Journal of Food Science and Technology (Iran)

Homepage:www.fsct.modares.ir

Scientific Research

Evaluation of probiotic and antimicrobial properties of *Levilactobacillus brevis* KKP 3945 Isolated from Local Yogurt

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ARTICLE INFO

Article History:

Received:2024/2/12

Accepted:2024/4/22

Keywords:

Levilactobacillus brevis,

Antimicrobial activity,

Resistance to acid,

Resistance to bile salts.

DOI: 10.22034/FSCT.21.157.82.

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ABSTRACT

This research was conducted to investigate the probiotic potential of the strain *Levilactobacillus brevis* KKP 3945 isolated from local yogurt. Initially, the strain was evaluated for probiotic characteristics such as acid resistance (pH 2, 3, and 4), hydrophobicity, bile resistance (0.3%, 0.5%, and 0.7%), antioxidant activity (DPPH and ABTS), and cholesterol assimilation. The antimicrobial activity of the strain against six indicator foodborne pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Shigella dysenteriae*) was assessed using disk diffusion agar and well diffusion agar methods. The ability to produce biogenic amines, absence of hemolytic and DNase activity was also evaluated. According to the results, based on the results, Growth of the strain was inhibited at a bile salt concentration of 0.5%. A significant decrease in the number of viable cells was observed when the pH was reduced from 4 to 2, decreasing from 9.30 to 8.78 Log CFU/mL. Additionally, the number of viable cells of the strain at pH 4 decreased from 9.30 to 8.25 Log CFU/mL with an increase in time from 0 to 3 hours. The highest inhibitory effect of the strain was observed against *S. aureus*, while the lowest inhibition was observed against *E. coli*. The strain showed negative results for hemolytic and DNase activity, and its ability to reduce cholesterol levels was determined to be 44.44 ± 0.6%. The percentage of free radical inhibition in the DPPH and ABTS methods was 48.3% and 50.5%, respectively. *Lev. brevis* KKP 3945 was found to be sensitive to antibiotics such as nitrofurazone, nalidixic acid, penicillin, and ciprofloxacin. The results indicated that *Lev. brevis* KKP 3945 possessed acceptable probiotic characteristics and could be utilized as a probiotic bacterium in food products.

[DOI: 10.22034/FSCT.21.157.82]

1- Introduction

In recent years, fermented products have garnered significant attention from researchers due to their high nutritional value and positive bioactive effects. Fermented products typically contain many lactic acid bacteria (LAB) and produce lactic acid as the final product [1]. LAB are Gram-positive, microaerophilic, and non-spore-forming bacteria with a DNA base composition of less than 50% mol guanine and cytosine (G+C) and generally lack catalase. However, instances of pseudocatalase have been observed in cultures with low sugar concentrations [2]. Most LAB strains are non-pathogenic and play a significant role in the fermentation of food products both industrially and traditionally, and are considered beneficial bacteria [3]. Today, more than 20 genera are recognized within this large family, the most important being Leuconostoc, Lactobacillus, Streptococcus, Lactococcus, and Pediococcus. These bacteria are abundant in traditional fermented products and used as starter or adjunct cultures in controlled food fermentation processes. These microorganisms create unfavorable conditions for the growth of potentially pathogenic or spoilage microorganisms by converting fermentable sugars into organic acids, ethanol, and other metabolites with antimicrobial potential [4].

Given the application of lactobacilli in fermentation industries, especially the dairy industry, and their presence in the gastrointestinal tract of mammals, extensive research has been conducted on the effects of these bacteria on pathogens and their impact on host health. These studies have shown that various Lactobacillus strains can effectively inhibit the growth of pathogenic bacteria, including *Staphylococcus aureus* and invasive *Escherichia coli* strains [5].

Traditional dairy-based fermented foods are rich sources of native lactic flora [6]. Native strains of LAB are critical in the dairy industry because these strains are adapted to local conditions and possess unique abilities to produce desirable flavors and aromas in various fermented products. These strains exhibit inherent Resistance to destructive phages and antimicrobial effects [7]. With the development of the dairy industry in the country and the promotion of food hygiene culture, the consumption of dairy products from this industry has replaced traditional dairy products in various regions, significantly reducing their production. Although many commercial probiotic species are available on the market, preventing the gradual elimination of native LAB strains and isolating and preserving them for future applications that can offer unique properties are crucial [8].

Considering a microorganism as a probiotic strain requires in vitro tests and confirmation through in vivo tests. According to the regulations of the Food and Agriculture Organization, initial evaluation of the probiotic characteristics of any microorganism must be conducted under laboratory conditions and within the framework of these regulations before in vivo tests are performed. Characteristics considered for a strain to be regarded as a probiotic include bacterial viability during the preparation of functional products, survival in the gastrointestinal tract, the ability to adhere to intestinal epithelial cells, and the ability to counteract pathogens. Given the importance of describing new probiotic strains, this laboratory study aims to investigate the probiotic potential (acid resistance, bile salt resistance, cholesterol absorption, hydrophobicity, biogenic amine production, DNase activity, lack of hemolytic activity, antioxidant activity assessment, cholesterol absorption, and sensitivity to common therapeutic antibiotics) and antimicrobial activity of the strain *Levilactobacillus brevis* KKP 3945 isolated from local yogurt from Tashan against key food-borne pathogens (*Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Listeria monocytogenes, Salmonella typhimurium, and Shigella dysenteriae*).

