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Research Article

# The Investigation of Probiotic Potential of Lactic Acid Bacteria Isolated from Traditional Dairy Products and Their Genomic Analysis in Northern Iran, Gilan

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ABSTRACT

Isolation, identification and selection of the best Lactobacillus with probiotic activity from local dairy products of Gilan province. In this study, 30 isolates obtained from local dairy products in rural areas of Gilan were identified microbiologically and biochemically. The probiotic characteristics of the strains were determined and antagonistic activity of the extract of Lactobacillus isolates against five pathogenic bacteria by the well method in agar, and antibiogram by the disk diffusion method and finally the isolates were identified based on the 16SrRNA sequence. Among the isolates, four strains with the best results in terms of probiotic performance were identified. In the investigation of antimicrobial activity, the isolates were able to inhibit the growth of Staphylococcus aureus, Shigella flexneri, Pseudomonas aeruginosa and Uropathogenic Escherichia coli, but they had no significant inhibitory effect on the growth of Bacillus cereus. Antibiogram results showed that all four strains were resistant to vancomycin and gentamicin but sensitive to ampicillin. None of the isolates showed hemolytic activity. Only two strains showed a clear band in the genomic analysis of 16srRNA by PCR method. The affinity of the strains indicated Lactobacillus delbrueckii subspecies bulgaricus. The extract of these two strains without neutralization and catalase enzyme showed the best activity against common pathogenic bacteria. According to the available results, our strain can be used as a suitable candidate as a supplement in the prevention and biological treatment of some clinical cases in laboratory and in vivo conditions with further research.

## 1. Introduction

Lactic acid bacteria are nutritionally important microorganisms. These bacteria are generally present in milk and other dairy products, in plants, and in the mucosa of the digestive system of humans and animals. These bacteria are commonly found in fermented foods, and they use carbohydrates as their sole source of energy (Carr et al., 2002; Mathur et al., 2005; George et al., 2018). The number of these bacteria, which are generally coccid or bacilli,

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includes more than 60 genera, which can be mentioned Lactobacilli and streptococci (Mokoena, 2017). Lactobacilli are more than 260 species based on morphological traits and fermentation products, but based on the 16sRNA sequence; they are officially classified into 26 genera of the *Lactobacillaceae* family (Zheng et al., 2020). As a fermenting strain, lactic acid bacteria have several important metabolic properties, including the production of acid and aromatic compounds, the ability to hydrolyze protein, the ability to produce sticky exopolysaccharides, and the inhibition of bacteria and fungi. These bacteria are not only capable of decomposing nutritional macromolecules such as protein and polysaccharides, but they can also decompose a number of undesirable substances, especially they can prevent the accumulation of mycotoxins too (Taheur et al., 2019). *Lactobacillus* species exist symbiotically with Oxalobacter in the human digestive system and can prevent the formation of oxalate stones (Sadaf et al., 2017). In addition to their use as starter cultures in dairy products and the presence of environmental non-starter lactic acid bacteria, their other field of study is the presence of probiotic non-starters (Bintsis, 2018). These strains rely on their ability to survive and colonize the gastrointestinal tract, which includes host protection against gastrointestinal pathogens. Probiotics are generally of interest in many fields such as pharmacology and therapeutic supplements (Granato et al., 2010; Ranadheera et al., 2019). At the same time, it should be kept in mind that the use of lactic acid bacteria in the effective transformation of unwanted substances in food is still considered a challenge (Perczak et al., 2018). Probiotics are not only useful in maintaining health or controlling infectious diseases, but also important in the treatment and management of diseases (Amara & Shibl, 2015). Some *Lactobacillus* species, including delbrueckii, are one of the most common starter bacteria and suitable for the production of fermented milk in the world. The primitive researches have indicated that the consumption of dairy products with this species was related to the quality of life and longevity of the Bulgarian population. For this reason, the focus of this study is on showing the probiotic function of Lactobacilli isolated from local dairy products of Gilan province.

## 2. Materials and Methods

### *2.1. Sampling and microbial growth conditions*

The present study was conducted by random cluster sampling method from the beginning of 2020 and for a year and a half in the rural areas of Rasht, Bandar Anzali and Lahijan. The reason for choosing these areas is the ability to produce high quality homemade dairy products. A total of 30 samples of dairy products were collected (Tables 1 and 2). All the samples were immediately transported to the laboratory in aseptic conditions with dry ice and kept at 4°C until the test.

At first, twenty-five grams of curd sample was dissolved in 225 ml of 0.1% (w/v) peptone water and then homogenized for three minutes by a grinder (Memmert, Germany) (Yang et al., 2012). To prepare serial dilutions, 0.1% peptone water was used to reduce the concentration and obtain the number of colonies that could be examined ($10^{-1}$ to $10^{-8}$). 100 µl of each dilution was inoculated in MRS agar bed (de Man Rogosa Sharpe agar-Merck, Germany) with a pH 6.5 (Yang et al., 2012). For yogurt samples, 1 ml of each sample was added to 99 ml of 0.1% peptone water and then from the serial dilutions, 100 µl was inoculated in the MRS agar (Yang et al., 2012). For milk, yogurt drink (doogh) and whey samples, 1 ml of each sample was dissolved with 9 ml of 0.1% peptone water and then 100 µl of the prepared dilution was cultured in MRS agar (Yang et al., 2012, Beukes et al., 2001). Nystatin (µ5 g/mL) and sodium azide 0.01% (w/v) were used to prevent the possible growth of yeast and gram-negative bacteria, respectively (Beukes et al., 2001). At the same time, direct culture of the sample was done without preparation of dilution. After purification, all isolates were stored in cryovials containing MRS broth containing 20% sterile glycerol (1/3 ratio) at -20°C and -70°C. For the temporary maintenance of isolates, consecutive cultures were used in MRS broth medium. At the same time, the isolates were kept at 4°C in the refrigerator for short-term storage on the surface and depth of MRS agar.

### *2.2. Analysis of Phenotypic Characteristics*

The obtained pure colonies were analyzed according to Bergey's manual for colony morphology, cell and biochemical characteristics

(Vos et al., 2011). Gram staining, catalase, oxidase and spore forming ability tests were performed for the isolates (Figure 1). In continue, the ability of the isolates to grow in aerobic, anaerobic and microaerophilic conditions with and without $CO_2$(5%) at temperatures of 30°C, 37°C and 42°C was investigated to show mesophilic and thermophilic lactic acid bacilli (Hammes & Vogel, 1995). Fermentation tests of glucose, maltose, mannitol, lactose, trehalose and sucrose and biochemical tests including TSI, SIM, urea, citrate and MR-VP were performed (Erkuş, 2007). In all stages of the experiment, in order to avoid possible contamination, gram staining was done. After obtaining the results, the colonies that were similar to lactic acid bacteria in terms of characteristics were purified and stored. Genomic identification of the best probiotics was done by PCR method along with phylogenetic tree drawing (Table 3).

