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# Isolation and characterization of lactic acid bacteria with probiotic potential from traditional fermented special Kurdish cheese (Zhazhi) in Kurdistan region, Iraq

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**ABSTRACT**

Zhazhi cheese is a unique farmhouse traditional fermented dairy of the Kurdistan Region in Iraq for its desired aroma and flavor. Undoubtedly, the lactic acid bacteria (LAB) are the critical factors in developing the aroma, flavor, and texture of Zhazhi cheese but it has not been studied or characterised. LAB has many important nutritional benefits, including increasing the nutritional value of food. Therefore, this research was performed to isolate and identify the potential probiotic LAB from traditional homemade Kurdish cheese. Then, the identified strains were tested to determine their probiotics traits, which include acid resistance, bile-salt tolerance, haemolytic, DNase, hydrophobic, autoaggregation, antimicrobial and antibiotic activities. The isolated five LAB strains comprised Lactobacillus casei, Lactobacillus rhamnosus, Enterococcus faecium, Pediococcus pentocaseus and Lactobacillus helvaticum were recognized as promising and the most potential probiotics for further applications. This is the first report on the direct selection of potentially probiotic LAB from Kurdish special cheese (Zhazhi).

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## Introduction

Lactic acid bacteria are quite widespread and the most beneficial microbes for society. They are beneficial microflora of the gastrointestinal tract (GIT) and are hence generally considered safe that boost digestion and immunity (1). They also participate in fermentation and are the main microflora in fermented foods. The lactic acid fermentation that these bacteria undertake has traditionally been understood and used by humans because they enhance the nutritional value, and bioavailability of essential micronutrients, and possess antioxidant properties (2).

Lactic acid bacteria are regarded as the most significant and diverse group, gram-positive cocci or rods, catalase-negative and capable of producing lactic acid as their primary product, which prevents the spread of pathogens and bacteria that cause food spoilage. However, rod-shaped bacteria like lactobacilli and cocci like streptococci, lactococci, enterococci, pediococci, or leuconostoc. Furthermore, they can secrete a variety of antimicrobial compounds like organic acids and bacteriocins (3). The probiotic properties of lactic acid bacteria have received more attention recently. To determine whether novel LAB isolates are probiotics, several criteria have been applied which include acid resistance, bile-salt tolerance, heat sensitivity, salinity, haemolytic, DNase, and antimicrobial antibiotic activities (4). There has been a great deal of medical and industrial attention in isolating novel probiotic strains with safety features. Probiotics are live microbes that impart the host a health advantage when administered in sufficient quantities via several modes of action and they are often referred to as friendly, healthy, and stable bacteria. Probiotic microorganisms may be isolated, tested, categorized, and characterized from a wide variety of natural substrates (5).

Traditional fermented dairy products are an excellent source of LAB with high probiotic potential that can be isolated. Special Kurdish Zhazhi cheese belongs to the most popular and important traditional raw milk cheeses in Iraq because they have low fat or non-fat contained excess amounts of casein and whey proteins (6). Zhazhi is a semi-hard made from goat and sheep milk, primarily produced by Kurdish tribes. The soft cheese balls that have been pressed are placed in bags made of sheep stomachs. The bags are preserved with a local herb known as the Jaje, which is a member of the thyme (*Thymus capitatus*) family. Other herbs (BeZaw, Kurda, and Kangir) are also added to improve the flavor of Zhazhi. The bags are transported to the low-temperature, high-humidity caves at the top of the mountains where it ripens in goat or sheep skin. The cheese is aged for six to eight months (7). The cheese acquires a unique flavor and aroma as it ages. Due to prolonged and the addition of herbs, ripened Zhazhi cheese has a yellowish color and a very strong, "sharp" aroma. The microflora contributing to the ripening of cheese has a great influence on the quality of the cheese. The metabolic activities of various microorganisms in cheese result in the production of aromatic substances in different cheeses (8).