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2-Materials and Methods

1.2. Isolation and Identification of Lev. brevis KKP 3945

The isolation and identification method for the target strain was performed according to the study by Sebektek and colleagues (2021). Five grams of local yogurt sample was added to peptone water (0.1%; 45 mL) and homogenized. After preparing dilutions (10^{-1} to 10^{-6}), cultures were performed on MRS agar. The strain was isolated from the culture medium and subjected to Gram staining and catalase testing. Genomic DNA was extracted using the Genomic DNA isolation VI kit and cultured overnight in MRS broth. After creating a pellet in microtubes containing the microbial suspension dissolving in 200 µL phosphate buffer and adding the appropriate enzyme solution to reach a final volume of 50 µL, the procedure was carried out according to the manufacturer's protocol. Universal primers based on conserved regions of 16S rRNA were used. Polymerase chain reaction (PCR) was performed in a final volume of 25.15 µL. The PCR microtube was placed in a thermocycler, and the temperature program was set according to Sebektek et al. (2021), including:

1. Activation: 5 minutes at 25°C, one cycle
2. Amplification, consisting of denaturation (30 seconds at 94°C), primer annealing (30 seconds at 94°C), and extension (2 minutes at 72°C), 35 cycles
3. Final extension: 10 minutes at 72°C, one cycle

Electrophoresis was performed at 95 volts for 45 minutes. The gel was then visualized using a gel documentation system [9]. The results showed that the isolate with catalase-negative and Gram-positive properties, with 98% similarity, belonged to the strain *Lev. brevis* KKP 3945.

2.2. Acid Resistance

The acid resistance test was performed according to the method of Berzegar et al. (2021). The target strain was cultured separately in 5 mL of MRS liquid medium for 18 to 24 hours at 37°C under anaerobic conditions to assess acid resistance. The grown bacteria were centrifuged at 6000g for 10 minutes at 4°C. The resulting pellet was washed twice with phosphate-buffered saline (PBS; Sigma-Aldrich), and the supernatant was discarded. The microbial suspension was adjusted to an optical density of 0.6 at 620 nm using a spectrophotometer. 50 µL of the microbial suspension was added to microtubes containing 450 µL of PBS solution, which served as the control sample. The control sample was incubated anaerobically at 37°C for 3 hours [10]. HCl (0.1 N) was used to prepare acids with pH values of 2, 3, and 4 in PBS. 50 µL of the microbial suspension was added to microtubes containing 450 µL of PBS with the desired pH and incubated with the control sample. After 3 hours, the samples were diluted to 10^-9. Finally, 100 µL of the desired dilutions was streaked on MRS agar plates and incubated anaerobically at 37°C for 24 hours. The number of *Lev. brevis* KKP 3945 bacteria was counted.

3.2. Bile Resistance Test

Bile salts react with the cell membrane of living cells, making them antimicrobial agents. Therefore, assessing the strain's Resistance to bile salts is essential to indicate the microorganism's viability. After activating the test strain in a 24-hour incubation at 37°C, the microbial suspension was centrifuged (9000g for 5 minutes at 4°C). After removing the culture medium, the remaining pellet was washed with a sterile phosphate buffer solution and centrifuged. 100 µL of the microbial suspension was cultured on MRS agar containing 0.3%, 0.5%, and 0.7% bile salts. The plates were incubated anaerobically at 37°C for 24 hours. The results were visually observed, with the plate without bile salts as a positive control [11].

4.2. Cell Surface Hydrophobicity

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Surface hydrophobicity indicates bacteria's tendency to adhere to non-polar solvents. High hydrophobicity allows them to move from an aqueous environment to an organic or non-polar environment, enabling bacteria to adhere to hydrocarbon particles on cells or mucosal surfaces. After growing bacteria on MRS agar at 37°C for 16 to 18 hours, they were centrifuged at 6000 rpm for 12 minutes. They were then washed with sterile, cold phosphate buffer solution and resuspended, with their absorbance adjusted to 0.6 at 600 nm (A1). Four milliliters of each bacterial suspension were mixed with 2 mL of n-hexadecane and vortexed for 2 minutes. The mixture was left at room temperature for 1 hour for bacteria to transfer between the two phases, and the absorbance of the aqueous solution was read at 600 nm (A2). Hydrophobicity was calculated using the following equation [12]:

$$\text{Hydrophobicity \%} = \left[\frac{A_1 - A_2}{A_1}\right] \times 100$$

5.2. Biogenic Amine Production

Biogenic amines are essential compounds for physiological and metabolic functions in all living organisms, produced by the metabolism of specific amino acids and decarboxylation reactions during fermentation or spoilage of foods. Controlling amine accumulation in fermented products, where histamine constitutes the majority of biogenic amines in foods, is a current challenge in the food industry. The ability of Lactobacillus strains to produce biogenic amines was assessed using the method by Berzegar et al. (2020), which includes precursor amino acids such as L-histidine monohydrochloride, disodium tyrosine, L-ornithine monohydrochloride, and L-lysine monohydrochloride. The Lev. brevis strain was streaked onto MRS agar containing 1 g/L of the precursor amino acids. Plates were incubated at 37°C for 48 hours. Colonies with purple halos indicated biogenic amine production [13].

