this is one of the isolate's probiotic highlights. The absence of hemolytic activity is regarded as a safety requirement forselecting a probiotic strain [29, 30]. In line with our results, *Lactobacillus plantarum* DU10 isolated from homemade fermented food products has been reported [10].

In this study, the antagonistic activity of isolates by the double-layer method showed that the most effective of isolates against all four pathogens *S. aureus*, *E. faecalis*, *E. coli*, and *P. aeruginosa* was related to the K2 isolate. Probiotic microorganisms exhibit inhibitory activity due to the bactericidal action of protease-sensitive bacteriocins [31]. In addition, lactic acid bacteria having probiotic potential can suppress the growth of pathogenic bacteria by pH reduction due to the secretion of antimicrobial compounds such as ammonia, bacteriocins, diacetyl, hydrogen peroxide, and organic acids such as acetic and lactic acids [30, 32]. Several studies have shown the effectiveness of LAB isolates in inhibiting the growth of pathogenic bacteria. In Ogunbanwo's study, it was shown that LAB isolates inhibit the growth of *S. aureus* through the production of organic acids, especially lactic acid. Kim et al. (2016) isolated LAB strains that showed strong antimicrobial activity against *E. faecalis*. The antimicrobial mechanism was attributed to the production of bacteriocins, which can disrupt the integrity of the cell membrane of *E.faecalis* and lead to cell death [33]. In a study by Shijia et al. (2020), LAB isolates inhibited the growth of two bacteria *E. coli* and *P. aeruginosa* through hydrogen peroxide production. Hydrogen peroxide can damage the DNA and protein of bacteria and lead to their death [34].

The results suggest that the lactic acid bacteria derived from curd samples have the potential to inhibit the binding of the tested pathogen strains. These findings are consistent with other research that highlighted the anti-binding properties of lactic acid bacteria strains derived

from different sources [30, 35]. The ability of lactic acid bacteria to inhibit pathogen attachment is of great significance in the context of preventing colonization and infection. When pathogens are unable to bind to host tissues or surfaces, their ability to establish infection is significantly diminished. The antimicrobial capabilities of lactic acid bacteria have been attributed to various mechanisms, including the production of antimicrobial substances (e.g., organic acids, bacteriocins) and competitive exclusion through adhesion to host surfaces [36, 37]. Therefore, the observed anti-adhesion effect of lactic acid bacteria, especially the potent isolate K2, suggests a potential role for these bacteria in reducing the risk of pathogen colonization and subsequent infection.

In this study, the best isolated lactic acid bacterium with probiotic potential (K2), identified based on molecular diagnosis and phylogeny tree, was *Lactobacillus plantarum*. In many research identifications of genera and species of probiotic lactic acid bacteria using biochemical tests has been well documented but today, ribotyping and phylogeny studies using the 16S rRNA gene which is conserved between different species of bacteria are more reliable. Chaudhary et al. (2018) obtained six LAB strains from traditional fermented food products of Himachal Pradesh and identified by 16S rRNA gene technique as various Lactobacillus spp. Also, in the study of Srinivash et al. (2023), 4 strains of LAB with probiotic potential were identified from homemade fermented food products with the same method [38].

CONCLUSIONS

Overall, the findings of this study showed that *Lactobacillus plantarum* had the best probiotic potential. Therefore, it can be consumed as an indigenous source of potentially beneficial LAB. In addition, according to the obtained results, LAB strains that have suitable properties to act as probiotics can be used to develop new probiotic starter cultures for curd production. Therefore, using probiotic lactic acid bacteria can ameliorate health and is important in the food and medical industry.

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Conflict of interests

The authors declare no conflict of interest

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