Although LAB probiotic strains from dairy fermented products have been isolated, examined, and identified by

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numerous researchers (1,2,5,9), none of these researchers has studied special Kurdish cheese Zhazhi in Kurdistan, Iraq. Isolation and identification of these bacteria are essential in both basic and applied research [10]. 16S ribosomal RNA (rRNA) nucleotide base sequences provide an accurate foundation for phylogenetic analysis and identification (11,12).

Consequently, the purpose of the current investigation was to isolate and identify novel LAB from traditionally fermented Kurdish cheese Zhazhi using molecular methods following the phylogenetic tree of the isolated LABs generated based on the partial sequences of the 16s rRNA gene and Assessing the in vitro probiotic potential and qualities of LAB in term of their prospective properties.

## Materials and Methods

### Sample collection and bacterial isolation

Zhazhi cheese was aseptically collected at random from various Kurdistan villages at various intervals, transported to the laboratory in sterile plastic bags, and stored at 4°C until further processing. Calcium carbonate has been used as an indicator for acid-producing strains when interacting with acid, and then a clear zone was observed. One gram or one millilitre of the sample was appropriately diluted and spread on Man Rogosa Sharpe (MRS) agar, which added 0.5% (w/v) CaCO3 were incubated at 37°C for 24-48 hours in anaerobic conditions. Fresh MRS agar plates were used to subculture the colonies that had a clear zone on the plate. For future use, all of the isolates were stored at -20°C in 50% glycerol stocks (13).

### Phenotypic characterization

The morphology of the isolated colonies was examined, and Grams staining and catalase tests were used to make a preliminary identification of the isolates. To qualify for probiotics parameters, only those isolates that were Gram-positive cocci and bacilli that were catalase negative were chosen and tested. Physiological characteristics were also assessed by using different temperatures and NaCl concentrations for growth (14).

### Molecular identification

#### *Genomic DNA isolation*

The nucleic acids of the samples were extracted by kit according to the protocols provided by the manufacturer. A (Thermo Scientific) nanodrop spectrometer was used to measure DNA concentration and purity following isolations. Their purity was between 1.73 and 2.20. DNA samples were stored at - 20 ºC for further use.

#### *16S rDNA gene amplification and sequencing*

Polymerase Chain Reaction (PCR) amplification was performed in a DNA thermal cycler. For PCR, the universal primer pair consisted of the forward (5'- AGA GTT TGA TCM TGG CTC AG-3') and reverse (5'- CGG TTA CCT TGT TAC GAC TT-3') as described by (15). The PCR condition included an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 40 seconds, annealing at 50°C for 40 seconds, and extension at 72°C for 40 seconds, and then final extension at 72°C for 10 minutes. PCR products were analysed by electrophoresis on 1.5% agarose gel at 90 V for forty minutes and visualised under UV light.

Then PCR products were sent to Zheen Hospital Seqlab (Erbil, Kurdistan Region, Iraq) for purification and sequencing. All obtained sequences were compared with known sequences in GenBank using the BLASTN database (https://www.ncbi.nlm.nih.gov/blast) and deposited in NCBI GenBank.

#### *Phylogenetic analysis*

The evolutionary history was inferred by applying the Neighbor-Joining method using MEGA11software.

The tree was computed using the p-distance method which involved 20 nucleotide sequences,5 from the 16S rRNA gene amplified from bacterial isolates and 15which represented the most similar matches from the NCBI GeneBank database. All ambiguous positions were removed for each sequence pair (pairwise deletion option). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed with less than a 50% cutoff. There were a total of 1482 positions in the final dataset.

### Evaluation of potential probiotic and safety characteristics

#### *Acid tolerance*

At an inoculum size of 0.1 percent (v/v), active LAB cultures were inoculated into five-millilitre aliquots of MRS broth that had been pH adjusted with 2 N HCl to 2.0, 4.0 and 6.0. The absorbance values at 600 nm were used to measure bacterial growth after 48 hours of incubation at 37°C. After incubation, an OD600nm value greater than 0.5 indicated acid resistance.

#### *Bile tolerance*

Bacterial growth was measured using absorbance values at 600 nm after overnight cultures from the tested isolates were inoculated into MRS broth that had been prepared with various concentrations of bile salt (0.5, 1.0, and 2.0% w/v) for 24 hours at 37°C. MRS medium lacking bile salt was used as a control.