6.2. DNase activity and hemolytic activity

determination was performed according to the extensive study by Vasie et al. (2020). Initially, strain *Lev. brevis* KKP 3945 was streaked on culture media linearly. Enzyme production was assessed after incubation at 37 degrees Celsius for 48 hours. Plates showing transparent and pink zones around colonies were reported as DNase-positive. The significance of the hemolytic activity assay lies in investigating the non-hemolytic nature of the blood and, consequently, the non-pathogenicity by the isolated strain. The hemolytic activity of strain *Lev. brevis* KKP 3945 was evaluated by streaking on tryptic soy agar (Merck Germany) supplemented with 7% (v/v) sheep blood. Plates were incubated at 37 degrees Celsius for 24 hours. After the incubation period, plates were examined for clear zones around colonies. Color changes were also noted. The formation of clear zones, green zones, or no zone formation around colonies indicates β-hemolysis, α-hemolysis, and γ-hemolysis, respectively. *This assay used staphylococcus aureus* and *Escherichia coli* bacteria as control samples for β-hemolysis and α-hemolysis.

7.2. Antioxidant Activity Assessment

The antioxidant activity of the target strain was evaluated using a modified DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging method. *Lev. brevis* KKP 3945 was inoculated into 100 mL MRS broth and incubated at 37°C for 24 hours. The cells were collected by centrifugation at 6000g for 15 minutes, washed twice with PBS, and resuspended in 10 mL of PBS. The cell-free supernatant was obtained by centrifugation at 10000g for 10 minutes. For the DPPH assay, 1 mL of the supernatant was mixed with 2 mL of a 0.2 mM DPPH solution in methanol and incubated in the dark for 30 minutes. The absorbance was measured at 517 nm using a spectrophotometer. The percentage of DPPH radical scavenging activity was calculated using the formula [14]:

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$$\text{Scavenging Activity \%}\left(1-\frac{A_{control}}{A_{sample}}\right)\times 100$$

8.2. Cholesterol absorption

Cholesterol absorption was assessed based on the study conducted by Alizadeh Behbahani et al. (2023). For this purpose, the liquid MRS culture medium was separately sterilized. Subsequently, Oxgall (0.3%) and polyoxyethylene ethyl oleate cholesterol were added to the liquid MRS medium. A cholesterol solution with a final concentration of 1% was added to the MRS medium containing 0.3% bile salt. The culture (1%) was inoculated and then incubated anaerobically at 30 degrees Celsius for 24 hours. The uninoculated sterile medium served as a control. Finally, the remaining cholesterol level in the medium will be calculated using the following formula:

$$\%\text{Cholesterol assimilation} = \frac{C - T}{C}\times 100$$

9.2. Antibiotic Sensitivity

Antibiotic resistance is rapidly evolving and has become a growing concern in public health. Some lactic acid bacteria are resistant to one or more antibiotics. Antibiotic resistance may occur naturally or be acquired through genetic mechanisms such as gene transfer via plasmids. To assess the sensitivity of *Lev. brevis* KKP3945 to common therapeutic antibiotics, a standard half-McFarland suspension equivalent to a 24-hour solid culture was prepared. 100 microliters of this suspension were streaked on agar MRS medium. Following this step, antibiotic discs of gentamicin (10 µg/mL), chloramphenicol (30 µg/mL), penicillin (10 µg/mL), vancomycin (30 µg/mL), nitrofurazone (300 µg/mL), nalidixic acid (30 µg/mL), imipenem (10 µg/mL), and ciprofloxacin (5 µg/mL) were placed on the culture medium using sterile forceps and gently pressed. The plates were incubated anaerobically at 37 degrees Celsius. After 24 hours, the diameter of the growth inhibition zone around the antibiotic discs was measured using a ruler and reported in millimeters.

10.2. Evaluation of Antimicrobial Activity of *Lev. brevis* KKP 3945

In this study, pathogenic bacteria, including *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Listeria monocytogenes*, *Shigella dysenteriae*, and *Salmonella typhimurium* were used to evaluate the antimicrobial activity using the agar well diffusion and agar disk diffusion methods.

10.2.1. Preparation of *Lev. brevis* KKP 3945 Supernatant

To prepare the supernatant, *Lev. brevis* KKP 3945 strain was cultured in 10 mL of liquid MRS medium and incubated at 37 degrees Celsius for 24 hours. Then, the active culture was centrifuged (at 5000 g for 15 minutes), and the obtained supernatant was passed through a syringe filter with a diameter of 0.22 micrometers to ensure complete removal of bacterial cells. The resulting supernatant was kept at 4 degrees Celsius until the assay was performed.

10.2.2. Evaluation of Antimicrobial Activity by Agar Well Diffusion Method

50 microliters of active bacterial culture of pathogenic bacteria with a concentration equivalent to half McFarland standard (1.08 × 10^8 CFU/mL) was evenly spread on MHA agar plates. Wells with a diameter of 6 mm were created using a sterile pipette tip, and the *Lev. brevis* strain supernatant was injected into the wells. To facilitate better absorption and diffusion of antimicrobial compounds from the wells, the plates were refrigerated at 4 degrees Celsius for one hour and then transferred to an incubator at 37 degrees Celsius, suitable for the growth of indicator strains. After 24 hours, the growth inhibition zone around the wells was measured using a ruler and reported in millimeters.

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10.2.3. Evaluation of Antimicrobial Activity by Agar Disk Diffusion Method

To evaluate the antimicrobial properties of the strain against the mentioned pathogenic bacteria using the agar disk diffusion method, the population of indicator bacterial strains was adjusted to half-McFarland standard using an absorbance reading. In the next step, sterile paper disks (PadtanTeb, Iran) containing the *Lev. brevis* strain supernatant were placed on the agar plates containing the indicator bacteria at a specific distance from the edge of the plate surface. Finally, the plates were incubated at 37 degrees Celsius for 24 hours, and the diameter of the growth inhibition zone was measured using a ruler.