### *2.3. In Vitro Assessment of probiotic potential of Lactobacillus isolates*

To evaluate the probiotic performance of the isolates, a blank sample (containing 4 ml of culture medium without bacteria at pH 8) and a standard strain of *Lactobacillus delbrueckii* subspecies *bulgaricus* (PTCC 1737), prepared from the scientific and industrial research organization of Iran (control strain) were used. The results of the probiotic performance of the standard strain for comparison with *Lactobacillus* isolates are only given in the form of a graph.

### *2.4. Salt Tolerance Test*

10 microliters of the growth broth of the strains were inoculated in 4 ml of MRS broth containing different concentrations of salt (4%, 8%, and 12%) and after 24 incubation at 37°C; the results were recorded at 620 nm wavelength (Matijašic & Rogelj, 2000).

### *2.5. Bile Salt Tolerance Test*

100 µl of the growth of the isolates was inoculated in 20 ml of MRS broth with different concentrations of bile salts (0.3%, 0.5% and 0.8% w/v) (Oxoid Co., England). After incubation at 37°C for 3 hours, the results were read at 620 wavelengths (Matijašic & Rogelj, 2000).

### *2.6. The Effect of pH on Microbial Growth*

The amount of 40 µl of the 24-hour culture of isolates was inoculated in 4 ml of MRS broth at different pH ranges (2, 3, 4 and 5) and the results were read after three hours of incubation at 37°C and at a wavelength 620 nm. The number of bacteria in the MRS agar medium was counted after three hours of incubation at 37°C and the survival rate was calculated as the logarithm of cfu/ml values (Grosu-Tudor & Zamfir, 2012, Zhang et al., 2016).

### *2.7. Phenol Tolerance Test*

The ability to grow isolates was done by inoculating 2% in 10 ml of MRS broth in the presence of different concentrations of phenol (0.2%, 0.3%, 0.4% and 0.5%) and the results after three hours of growth at 37°C was recorded at wavelength 620 nm (Xanthopoulos et al., 2000).

**Table 1.** Dairy samples collected from rural areas of Gilan province

| Sample | Yogurt | Dough | Curd | Whey | Milk |
|---|---|---|---|---|---|
| Number | 15 | 5 | 1 | 5 | 4 |

**Table 2.** Sampling areas of dairy products in Gilan province

| Region<br>Sample | Sangachin (Anzali) | Loleman (Rasht) | Pirbazar (Rasht) | Barmacheh (Khomam) | Katehshal (Lahijan) | Soustan (Lahijan) | Kateshal (Khomam) | Salekdeh (Kiashahr) |
|---|---|---|---|---|---|---|---|---|
| Yogurt | 2 | 3 | 2 | 2 | 1 | 1 | 2 | 2 |
| yogurt drink | - | 1 | 1 | - | 1 | - | 1 | 1 |
| Curd | - | - | - | 1 | - | - | - | - |
| Whey | 1 | - | 1 | - | 1 | 1 | 1 | - |
| Milk | 1 | - | - | 1 | 1 | - | - | 1 |

*2.8. In vitro antagonistic activity of cell free supernatants of Lactobacillus isolates against pathogenic bacteria*

To detect the inhibitory activity of the supernatant solution of isolates against selected clinical isolates, Agar well diffusion method was used. First, the strains were cultured in MRS broth medium and a standard turbidity with a concentration of $10^8$ to $1 \times 10^7$ cfu/ml (OD600 ± 0.2) was prepared. After incubation, the cultures were centrifuged (Centrium, England) at 10,000 rpm for 10 min at 4 °C to obtain cell-free culture supernatant (CFCS). After filtering the solution to ensure the absence of cells, the inhibitory effect of the supernatant of the isolates against selected bacteria including *Staphylococcus aureus*, *Bacillus cereus*, *Shigella flexneri*, *Pseudomonas aeruginosa* and *Uropathogenic E.coli* was investigated. At first, from active culture of the selected bacteria in Mueller Hinton agar, dense culture was given in three directions in the agar medium, and then wells with a diameter 6 mm were created on the agar and at certain distances from each other. In order to avoid the inhibitory effects of the acidic pH of the supernatant of the isolates, 100 µl of the supernatant solution of the treated isolates (neutralized by sodium hydroxide 1 N at pH 6.5) was added to the first well and the untreated supernatant was added to the second well (control). In order to check the inhibitory effect of the existing hydrogen peroxide, the remaining solution was treated with catalase enzyme (5 mg/ml) and then it was neutralized with sodium hydroxide 1 M to remove the effect of organic acids, acidic pH and hydrogen peroxide, and 100 µl of it was added to the third well. MRS broth without supernatant was used as a negative control for the fourth well. In order to better diffusion the solution inside the wells and inhibit microbial growth before the solution penetrates into the agar, the plates were placed at 4 °C for two hours, and after 24 hours of incubation at 35 °C, the diameter of the growth inhibitory halo of each strain was measured in millimeters and the results were recorded. In order to reduce the error, each test was repeated three times. The diameter of the inhibitory zones of the growth (after deducting the diameter of the well) as weak, + (≤14 mm), moderate, ++ (15-19mm) and strong, +++ (≥20mm) was recorded for each isolate (Reuben et al., 2020). MRS growth broth alone was used as a negative control. For further confirmation, a swab was taken from zones of bacterial growth inhibition (halo) and inoculated in the MRS agar medium. Depending on growth, bacteriostatic and bactericidal activity was defined. As, the growth of the reagent organism was considered as bacteriostatic activity and the lack of growth was considered as bactericidal activity.

*2.9. In vitro safety assessment of Lactobacillus strains*

i. Antibiotic susceptibility of *Lactobacillus* isolates against selected antibiotics

Antimicrobial resistance of isolates to selected antibiotics (Padtan Teb co., Iran) including ampicillin (10 µg), vancomycin (10µg), gentamicin (10µg), clindamycin (20µg), tetracycline (30 µg) and streptomycin (10 µg) was done by agar disk diffusion method. In summary, the 100 µl of the active growth broth of each isolate was uniformly inoculated on a lawn of bacteria seeded on the surface of an agar medium and then, the plates were placed in the refrigerator for antibiotic diffusion for 30 minutes. The plates were incubated at 37 ° C for 24 hours. The results were compared with the standard values of CLSI (CLSI-2018). Growth inhibitory diameter was measured by caliper and the results were recorded as resistance (≤15 mm), semi-sensitive (16-19 mm) and sensitive (≥20 mm) (23-26).

ii. Hemolysis Test

From the 24-hour growth broth of isolates were cultured on blood agar containing sheep blood (5%) and after 24 hours of growth at 37°C, the type of hemolysis reaction was assessed. Isolates without hemolysis were used in further experiments.

### *2.10. PCR-based genotyping of isolated Lactobacilli*

For the molecular confirmation of *Lactobacilli* , DNA extraction was initially done using kit (Karmania Pars Gene, KPG) and then replication of fragment approximately 1700 bp of 16SrRNA gene region using primers prepared in PCR reaction with the thermocycler (BioRad -USA) according to a specific time and temperature schedule was performed (Table 3). Universal primers were designed with Oligo 7 software (Table 4). The reaction mixture of a 20-µL final volume analyzed using conventional PCR technique with 10 µl master mix (Ampliqon, Denmark), 1 µl forward primer, 1 µl reverse primer (SinaClon company product), 3 µl from DNA and 5 µl ddH2O. After PCR completed, standard DNA Marker (Ladder 2kb, product of SinaClon, Iran) was used to determine the sample band size by Agarose gel electrophoresis. The bands were then seen by Bio-Rad's gel documentation system.