#### *Phenol tolerance*

Bacterial growth was measured using absorbance values at 600 nm after overnight cultures from the tested isolates were inoculated into MRS broth that had been prepared with (0.1, 0.3 and 0.4%) of phenol for 24 hours at 37°C.

#### *Adhesion and hydrophobicity properties*

Microbial adherence to solvents provides information about the isolates' hydrophobicity and surface properties with minor adjustments. The cultures that had grown overnight were harvested (4000 g for 5 minutes). The cells were then resuspended in 10 mL of PBS pH 7.4 solution after being washed twice. The concentration of the cells was increased to 108 cfu/ml. The cell suspension was then mixed with 3 millilitres of organic solvent (chloroform, xylene, and ethyl acetate). Phase separation was completed, the upper phase was removed, and the absorbance value of the cell suspension was measured at 600 nm after they were vortexed for one minute and left at room temperature for one hour (8). The following equation can be

used to evaluate hydrophobicity:
%Hydrophobicity=(1 - A1/ A0)*100
where the absorbance values at 600 nm before and after organic solvent extraction are A0 and A1, respectively.

***Auto-aggregation***

Auto-aggregation properties were also evaluated to determine the adhesion abilities of isolates. After adjusting the concentration of the cells to approximately 108 cfu/ml, 3 ml of resuspended cells have been added to the test tube, where they were incubated at 37 °C for 24 hours. The cell density was measured by spectrophotometer at 600nm using 1 ml of the upper part of the cell suspension (8).

% Autoaggregation =(1 - A1/ A0)*100

where the initial and final absorbance values at 600 nm, respectively, are A0 and A1.

***Hemolytic activity***

Test cultures were streaked onto blood agar plates containing 5% (v/v) sheep blood and incubated at 37°C for 48 hours in order to evaluate the isolates' haemolytic ability. The plates were examined for the signs of -haemolysis (green-hued zones around colonies), -haemolysis (clear zones around colonies), or -haemolysis (no zones around colonies). For further investigation, non-hemolytic bacterial isolates were chosen (5).

***DNase activity***

Bacterial isolates were streaked on DNase agar medium and incubated for 48 hours at 37°C to check for the production of the DNase enzyme. Clear and pinkish areas around the colonies indicated positive DNase activity (16).

***Antagonistic activity***

The antimicrobial activity of the strains toward pathogens has been evaluated using the agar-well diffusion method. The pathogenic strains were *Escherichia coli*, *Salmonella enteric*, *Staphylococcus aureus*, *Bacillus subtilis* and *Candida albican*. After incubation at 37 °C for 24 hours, wells were filled with 50 L suspension Lactobacillus strains on swabbed plates containing a pathogen suspension. Each experiment was carried out in triplicate (8).

***Antibiotic susceptibility***

Isolates were tested for antibiotic susceptibility using the Kirby-Bauer disc diffusion method. Adjustment of overnight cultures grown in MRS broth to 0.5 McFarland was done. A sterile cotton swap was then used to spread the cells across the Mueller Hinton agar surface. An antibiotic disk was placed on it to allow the diffusion of antibiotics into the medium and then incubated at 37°C for 24 h. The inhibition zone around each antibiotic disk was measured in mm to check the susceptibility of the isolate. The results have been expressed in terms of resistance (below 15 mm), (16–20 mm), or (above 21 mm) (8).

## Results

### Isolation and phenotypic characterization

Physiological and biochemical tests were first performed on a total of 62 bacterial cultures that were isolated from traditional fermented Kurdish cheese samples from various parts of Kurdistan. Twelve LAB were selected based on the different colonies characteristics. All isolates showed a typical appearance of LAB under anaerobic conditions (small, pinpointed, round, matte and white colonies), which exhibited a clear zone and growth on MRS agar supplemented with CaCO3.

All the selected 12 LAB isolates were Gram-positive, cocci, oval and rod-a shaped, and showed negative results for catalase. Cocci isolates were presumptively identified as Enterococcus, Pediococcus and Lactococcus and oval-shaped bacteria as Leuconostoc. The remaining 6 rod-shaped isolates were presumptively identified as the Lactobacilli group as shown in (Table 1). Fifty of the catalase-positive isolates would be eliminated.