11. Statistical Analysis of Data

Data analysis was performed using a one-sided analysis of variance with version 22. To compare means, the Duncan multiple range test was used with a confidence level of 95%, and graphs were plotted using Microsoft Excel 2016.

3-Results and Discussion

1-3- Acid Resistance

The viability of *Lev. brevis* KKP3945 at different pH levels is shown in Figure 1. Lactic acid bacteria must have the ability to withstand the low pH of the digestive system to perform their probiotic functions. Therefore, acid resistance in lactic acid bacteria is crucial for their growth and the fermentation and production of probiotic products. The survival of *Lev. brevis* KKP3945 was significantly influenced by pH and exposure time to pH. With a decrease in pH from 4 to 2, a considerable reduction in the number of viable cells was observed, with the viability decreasing from 9.30 to 8.78 Log CFU/mL.

Additionally, the number of viable cells of the strain decreased from 9.30 to 8.25 Log CFU/mL with an increase in exposure time from zero to 3 hours. One of the critical criteria for selecting probiotic bacteria is viability at low pH of the digestive system. Bacteria tolerate low pH conditions through various mechanisms, with one of the most important mechanisms for lactobacilli species being the F0F1-ATPase system. Noshad et al. (2021) reported that acid resistance is strain-dependent and needs to be individually evaluated for each bacterium. It was also reported that *Lactiplantibacillus plantarum* and *Lev. brevis* have higher Resistance to low pH than pediococci. In a similar study, Singh et al. (2020) reported that *Lev. brevis* ATCC14869 isolated from Sauerkraut is capable of growth and survival at low pH.

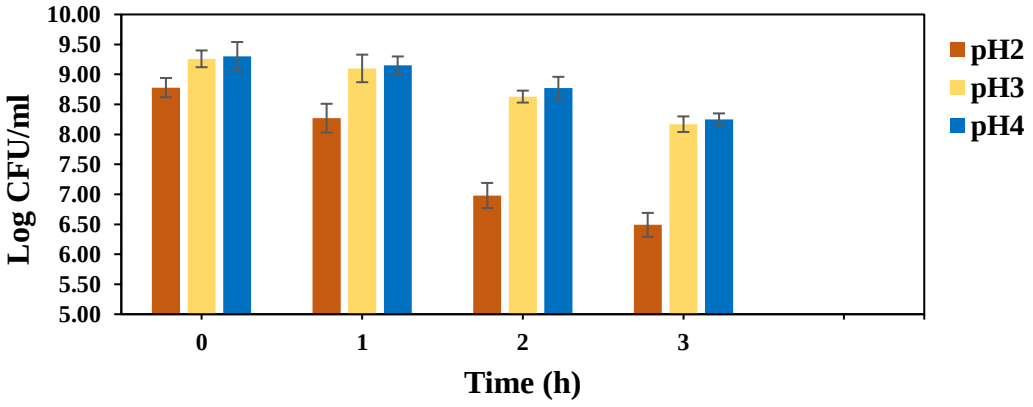

Fig. 1. The capacity of *Levilactobacillus brevis* KKP 3945 to endure in an acidic pH environment.

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2-3- Bile Resistance Test

Bile salts play a crucial and fundamental role in the specific defense of the intestine by eliminating microorganisms. Therefore, assessing the viability of strains at various concentrations of bile salts is essential to demonstrate probiotic potential. Bile salts aid in the digestion of fat compounds but sometimes exhibit a specific antibiotic property, inhibiting the growth of gut microbiota. In this study, concentrations of 0.3%, 0.5%, and 0.7% of bile salts were used to examine the Resistance of the tested strain. The results of the Resistance to bile salts are presented in Table 1. The results showed that *Lev. brevis* KKP 3945 shows growth at low concentrations of bile salts. In this study, the growth of the tested strain was halted as the percentage of bile salts increased from 0.3% to 0.5%. Considering that the concentration of bile salts in the body usually does not exceed 0.3%, the robust stability of the strain could be due to its capacity to convert bile salts into cholesterol and amino acids. This finding is consistent with the results of Somashakaraya and colleagues (2021), who reported that *Lev. brevis* MYSN105 possesses significant probiotic properties, especially stability in acidic environments and environments rich in bile.

Table 1. The ability of *Levilactobacillus brevis* KKP 3945 to endure varying concentrations of bile salts

Survivability	0.3%	0.5%	0.7%	Control
	Growth	Not Growth	Not grown	Growth

3-3- Cell Surface Hydrophobicity

Hydrophobicity is one of the critical factors for bacteria to adhere to various surfaces. Hydrophobicity depends on various factors such as van der Waals forces, Brownian motion, surface electric charge, and gravitational force. Therefore, this feature should be investigated separately for bacteria. Hydrophobicity is directly attributed to the strain's adhesive ability. Various compounds, including n-hexadecane, xylene, ethyl acetate, and chloroform, can diagnose hydrophobicity. Hydrophobicity results from the interaction between bacteria and host cells and can be assessed in laboratory conditions by mixing bacterial suspensions with one of the mentioned non-polar compounds and measuring the absorption of the aqueous solution (OD600nm) before and after mixing. The reduction in absorption of the suspension after exposure of cells to hydrocarbons indicates the hydrophobic property of the bacteria. For Lev. brevis KKP 3945, the level of hydrophobicity was 60.0 ± 5.58%. In a similar study, Lev. brevis isolated from pickled cucumber exhibited surface hydrophobicity of 29.46% for n-hexadecane. Additionally, research conducted by Fadri and colleagues (2023) showed that the hydrophobicity of Lev. brevis derived from Sauerkraut was 51%. The hydrophobic properties of probiotic bacteria can serve as an initial assessment of their potential for attachment to epithelial cells.