### *2.11. Sequencing and drawing the phylogeny tree*

Among the four probiotic isolates, only two clear bands were seen in PCR. After PCR, 50 µl of the product along with 16SrRNA 1700 bp primers designed with MEGA 7 software were sent to Pishgam Corporation for sequencing by maintaining the cold chain. A phylogenetic tree was also drawn using MEGA 7 software for the 16srRNA sequence (Figure 1).

### *2.12. Statistical analysis*

The tests were performed in three replicates and the data obtained by statistical software of Minitab ver.16 and GraphPad Prism ver.9 are shown as mean ± standard deviation ($P<0.05$).

**Table 3.** PCR temperature and time schedule

| Number of cycles | Time | Temperature | |
|---|---|---|---|
| 1 | 5 min | 94°C | Initial Denaturation |
| 35 | 40 sec | 94°C | Denaturation |
| | 45 sec | 57°C | Annealing |
| | 45 sec | 72°C | Extension |
| 1 | 5 min | 72°C | Final extension |

**Table 4.** Primer sequence of 16SrRNA gene

| Gene | Primer | Sequence (5′ to 3′) |
|---|---|---|
| 16srRNA | Forward | 5-`AGAGTTTGATCCTGGCTCAG-3` |
| | Reverse | 5`-CTAGTACCAAGGCATTCACC-3` |

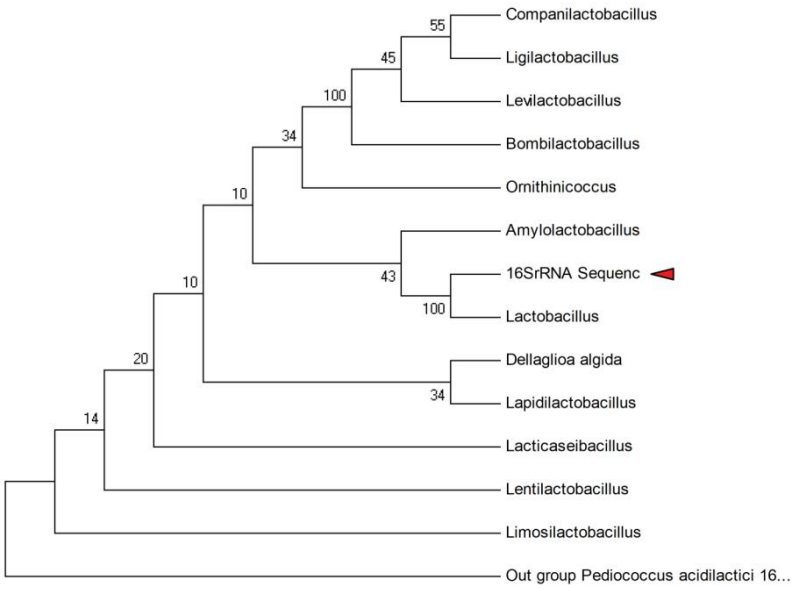


**Figure 1.** Phylogeny tree image, showing the relationship between the 16SrRNA gene sequence of the Lactobacillus isolate and the reference sequences in the GenBank

## 3. Results

### *3.1. Microbial findings*

Among the collected samples, four species showed the most similarity to Lactobacilli, according to the phenotypic characteristics and the probiotic potentials. All four isolates were gram-positive bacilli without spores and without movement. Catalase, oxidase, MR-VP, urease and citrate tests were negative. Investigation of carbohydrate fermentation showed no gas production among four isolates. The results of carbohydrates fermentation were somewhat different from each other. Glucose, lactose and sucrose tests were positive in all isolates, but different results were seen in other sugars including mannitol, maltose and trehalose. The result of TSI test for all four isolates was A/A.

The size of the colonies was about three millimeters. Violent colonies were not seen. Growth in broth was uniform for isolates that settled over time, indicating cell death (pH less than 4). At the same time, the sedimentary accumulations were homogeneous. Colonies did not indicate a distinct odor on MRS agar. The colony morphology of the isolates showed similarity to this group despite the variability in the shape and arrangement of lactic acid bacilli and the multiplicity of species and strains and culture conditions. (Figure 2, Table 5). Although the majority of investigated isolates were relatively oxygen tolerant, their proper growth occurred in microaerophilic and anaerobic conditions.

Examining the probiotic properties of the isolates in different salt concentrations (4%, 8% and 12%) showed that the best growth condition was seen in 4% and weaker growth was seen in 8% and 12% and the temperature growth results of the isolates were determined (Figure 3, Table 6). Investigating the pH tolerance (5, 4, 3 and 2.5) among the isolates showed that at pH=5 proper growth was observed and with increasing acidity, growth decreased at pH values of 4 and 3, and at pH 2.5 weak growth was seen. The growth of all isolates was significant (70% to 95%) at pH values (3, 4, 5) and varied from 70% to 83% at pH 2.5 (Figure 4, Table 7).

The tolerance results of the isolates in different concentrations of phenol showed the relatively favorable growth ability of the first and second isolates in the amounts of 0.2%, 0.3% and 0.4% of phenol and relatively weak growth in 0.5%. Other isolates showed better growth only in 0.2% and 0.3% amounts of phenol, but the growth was weak in 0.4% and 0.5% amounts.

**Table 5.** Bacteriological and biochemical analysis of isolates from dairy samples collected from different geographical regions of Gilan province

| Isolates | Isolate 1 | Isolate 2 | Isolate 3 | Isolate 4 |
|---|---|---|---|---|
| Morphology of colonies | Cream colored, irregular, medium and flat | Cream color, round, medium and raised | Cream color, round, medium and raised | Cream-yellow, round, small and raised |
| Microscopic examination | Long rod shaped | Rod shaped and medium size | Short to medium rod shaped | Rod shaped and medium size |
| Gram coloring | + | + | + | + |
| Catalase test | - | - | - | - |
| Oxidase test | - | - | - | - |
| Indole test | - | - | - | - |
| MR test | - | - | - | - |
| VP test | - | - | - | - |
| Citrate test | - | - | - | - |
| Glucose | + | + | + | + |
| Mannitol | + | + | - | - |
| Maltose | - | + | - | + |
| Sucrose | + | + | + | + |
| Lactose | + | + | + | + |
| Trehalose | - | + | - | - |
| TSI | A/A | A/A | A/A | A/A |