All the selected LAB isolates were able to survive at temperature ranges 15, 37, and 45°C but only two isolates MED17 and MED22 had not grown at 15°C and 45°C respectively. They revealed high growth at 4% and 6% NaCl, but only two isolates MED17 and MED22 strain showed no growth at 8% NaCl.

### Molecular identification by 16S rDNA sequencing and phylogenetic analysis of isolates

Molecular identification of the selected isolates was

**Table 1**. Phenotypic characteristics and biochemical ability of isolated LAB strains.

| Isolates | | | | Temperature | | | NaCl | | |
|---|---|---|---|---|---|---|---|---|---|
| | Gram stain | Shape | Catalase activity | 15°C | 35°C | 45°C | 4 | 6 | 8 |
| MED5 | + | Rod | - | + | + | + | + | + | + |
| MED7 | + | Rod | - | + | + | + | + | + | + |
| MED12 | + | Cocci | - | + | + | + | + | + | + |
| MED13 | + | Cocci | - | + | + | + | + | + | + |
| MED16 | + | Ovoid cocci | - | + | + | + | + | + | + |
| MED17 | + | Cocci | - | - | + | + | + | + | - |
| MED20 | + | Cocci | - | + | + | + | + | + | + |
| MED22 | + | Rod | - | + | + | - | + | + | - |
| MED26 | + | Rod | - | + | + | + | + | + | + |
| MED31 | + | cocci | - | + | + | + | + | + | + |
| MED32 | + | Rod | - | + | + | + | + | + | + |
| MED34 | + | Rod | - | + | + | + | + | + | + |

(+): growth, (-): no growth.

**Table 2**. Identification of Zhazhi cheese isolates according to the sequencing of the 16S rRNA gene.

| Isolates | Source | Species | Accession number |
|---|---|---|---|
| MED5 | Cheese | Lactobacillus casei | OQ117232 |
| MED7 | Cheese | Lactobacillus rhamnosus | OQ117234 |
| MED12 | Cheese | Enterococcus faecium | OQ130176 |
| MED13 | Cheese | Enterococcus durans | OQ130177 |
| MED16 | Cheese | Leuconostoc lactis | OQ130180 |
| MED17 | Cheese | Streptococcus thermophilus | OQ130181 |
| MED20 | Cheese | Pediococcus pentocaseus | OQ130184 |
| MED22 | Cheese | Lactococcus lactis | OQ130186 |
| MED26 | Cheese | Lactobacillus helvaticum | OQ150531 |
| MED31 | Cheese | Enterococcus faecium | OQ150536 |
| MED32 | Cheese | Lactobacillus plantarum | OQ165193 |
| MED34 | Cheese | Lactobacillus herbarum | OQ253518 |

carried out by amplification and sequencing of their 16 S rRNA gene. The amplified PCR products were purified, sequenced, and aligned using a blast with the published sequences of the 16 S rRNA gene of other strains deposited in NCBI databases. The 16S rRNA nucleotide sequences described in this report have been submitted to NCBI's GenBank database with their accession numbers shown in Table 2. As illustrated in Figure 1 the phylogenetic positions of the potential LAB species.

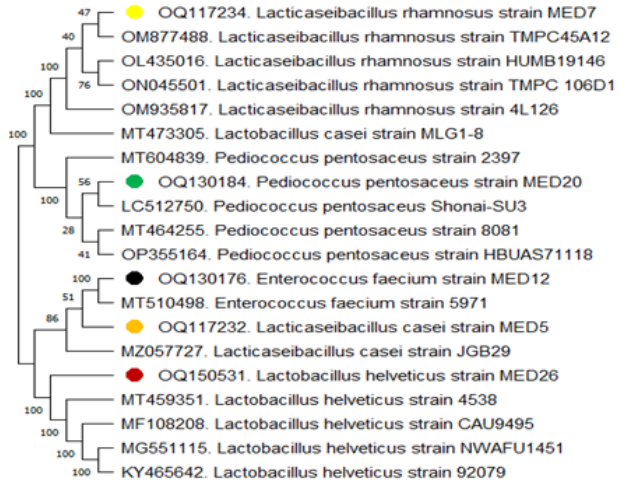


**Figure 1.** Phylogenetic tree based on 16S rRNA gene sequences of the potential strains from Zhazhi cheese. The isolates sequenced in the study are presented in bold type with the GenBank accession.