4-3- Cholesterol Absorption

Although cholesterol is essential for body tissues, elevated cholesterol levels are the leading cause of cardiovascular diseases and the most common cause of death worldwide. Pharmacological treatment to reduce cholesterol levels is usually adequate, but long-term medication use may pose risks to the body. One of today's most common methods is consuming fermented foods containing

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probiotic bacteria. Lactic acid bacteria have been recognized for their ability to improve intestinal transit, maintain gut flora balance, and regulate the intestinal acid-base balance, leading to the regulation of the immune system and reduction of serum cholesterol levels. This study showed *Lev. brevis* KKP 3945 could reduce cholesterol levels by 6.0 ± 44.44%. The findings are consistent with similar studies indicating the ability of *Lev. brevis* strains to reduce cholesterol. Hojati et al. (2020) also reported a cholesterol degradation rate of 41% for *Lev. brevis* gp104 in a medium devoid of bile salts and 58% in a medium containing bile salt. Various laboratory studies have proposed different mechanisms to explain probiotic bacteria's cholesterol-lowering effects. These mechanisms include cholesterol absorption, cholesterol binding to bacterial cell walls, microbial conversion of cholesterol to coprostanol, and the enzymatic process of bile salt deconjugation.

5-3- Evaluation of DPPH and ABTS Antioxidant Potential

The percentage of free radical scavenging activity by *Lev. brevis* KKP 3945 is shown in Figure 2. Li et al. (2012) stated that the ability of intact cells to eliminate free radicals is related to substances present on the surface of bacterial cells, such as proteins, polysaccharides, and lipoteichoic acid. It has been shown that antioxidant substances produced by lactic acid bacteria include NADH, NADPH, Mn+2, antioxidant enzymes, exopolysaccharides, and bioactive compounds. Based on similar studies, all lactic acid bacteria exhibit free radical scavenging activity. The protective ability of probiotics against oxidative stress involves inhibiting oxygen species, chelation of metal ions, and reduction of ascorbate autoxidation. Antioxidant components in lactic acid bacteria include bacterial exopolysaccharides, bioactive peptides, antioxidant enzymes, and manganese. The gut microbiota can also produce bioactive antioxidant substances through microbial transformation processes using various enzymatic reactions.

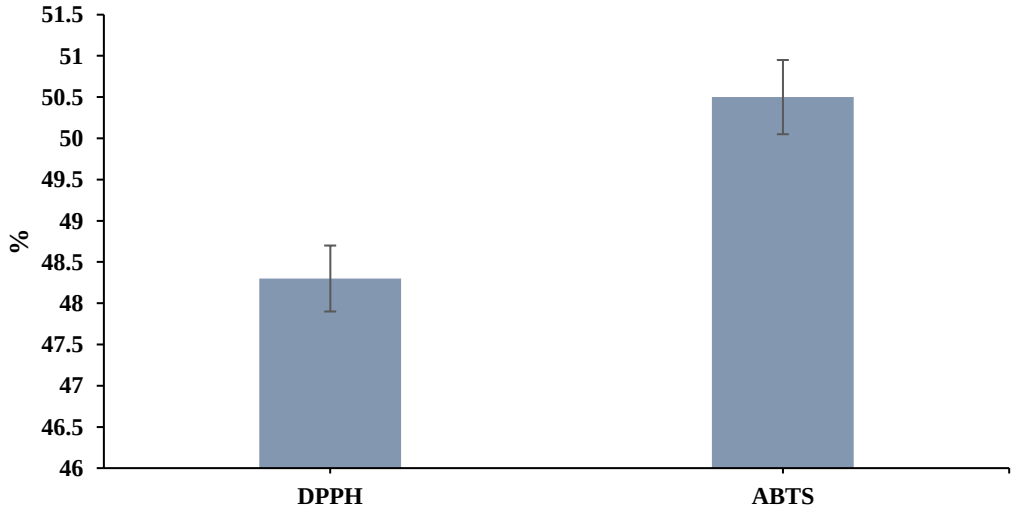

Fig. 2. The antioxidant activity (DPPH & ABTS) of *Levilactobacillus brevis* KKP 3945.

6-3. DNase Test and Hemolytic Activity

Based on the obtained results, DNase production by *Lev. Brevis* KKP 3945 was negative, and no clear zones around the colonies were observed on the culture medium. This indicates its potential for probiotic

[DOI: 10.22034/FSCT.21.157.82]

preparation. Additionally, the response obtained from the hemolytic activity was also negative. The importance of this test lies in assessing the lack of blood degradation and, consequently, the non-pathogenicity of the isolated strain. Similar results have shown that most lactic acid strains do not have hemolytic activity [27]. In the study by Alizadeh Behabani et al. (2023), the probiotic potential, safety, and anti-pathogenicity of *Lev. brevis* HL6, as well as its potential use as a bio preservative in peach juice, were evaluated, indicating that the tested strain did not produce DNase or biogenic amines [16]. The *Lev. brevis* AcCh91 strain isolated from chhurpi (a traditional fermented milk product from India) in the study by Shangpliang and Tamang (2023) also showed similar results, and they did not exhibit any signs of hemolysis [28]. The lack of hemolytic activity is crucial for probiotics because hemolysis can increase iron availability to the organism, leading to conditions such as anemia and edema in humans [29].