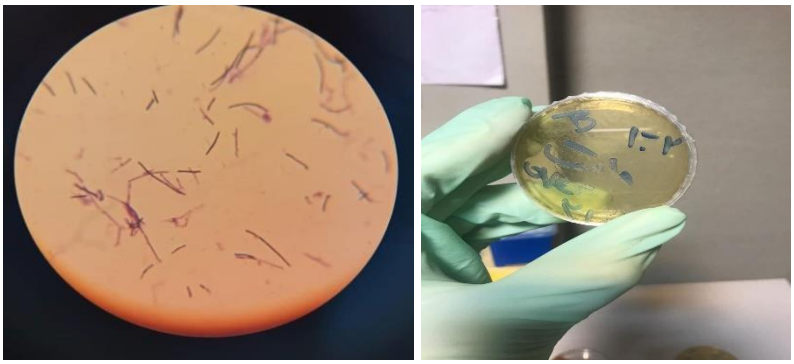

**Figure 2.** Microscopic view and colony of pure isolate of Lactic acid bacteria

The percentage of survival for the first and second isolates in all concentrations ranged from 91% to 68% and for the rest of the isolates from 85% to 61% (Figure 5, Table 8). Bile salt tolerance test showed that all the isolates had relatively good growth in 0.3% amount and a sensible growth reduction was seen gradually in 0.5% and 0.8% amounts. The survival percentage of the first and second isolates varied from 91% to 70%. Other isolates showed weaker growth (85% to 64%) (Figure 6, Table 9). In total, among the four *Lactobacillus* isolates, only isolate 1 was able to survive and show significant growth in pH 3, phenol 0.4%, salt 8% and bile salt 0.8%. The results were calculated using the following formula.

Survival rate :(%) = Biomass (LogCFU/mL) at time (t)/Biomass (LogCFU/mL) at initial time (0) × 100

The samples were incubated at 37°C and retrieved for enumeration at respective end points. The biomass (CFU/mL) of each culture obtained in the assays, made in triplicate, was enumerated on MRS agar incubated at 37°C for 24h (Tables 6, 7, 8 and 9).

**Table 6.** Tolerance of isolated Lactic acid bacteria to temperature and salt concentrations

| | **Sodium chloride concentrations** | | | **Growth rate*** | | |
|---|---|---|---|---|---|---|
| | Optical density (OD) at 620 nm | | | | | |
| Isolates | **4%** | **8%** | **12%** | **15** ˚C | **37** ˚C | **42** ˚C |
| **1** | 1.49 ± 0.41 | 1.39 ± 0.16 | 0.48 ± 0.30 | - | ++ | ++ |
| **2** | 1.44 ± 0.18 | 1.33 ± 0.12 | 0.32 ± 0.48 | - | ++ | ++ |
| **3** | 0.81 ± 0.25 | 0.74 ± 0.19 | 0.11 ± 0.61 | - | ++ | + |
| **4** | 0.75 ± 0.10 | 0.62 ± 0.35 | 0.07 ± 0.53 | - | ++ | + |

*+/ normal growth; ++/ good growth; -/ No growth

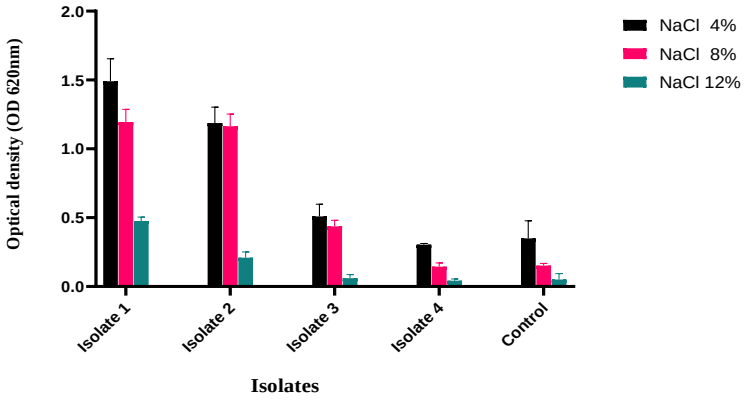


**Figure 3.** The effect of salt concentration on the growth of Lactobacillus isolates

**Table 7.** Survival (Log CFU/mL) and the percentage of survival of Lactobacillus isolates at different pH concentrations

| **Isolates** | **Time (hour)** | **Control (PBS, pH 7.2)** | **pH and percentage of survival** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 2.5 | survival % | 3 | survival % | 4 | survival % | 5 | survival % |
| 1 | 0 | 9.55 ± 0.24 | 7.08 ± 0.30 | 79 | 8.09 ± 0.04 | 89 | 8.14 ± 0.14 | 90 | 8.31 ± 0.03 | 94 |
| | 3 | 9.26 ± 0.18 | 8.91 ± 0.28 | | 0.09 ± 0.06 | | 8.95 ± 0.02 | | 8.87 ± 0.02 | |
| 2 | 0 | 9.55 ± 0.24 | 7.11 ± 0.07 | 70 | 7.29 ± 0.09 | 82 | 8.37 ± 0.06 | 94 | 8.43 ± 0.04 | 95 |
| | 3 | 9.26 ± 0.18 | 8.98 ± 0.01 | | 8.92 ± 0.10 | | 8.90 ± 0.07 | | 8.79 ± 0.04 | |
| 3 | 0 | 9.55 ± 0.24 | 7.19 ± 0.09 | 81 | 7.36 ± 0.21 | 84 | 7.29 ± 0.17 | 82 | 7.64 ± 0.05 | 86 |
| | 3 | 9.26 ± 0.18 | 8.90 ± 0.16 | | 8.79 ± 0.07 | | 8.81 ± 0.12 | | 8.83 ± 0.07 | |
| 4 | 0 | 9.55 ± 0.24 | 7.26 ± 0.03 | 83 | 7.60 ± 0.19 | 86 | 7.65 ± 0.23 | 88 | 8.04 ± 0.05 | 91 |
| | 3 | 9.26 ± 0.18 | 8.80 ± 0.29 | | 8.87 ± 0.01 | | 8.67± 0.07 | | 8.85 ± 0.08 | |

**Table 8.** The survival rate (%) of Lactobacillus isolates at different concentrations of phenol

| Isolates | Time (hour) | Control (PBS, pH 7.2) | Phenol (pH 8) and the percentage of survival | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0.2 | survival % | 0.3 | survival % | 0.4 | survival % | 5 | survival % |
| 1 | 0 | 9.70 ± 0.16 | 7.24 ± 0.05 | 91 | 7.13 ± 0.05 | 86 | 8.55 ± 0.01 | 72 | 8.31 ± 0.03 | 70 |
| | 3 | 9.55 ± 0.18 | 7.98 ± 0.01 | | 8.29 ± 0.01 | | 6.14 ± 0.02 | | 8.87 ± 0.02 | |
| 2 | 0 | 9.70 ± 0.16 | 6.77 ± 0.32 | 91 | 6.82 ± 0.32 | 85 | 8.43 ± 0.33 | 70 | 8.43 ± 0.04 | 68 |
| | 3 | 9.55 ± 0.18 | 7.43 ± 0.33 | | 8.01 ± 0.33 | | 5.87 ± 0.32 | | 8.79 ± 0.04 | |
| 3 | 0 | 9.70 ± 0.16 | 6.55 ± 0.32 | 80 | 6.01 ± 0.32 | 73 | 5.37 ± 0.32 | 64 | 7.64 ± 0.05 | 61 |
| | 3 | 9.55 ± 0.18 | 8.13 ± 0.33 | | 8.22 ± 0.33 | | 8.39 ± 0.28 | | 8.83 ± 0.07 | |
| 4 | 0 | 9.70 ± 0.16 | 6.29 ± 0.21 | 85 | 6.79 ± 0.21 | 80 | 5.69 ± 0.21 | 67 | 8.04 ± 0.05 | 65 |
| | 3 | 9.55 ± 0.18 | 7.39 ± 0.16 | | 8.47 ± 0.16 | | 8.51± 0.46 | | 8.85 ± 0.08 | |

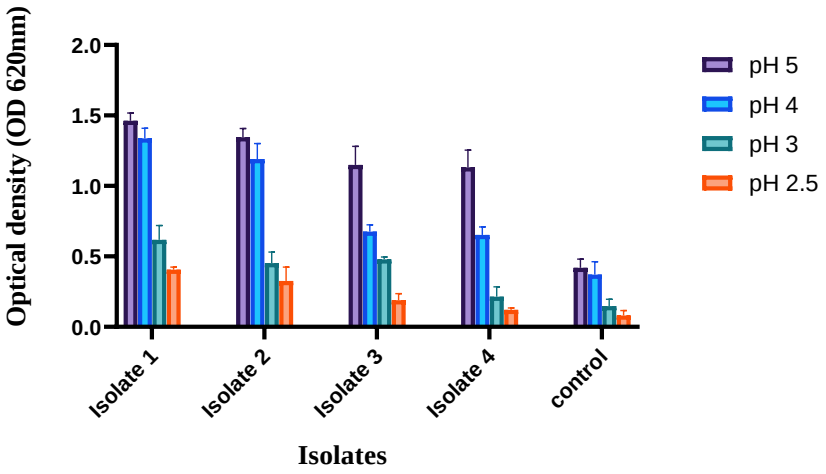


**Figure 4.** The ability to grow Lactobacillus isolates in different pH values

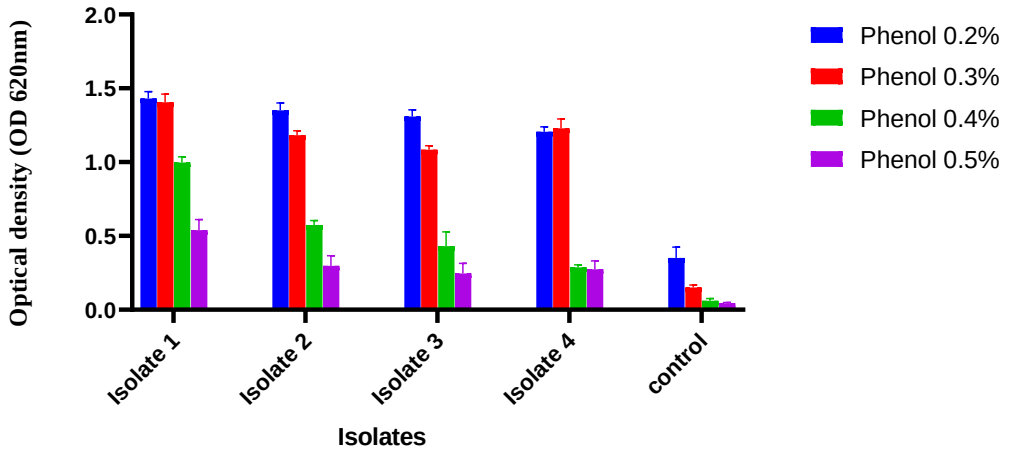


**Figure 5.** Phenol effect on the growth of Lactobacillus isolates

**Table 9.** The survival rate (%) of Lactobacillus isolates in different concentrations of bile salt

| Isolates | Time (hour) | Control (PBS, pH 7.2) | Bile salt (pH 8) and the percentage of survival | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0.3% | survival % | 0.5% | survival % | 0.8% | survival % |
| 1 | 0 | 9.47 ± 0.2 | 7.98 ± 0.01 | 91 | 8.29 ± 0.01 | 86 | 8.55 ± 0.01 | 72 |
| | 3 | 9.02 ± 0.14 | 7.24 ± 0.05 | | 6.93 ± 0.05 | | 6.14 ± 0.02 | |
| 2 | 0 | 9.47 ± 0.2 | 7.43 ± 0.32 | 91 | 8.01 ± 0.33 | 85 | 8.43 ± 0.33 | 70 |
| | 3 | 9.02 ± 0.14 | 6.57 ± 0.32 | | 6.27 ± 0.32 | | 5.87 ± 0.32 | |
| 3 | 0 | 9.47 ± 0.2 | 8.13 ± 0.33 | 80 | 8.22 ± 0.32 | 73 | 5.37 ± 0.32 | 64 |
| | 3 | 9.02 ± 0.14 | 7.17 ± 0.32 | | 6.01 ± 0.32 | | 8.39 ± 0.28 | |
| 4 | 0 | 9.47 ± 0.2 | 7.39 ± 0.16 | 85 | 8.47 ± 0.16 | 80 | 5.49 ± 0.21 | 67 |
| | 3 | 9.02 ± 0.14 | 5.69 ± 0.21 | | 5.79 ± 0.21 | | 8.21± 0.46 | |

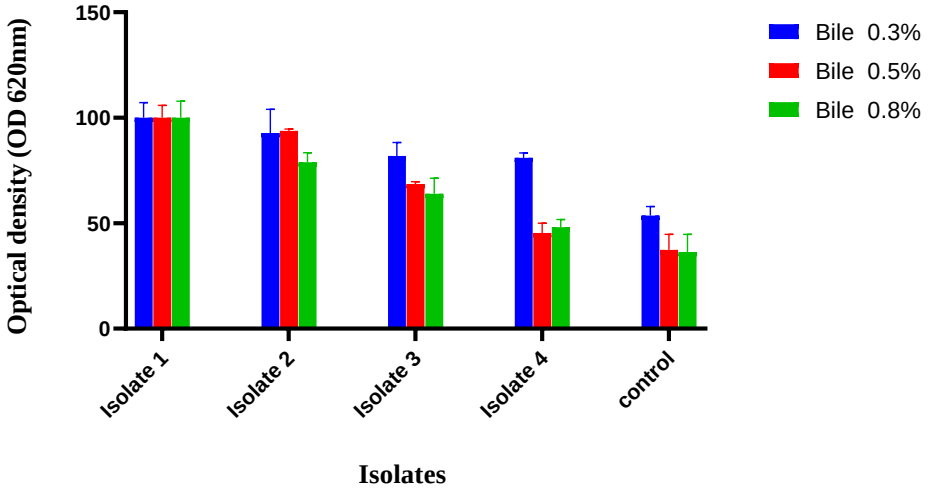


**Figure 6.** Effect of different bile salt concentrations on the growth of Lactobacillus isolates

Examining inhibitory activity of the untreated supernatant of the isolates against the selected pathogenic bacteria indicated that the first isolate against *Shigella flexneri*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and Uropathogenic *Escherichia coli* had a moderate inhibitory effect (inhibitory growth >70%). The second isolate was associated with less inhibitory effects (less than 70% inhibitory growth). In total, the fourth and third isolates, respectively, showed variable but much lower inhibitory power on the tested pathogens (52%