7-3. Biogenic Amine Production

This study did not observe biogenic amine production by *Lev. brevis* KKP 3945. Biogenic amines are primarily formed by the amination and transamination of aldehydes and ketones or the decarboxylation of amino acids. They are commonly found in foods that lack protein and amino acids and are affected by microbial or biochemical processes such as fermentation [10]. Biogenic amines may be present in fish-related foods, fermented sausages, cheese, and other fermented foods. The absence of histamine and tyramine is typically examined to assess their toxic and allergenic effects. Although biogenic amine production by some strains of lactic acid bacteria, such as Enterococcus and Lactobacillus, has been reported, their absence in food is an important principle.

In a similar study, Alizadeh Behabani et al. (2023) reported that *Lev. brevis* HL6 did not show biogenic amine production [16]. The formation of biogenic amines during food processing depends on the availability of free amino acids, microorganisms with appropriate catabolic pathways, and a conducive environment for decarboxylase activity. Biogenic amines serve as precursors for the synthesis of hormones, alkaloids, nucleic acids, and proteins, playing roles in body metabolism, such as blood pressure regulation and temperature control. Despite these favorable characteristics, consuming foods containing high amounts of biogenic amines can release adrenaline and noradrenaline, stimulate gastric acid secretion, increase cardiac output, cause migraines, and elevate blood sugar levels and blood pressure [30]. Generally, "excessive consumption" of an amine may put all individuals at risk. However, people with gastrointestinal issues, as their intestinal oxidase activity is usually lower than that of healthy individuals, are more at risk. Additionally, individuals with respiratory and coronary artery problems, high blood pressure, or vitamin B12 deficiency show greater sensitivity to lower doses of biogenic amines [31].

8-3. Antimicrobial Activity Test

The results of the antimicrobial activity evaluation of *Lev. brevis* KKP 3945 against pathogenic bacteria by agar well diffusion and disk diffusion methods are shown in Figures 3 and 4. Considering the potential of probiotics to modify and balance the gut microbiota, their antimicrobial properties against harmful pathogens are highly significant. The antimicrobial ability of probiotics is essential as the probiotic strain must suppress the growth of potential pathogenic bacteria [32]. The antimicrobial effect of lactic acid bacteria is mainly related to metabolites such as organic acids (primarily lactic, acetic, propionic, sorbic, and benzoic acids), hydrogen peroxide, diacetyl, ethanol, phenols, and protein compounds they produce during growth.

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Additionally, some strains of lactic acid bacteria can produce bacteriocins, which have significant antibacterial activity [33]. In the body, probiotic strains compete with pathogenic microorganisms for binding sites and nutrients, preventing the proliferation of pathogenic bacteria through a competitive exclusion mechanism [34].

The acidic and non-acidic antimicrobial effect of *Lev. brevis* KKP 3945 on pathogenic strains, as shown by agar diffusion using disk and well methods, is illustrated in Figure 2. In both methods, the highest inhibitory effect was observed against *S. aureus*, a Gram-positive facultative anaerobe that often causes skin infections but can also lead to pneumonia, endocarditis, and bone infections. The lowest inhibitory effect was seen on *E. coli*, a Gram-negative facultative anaerobe responsible for diarrhea and food poisoning. The presence of bacteriocins typically prevents the growth of Gram-positive pathogens, while for Gram-negative types, the main antimicrobial properties are attributed to the presence of hydroxy fatty acids, organic acids, and hydrogen peroxide. Therefore, in Lactobacillus strains, the primary reason for antimicrobial properties is the production of bacteriocins. However, compounds such as hydrogen peroxide, organic acids, ethanol, and competition for nutrients also play a role [35].

Vasei et al. (2018) showed that enterocins produced by Enterococcus species are peptides with the highest antimicrobial properties against food-borne pathogens, especially *L. monocytogenes* [36]. In the body, probiotic strains compete with pathogenic microorganisms for binding sites and nutrients, preventing the proliferation of pathogenic bacteria through a competitive exclusion mechanism [11]. The antimicrobial activity results of this study were consistent with those of Hojjati et al. (2023).