to 68% inhibitory growth). The supernatant of four untreated isolates were not able to inhibit the growth of *Bacillus cereus* (inhibitory growth 42% to 55%). The inhibitory activity observed for cell-free culture supernatant of neutralized isolates was significantly reduced compared to the untreated ones ($P < 0.05$). The inhibitory activity of the treated solution of isolates in the catalase test was similar to that of the solution of untreated isolates (Figures 7 and 8, Table 10). The results of the antibiotic sensitivity screening of the isolates were different with respect to the selected antibiotics, so that the first and second isolates were semi-sensitive to ampicillin and clindamycin antibiotics (16-17 mm) and were resistant to other antibiotics. In contrast, the third isolate was semi-sensitive to ampicillin and tetracycline but resistant to other antibiotics. The fourth isolate was resistant to vancomycin and gentamicin, was semi-sensitive to streptomycin but susceptible to clindamycin, ampicillin, and tetracycline (Figure 9, Table 11). There was no hemolytic reaction in the hemolysis examination of four isolates in blood agar medium.

### *3.2 Genomic findings*

Among four isolates, sequencing analyzes of two selected strains with better probiotic performance at http//www.ncbi.nim. nih.gov/ BLAST were compared and an isolate with the most similarity (96.46 % against 96.43 %) was used for subsequent experiences. The presence of sharp single bands in agarose gel electrophoresis indicated the accuracy of the reaction for the isolates under study. The strain with the highest similarity was *Lactobacillus delbrueckii* (Figures 10 and 11).The genome sequence of Lactobacillus strain delbrueckii KHRS22 16S ribosomal RNA gene, partial sequences available at NCBI GenBank under Sequence Read Archive (SRA) accession number OQ970641.1.

## 4. Discussion and conclusion

Relying on morphological characteristics, it was shown that the isolates were members of lactic acid bacteria, and based on the results of microbiological and biochemical tests, the phenotypic characteristics of *Lactobacillus* species were shown. The results regarding the tolerance of four isolates in different salt concentrations showed that growth was acceptable up to 8% salt concentration and all isolates showed weak growth against 12% salt.

This characteristic has been shown in studies of *Lactobacillus* strains of the human digestive system that tolerate high salt (Gilliland et al., 1984).

This result was consistent with other similar studies from samples of dairy products up to the growth range of 1% salt (Essayas et al., 2020). In contrast, other studies showed that most *Lactobacillus* strains did not grow much in the ranges of 6% and 8% salt (Reale et al., 2015). It can be concluded that the strains isolated in this study are osmophilic. Although salt tolerance is not a characteristic of a probiotic, in the food industry, due to the fact that some food items contain considerable salt, the presence of salt-sensitive strains can be considered an important technical challenge. The growth temperature study among isolates showed that only one isolate was able to grow well at 42 ºC. The growth of other strains was weak or did not grow at this temperature, so it can be concluded that our isolate is an osmo-thermotolerant bacterium and can be used in industrial processes (El-Gendy et al., 1983). Investigation of higher temperatures was not achieved for these isolates. Although a physiological overlap between osmotic stress tolerance and heat tolerance among strains has been shown in studies, but the response to heat stress can have different meanings than the salt tolerance response, or it can reflect the nature of the strain or population heterogeneity. (Lewis et al., 1995).

The results of the survival and growth of the isolates in bile amounts of 0.3%, 0.5% and 0.8% showed that almost all four isolates were able to grow in a concentration higher than the normal concentration of bile in the human digestive system, i.e. 3.5%. But since the first isolate showed good growth at a concentration of 0.8%, the use of such strains can be a suitable candidate in the food industry in cases where longer shelf life of probiotics is considered (Graciela et al., 2001).

**Table 10.** Inhibitory activity of the untreated and treated supernatants of Lactobacillus isolates on selected pathogens in terms of millimeter and percentage of growth inhibition

| **extracts of isolates** / **Organism** | **Isolates 1** | | | **Isolates 2** | | | **Isolates 3** | | | **Isolates 4** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | The percentage of growth inhibition | treated | untreated | The percentage of growth inhibition | treated | untreated | The percentage of growth inhibition | treated | untreated | The percentage of growth inhibition | treated | untreated |
| Pathogenic *Escherichia coli* | 75.0 | 12 | 16 | 62.50 | 10 | 16 | 60.0 | 9 | 15 | 57.14 | 8 | 14 |
| *Shigella flexneri* | 73.33 | 11 | 15 | 68.42 | 13 | 19 | 56.25 | 9 | 16 | 52.94 | 9 | 17 |
| *Staphylococcus aureus* | 78.60 | 11 | 14 | 66.66 | 8 | 12 | 56.25 | 9 | 16 | 68.42 | 13 | 19 |
| *Pseudomonas aeruginosa* | 76.92 | 10 | 13 | 57.89 | 11 | 19 | 57.14 | 8 | 14 | 58.82 | 10 | 17 |
| *Bacillus cereus* | 55.60 | 10 | 18 | 42.10 | 8 | 19 | 50.0 | 8 | 16 | 47.05 | 8 | 17 |