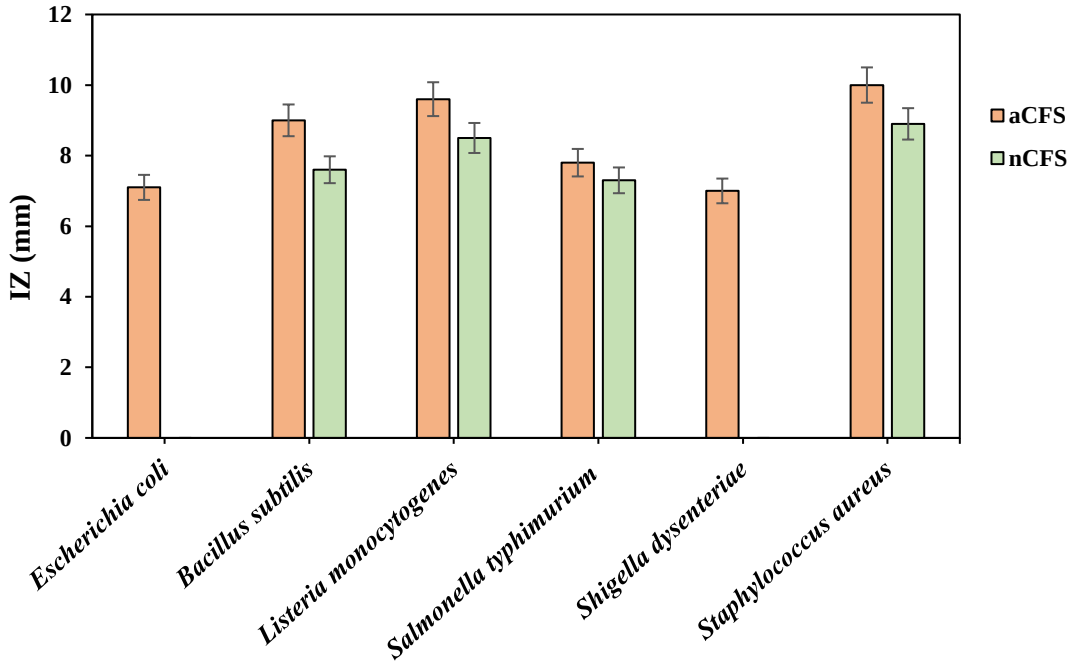

Fig. 3. The antimicrobial potency of *Levilactobacillus brevis* KKP 3945 using well diffusion agar. The abbreviations aCFS and nCFS are acid cell-free supernatants and neutralized cell-free supernatants, respectively.

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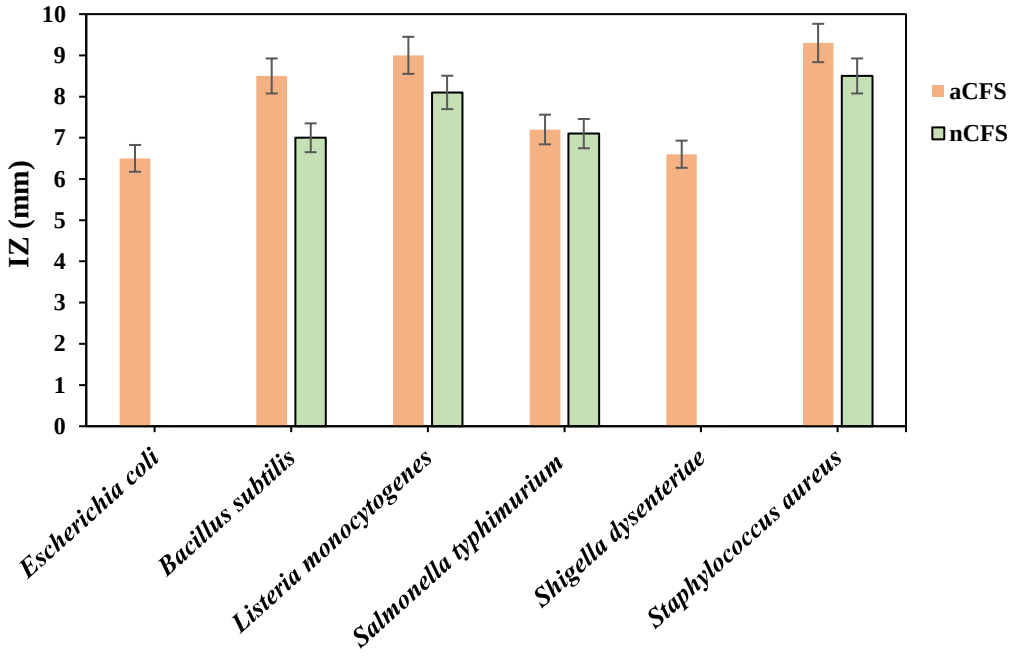

Fig. 4. The antimicrobial potency of *Levilactobacillus brevis* KKP 3945 using disk diffusion agar. The abbreviations aCFS and nCFS are acid cell-free supernatants and neutralized cell-free supernatants, respectively.

9-3. Antibiotic Resistance

Antibiotic resistance is developing at an alarming rate and has become a growing concern in public health. Some lactic acid bacteria are resistant to one or more antibiotics. Antibiotic resistance may occur naturally or be acquired through genetic mechanisms such as gene transfer via plasmids or transposons [1]. In this study, the resistance level of the strain under investigation against eight clinical antibiotics was examined. Table 2 shows the resistance level of *Lev. brevis* KKP 3945 to various antibiotics. The mechanism of action of antibiotics on the destruction of microorganisms varies. Some antibiotics, such as streptomycin, chloramphenicol, tetracycline, and erythromycin, inhibit protein synthesis, while others, like rifampicin and cotrimoxazole, prevent mRNA synthesis. Some antibiotics, such as ampicillin, vancomycin, and penicillin G, disrupt the bacterial cell wall. Beta-lactam antibiotics, such as penicillin and cephalosporins, affect cell permeability and lead to cell wall destruction [37]. According to existing research, Lactobacillus species are typically sensitive to antibiotics such as erythromycin, chloramphenicol, and tetracycline [38]. The significant sensitivity of the isolated strain to multiple antibiotics indicates that it may not carry genes that confer antibiotic resistance and potentially transfer it to pathogenic microorganisms.

In the study by Alizadeh Behabani et al. (2023), *Lev. brevis* HL6 was sensitive to almost all antibiotics, with the highest and lowest sensitivity observed for nitrofurantoin (26 mm inhibition zone) and ampicillin (10.5 mm inhibition zone), respectively [16]. The antibiotic sensitivity of lactic acid bacteria strains is a probiotic characteristic, and the level of antibiotic resistance can vary among different strains and even within subspecies [38]. The significant sensitivity of the isolated strain to multiple antibiotics suggests that it

[DOI: 10.22034/FSCT.21.157.82]

may not have genes that cause antibiotic resistance.