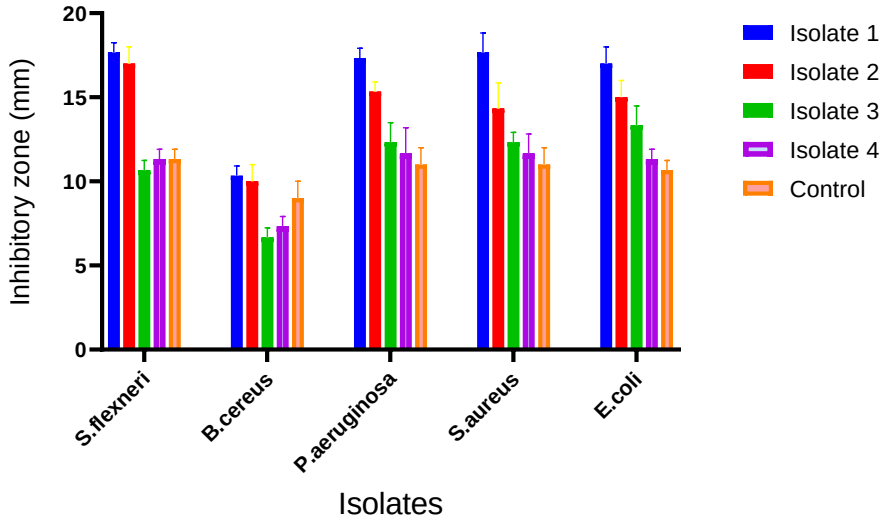


**Figure 7.** The inhibitory effect of the untreated supernatant of Lactobacillus isolates against selected pathogens

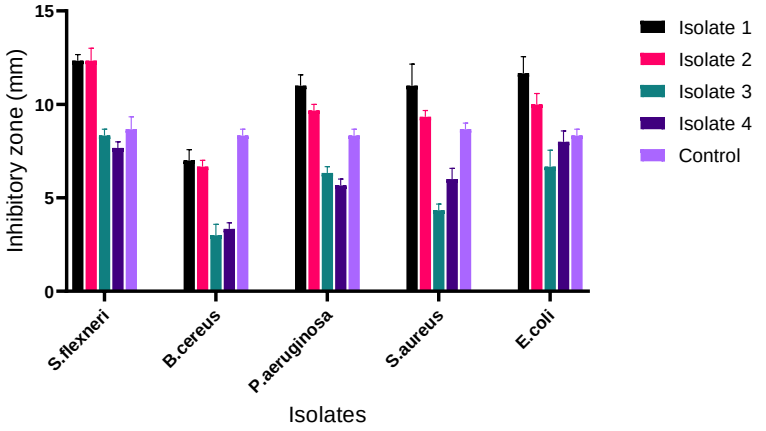


**Figure 8.** The antibacterial activity of the treated supernatant of Lactobacillus isolates against selected pathogens

**Table 11.** Antibacterial susceptibility profile of regional yoghurt Lactobacillus isolates to commercial antibiotics*

| Antibiotic / Organism | Ampicillin | Vancomycin | Streptomycin | Gentamicin | Clindamycin | Tetracycline |
|---|---|---|---|---|---|---|
| | Mean zone diameter (mm) [1] | | | | | |
| Isolate(1) | 16.7[1] | 13.3 | 14 | 14 | 16.3 | 15 |
| Isolate(2) | 16.3 | 11.7 | 13.3 | 14.3 | 17 | 14.7 |
| Isolate(3) | 16.7 | 11 | 14.3 | 14 | 13.3 | 16.3 |
| Isolate(4) | 18.3 | 11.7 | 16 | 13 | 16.7 | 18.7 |

* Minimum Inhibitory Concentration (MIC) breakpoint is defined for each bacterial isolate by CLSI-2018.

[1] Diameter of inhibition zone, converted to the nearest whole millimeter, the mean of three readings.

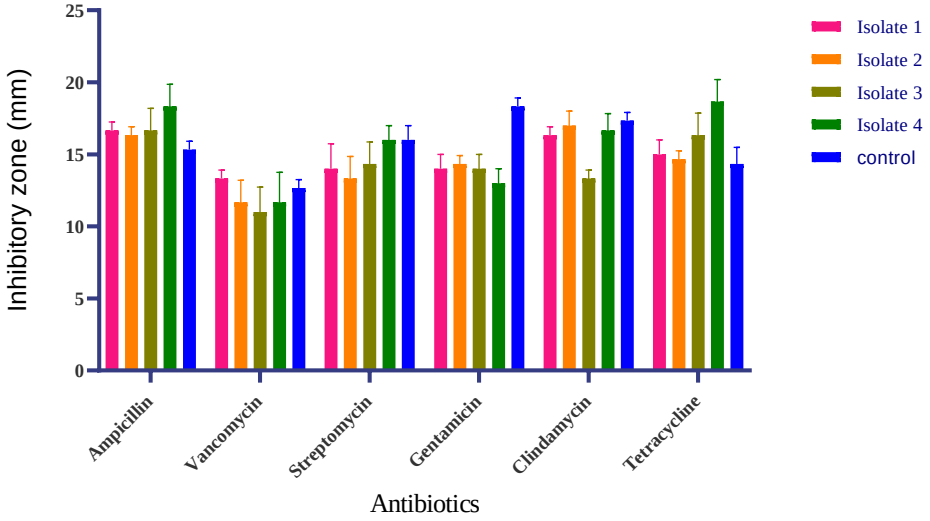


**Figure 9.** Antibiotic sensitivity pattern of indigenous Lactobacilli isolated from dairy products

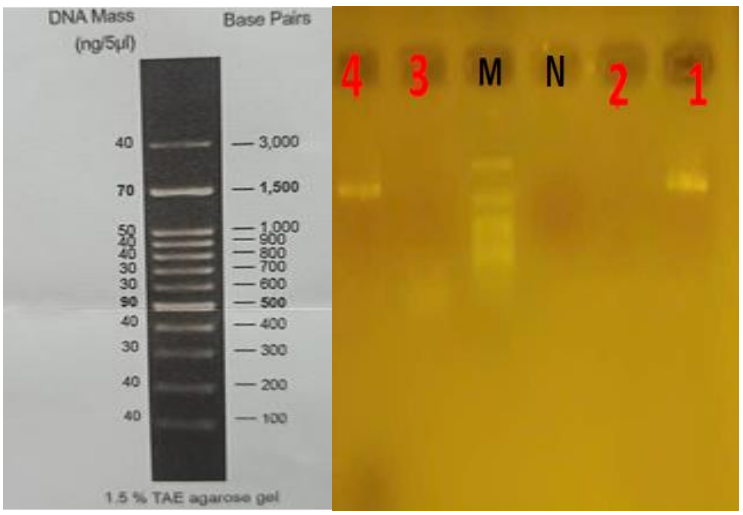


**Figure 10.** Display of bands of two isolates on agarose gel of PCR product (columns 1 and 4) M; Molecular marker, N; Negative control and 2kbp ladder display

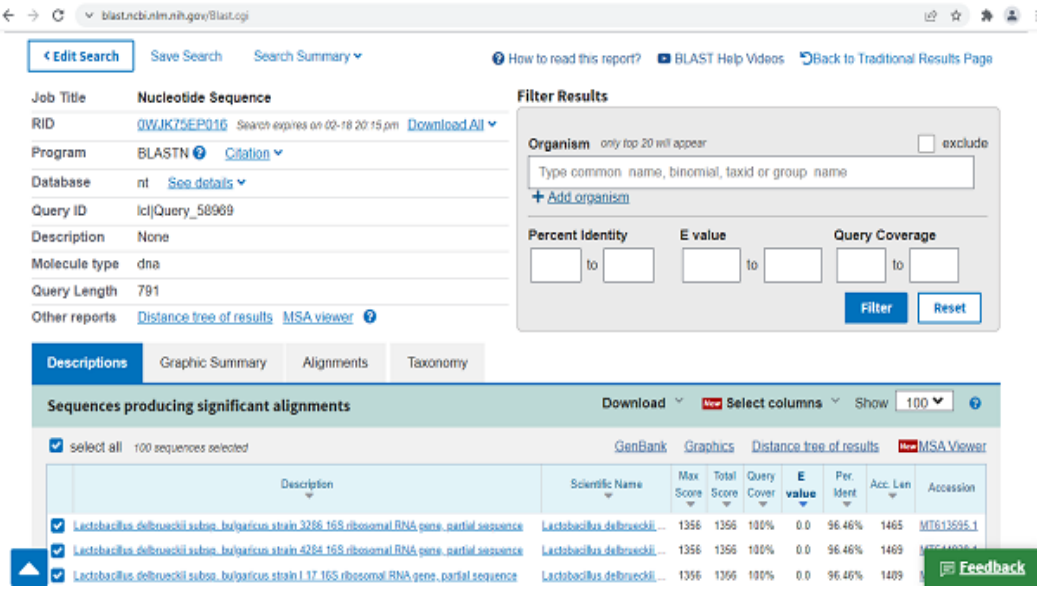


**Figure 11.** Blast sequencing result of 16SrRNA in NCBI database

The examination of research isolates regarding tolerance in different pH concentrations clearly showed acceptable growth at pH 4 and pH 5 values, but actually after 72 hours of incubation, very weak growth was seen at pH 3 and no growth was seen at pH 2.5, which is likely due to inhibiting metabolism and reducing growth or cell death. The findings of other researchers were also associated with similar results (Sahadeva et al., 2011). However, other research showed that *Lactobacillus* strains were able to survive and grow at pH 3 during 3 hours of incubation (Prasad et al., 1998). The results of this screening regarding the tolerance of isolates to different concentrations of phenol showed that after 24 hours of incubation at 37 ºC, the first and second strains showed significant growth in two values of 0.2% and 0.3%, but in contrast to phenol 0.5% growth was very weak. The third and fourth isolates both had relatively acceptable growth in concentrations of 0.2% and 0.3%, but no growth was seen in concentrations of 0.4% and 0.5%. Tolerance to phenol is considered another criterion in the selection of probiotics because intestinal microbiota produces this toxic compound in the digestive tract, so that intestinal bacteria can produce toxic substances such as ammonia, indoleamine and phenol by hydrolyzing urea and therefore Lactobacilli should be able to tolerate in this medium. There are many instances of phenol tolerance reported in lactic acid bacteria that were isolated from natural fermented food sources (Ghabbour et al., 2011). The results of culture of isolates on blood agar plate containing sheep blood 5% after 24 to 48 hours incubation at 37 ºC did not show any signs of α or β hemolysis. This result was in agreement with other studies that investigated the hemolytic activity of probiotics and Lactobacilli isolated from traditional fermented products (Halder et al., 2017), so that according to the definition of the European Food Safety Authority, even if the bacteria used in food products provide the required criteria for use in the food industry, the evaluation of their hemolytic activity is strongly suggested (Guido Rychen et al., 2017). The results of this research indicated that in the tests of probiotic action, especially the tolerance to bile and acidity, the native strains of the region had higher probiotic potential than the control strains ($P<0.05$). In general, the investigation of the inhibitory properties of lactic acid bacteria obtained from different sources and for different species with different antibiotics in research reports was associated with different results (AlKalbani et al., 2019). The results of Zommiti et al.'s research showed that lactic acid bacilli could not show much effectiveness on gram-negative or even gram-positive pathogens (Zommiti et al., 2018), on the other hand, the results of Bazireh et al.'s research show the importance of the effectiveness of selected strains on microorganisms with broad-spectrum resistance (Bazireh et al., 2020). The reasons for the antimicrobial effectiveness of isolates can be the production of organic acids, bacteriocins, the activity of antimicrobial peptides and other metabolites produced by the organism (Kivanc et al., 2011). Boris et al.'s study on the antimicrobial effectiveness of *Lactobacillus delbrueckii* subsp. *lactis* on the tested bacteria showed the narrow spectrum of function of bacteriocin UO004 in this species (Boris et al., 2001). In contrast, Ogunbanwo et al. showed that bacteriocin produced by *Lactobacillus plantarum* and *Lactobacillus brevis* had broad-spectrum antimicrobial activity against pathogenic bacteria and food spoilage organisms (Ogunbanwo et al., 2003). Smaoui et al.'s research on bacteriocin-producing strains showed that some strains were able to be effective on all gram-positive and gram-negative organisms and pathogenic fungi (Smaoui et al., 2010). Therefore, further investigation of these strains regarding their effectiveness against various pathogens seems necessary, so that the strains may be considered as safe probiotics in vivo through the competitive elimination of some pathogens (Schillinger et al., 1996). In summary, although small concentrations of acetaldehyde, acetone, acetoin and diacetyl can also be produced, the greater antimicrobial efficacy of the untreated supernatant to the treated supernatant solution in this screening can be attributed primarily to the role of lactic and acetic acids in the relative inhibition of microbial growth. This research did not show that hydrogen peroxide in the solution of the isolates had an inhibitory effect, and the reason for this could be the characteristic of the species or strains under investigation. Similar results have been reported in other researches (Arena et al., 2016). In this study, the antimicrobial sensitivity of four isolates against different antibiotics was also evaluated. The choice of these antibiotics was due to their common use in the

treatment of indicator microorganisms during clinical cases. The results of the antibiogram of isolates in this study against 6 selected antibiotics showed that all isolates, especially the first and second isolates, showed the typical intrinsic resistance pattern of Lactobacilli. Although the extent of the inhibitory effects was variable, the phenotypic results of the resistance of isolates cannot be a strong sign in defining a probiotic without examining the pattern of resistance genes, although the inherent resistance of *Lactobacillus* species to vancomycin and to the majority of aminoglycosides, trimethoprim and ciprofloxacin is common, but it is necessary to the MIC values of their resistance or sensitivity, taking into account the MIC range of reference quality control organisms at the national and global level, is regularly updated by working groups due to the increase in resistant strains, in order to confirm the health of probiotics used in the food chain or to prevent transmission and spread of resistance among pathogens and pathogenic opportunistic bacteria. At the same time, resistance to other antibiotics may represent important strain reservoirs for resistance genes which are acquired (Giraffa et al., 2010). In Campedelli et al.'s study, in the investigation of the phenotypic and genotypic evaluation of resistance among *Lactobacillus* species, out of 182 species, the most resistant to trimethoprim, vancomycin, and kanamycin were seen, and the role of mobile genetic elements in transmission potential was also investigated, but due to the small number of resistant isolates in this research cannot be referred it to all lactic acid bacteria (Campedelli et al., 2019). During several decades, a lot of research has been done to find new probiotics that can be valuable in terms of market needs or have multiple health benefits. For this reason, the other goal of this study was to find a new more effective probiotic that can be considered a suitable alternative in preventing or treating common infectious diseases or preventing food spoilage. Also, due to the diversity of species or strain characteristics among probiotics from different sources and the increasing demand of the world market for new and effective probiotics, more research is needed in this regard. Probiotics isolated from the human or animal gastrointestinal tract have certain characteristics that distinguish them from dairy products, for example, digestive types show greater resistance to bile salts and low pH levels than dairy types (Kılıc, 2013). This research showed that osmothermophilic isolates of *Lactobacillus delbrueckii* isolated from the local yogurt of Gilan province can be used as biological preservatives and due to the strain durability of them in food and dairy products as a supplement or preferably from the supernatant of the strain along with a peptide such as lactoferrin, they are effective during the expiration period of the product. Also, maltose fermenting strains in this study can be effective in product quality in the food industry and finally microbial stability (Back, 1988). Considering the harmful effects of chemical preservatives, it can be predicted that in the continuation of this study, more and more detailed research of probiotic strains from local sources along with biochemical investigations of their specific metabolites such as stereoisomers of 2,3 butanediol by MALDI-TOF analysis, diffusion gel, GC/Mass and chromatographic methods in line with biomedical and clinical research, along with prebiotics and symbiotics, can provide a new way to improve human health as much as possible.

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