Table 2. Effect of Common Therapeutic Antibiotics on *Levilactobacillus brevis* KKP 3945

Antibiotic	*L. brevis* KKP3945
Vancomycin	**Intermediate**
Gentamicin	**Sensitive**
Chloramphenicol	**Intermediate**
Nitrofurazone	**Sensitive**
Nalidixic	**Sensitive**
Penicillin	**Sensitive**
Imipenem	**Resistant**
Ciprofloxacin	**Sensitive**

4. Conclusion

Traditional dairy-based fermented foods are rich sources of indigenous lactic flora. Native strains of lactic acid bacteria have gained significant importance in the dairy industry because identifying and examining their technological characteristics can enable their industrial applications. This study *evaluated Lev. brevis* KKP 3945, isolated from local yogurt, for its probiotic and antimicrobial properties. This strain demonstrated the ability to withstand low pH levels and various concentrations of bile salts. It exhibited an excellent capability to inhibit pathogenic bacteria. *Lev. brevis* KKP 3945 was sensitive to common therapeutic antibiotics, alleviating concerns about the potential transfer of antibiotic-resistance genes to pathogenic bacteria. Overall, the results indicated that this strain possesses acceptable probiotic and antimicrobial potential and can be used for human consumption. Further tests in vitro and in vivo, including adhesion to intestinal epithelial cells, bacteriocin production, and the strain's effect on blood sugar levels in animal models, are necessary to utilize this bacterium as a probiotic and natural preservative in the production of functional food products.

5. Acknowledgements

The authors would like to thank the Vice-Chancellor for Research and Technology of Agricultural Sciences and Natural Resources University of Khuzestan for the financial support of the research project No. 1402.44, from which this article is derived.

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[DOI: 10.22034/FSCT.21.157.82]

مجله علوم و صنایع غذایی ایران

سایت مجله: www.fsct.modares.ac.ir

مقاله علمی_پژوهشی

بررسی ویژگی‌های پروبیوتیکی، ضدمیکروبی و ایمنی سویه *Levilactobacillus brevis* KKP 3945 جداسازی شده از ماست محلی

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اطلاعات مقاله

تاریخ های مقاله :

تاریخ دریافت: ۱۴۰۲/۱۱/۲۳

تاریخ پذیرش: ۱۴۰۳/۲/۳

کلمات کلیدی:

Levilactobacillus brevis،

فعالیت ضدمیکروبی،

مقاوت به اسید،

مقاومت به نمک‌های صفراوی.

DOI:10.22034/FSCT.21.157.82.

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چکیده

این پژوهش با هدف بررسی پتانسیل پروبیوتیکی سویه *Levilactobacillus brevis* KKP 3945 جدا شده از ماست محلی انجام شد. ابتدا سویه، از نظر ویژگی‌های پروبیوتیکی از قبیل مقاومت به اسید (pH ۲، ۳ و ٤)، هیدروفوبیسیتی، مقاومت به صفرا (۰/۳، ۰/۵و۰/۷)، فعالیت آنتی‌اکسیدانی (DPPH و ABTS) و جذب کلسترول مورد ارزیابی قرار گرفت. فعالیت ضدمیکروبی (دیسک دیفیوژن آگارو چاهک آگار) سویه در مقابل ٦ پاتوژن شاخص غذایی (*Escherichia coli*، *Staphylococcus aureus*، *Bacillus subtilis*، *Listeria monocytogenes*، *Salmonella typhimurium* و *Shigella dysenteriae*) بررسی شد. توانایی تولید آمین بیوژنیک، عدم فعالیت همولیتیک و DNase نیز ارزیابی شد. بر اساس نتایج، در غلظت ۰/۵٪ نمک‌های صفراوی از رشد سویه جلوگیری شد. با کاهشpH از٤ به ۲، کاهش قابل توجهی در تعداد سلول‌های زنده مشاهده شد و از ۹/۳۰ به ۸/۷۸ Log CFU/mL کاهش یافت. علاوه بر این، تعداد سلول‌های زنده سویه درpH ٤ با افزایش زمان از صفر به ۳ ساعت از۹/۳۰ به ۸/۲۵ Log CFU/mL کاهش یافت. بیشترین اثر بازدارندگی سویه در مهار باکتری *S. aureus* و کمترین بازدارندگی بر *E. coli* مشاهده شد. پاسخ به‌دست آمده از فعالیت همولیتیکی و DNase منفی بوده و میزان توانایی سویه در کاهش میزان کلسترول ۰/٦ ± ٤٤/٤٤ به‌دست آمد. میزان درصد مهار رادیکال‌های آزاد در روش DPPH و ABTS به ترتیب،۴۸/۳ و۵۰/۵۰ بود. *Lev. brevis* KKP 3945 نسبت به آنتی‌بیوتیک‌های نیتروفورازون، نالیدیکسیک، پنیسیلین و سیپروفلوکساسین حساس بود. نتایج نشان داد *Lev. brevis* KKP 3945 ویژگی‌های پروبیوتیکی قابل قبولی داشت و می‌توان از آن به‌عنوان یک باکتری پروبیوتیک در محصولات غذایی بهره برد.

[DOI: 10.22034/FSCT.21.157.82]