## Evaluation of the potential probiotic and safety properties of LAB isolates

### *Acid, bile, and phenol tolerance*

As presented in Table 3 indicate that all 12 isolates were having good potential to survive at pH 2, 4 and 6, and were able to survive at 0.5% and 1.0% bile salt, except isolate *Streptococcus thermophillus* not able to grow at 2% bile salt. Also, all the isolates were able to survive at 0.1% and 0.3% of phenol except isolate *Streptococcus thermophillus*, *Lactococcus lactis* and *Enterococcus faecium* not able to grow at 0.4% phenol.

### *Cell surface hydrophobicity and autoaggregation*

Percentages of hydrophobicity of the LAB isolates tested were greatly variable ranging from (13% to 90%) based on the bacterial cell. *Lactobacillus rhamnosus* had the highest hydrophobicity out of all tested isolates, with a value of 90.4±0.25%, while the *Streptococcus thermo-*

**Table 3.** pH, Bile salt and phenol tolerance with Haemoltic and DNAse activity.

| Isolates | pH | | | Bile salt | | | Phenol | | | Haemolytic | DNAse |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 4 | 6 | 0.2 | 1 | 2 | 0.1 | 0.3 | 0.4 | | |
| *Lactobacillus casei* | + | + | + | + | + | + | + | + | + | - | - |
| *Lactobacillus rhamnosus* | + | + | + | + | + | + | + | + | + | - | - |
| *Enterococcus faecium* | + | + | + | + | + | + | + | + | + | - | - |
| *Enterococcus durans* | + | + | + | + | + | + | + | + | + | - | - |
| *Leuconostoc lactis* | + | + | + | + | + | + | + | + | + | - | - |
| *Streptococcus thermophillus* | + | + | + | + | + | + | + | + | - | - | - |
| *Pediococcus pentocaseus* | + | + | + | + | + | + | + | + | + | - | - |
| *Lactococcus lactis* | + | + | + | + | + | - | + | + | - | - | - |
| *Lactobacillus helvaticum* | + | + | + | + | + | + | + | + | + | - | - |
| *Enterococcus faecium* | + | + | + | + | + | + | + | + | - | - | - |
| *Lactobacillus plantarum* | + | + | + | + | + | + | + | + | + | - | - |
| *Lactobacillus herbarum* | + | + | + | + | + | + | + | + | + | - | - |

(+): growth, (-): no growth. Calculated from the initial and final OD at 600 nm during incubation in MRS. Measurements above 0.5 were considered able to resist low pH conditions.

**Table 4**. Hydrophobicity and auto-aggregation ability of isolates.

| Isolates | xylene% | ethyl acetate% | Chloroform% | Autoaggregation% |
|---|---|---|---|---|
| *Lactobacillus casei* | 87.6±0.50 | 65.7±0.20 | 89.3±0.20 | 85.8±0.10 |
| *Lactobacillus rhamnosus* | 82.5±0.10 | 80.9±5.37 | 90.4±0.25 | 92.9±0.20 |
| *Enterococcus faecium* | 76.2±0.40 | 37.6±0.30 | 71.7±0.20 | 81.6±0.30 |
| *Enterococcus durans* | 74.8±0.30 | 23.9±0.20 | 80.2±0.45 | 41.1±0.10 |
| *Leuconostoc lactis* | 55.2±0.10 | 43.1±0.20 | 58.2±0.60 | 46.6±0.20 |
| *Streptococcus thermophilus* | 33.7±0.40 | 29.8±0.10 | 47.4±0.50 | 33.7±0.10 |
| *Pediococcus pentocaseus* | 86.4±0.30 | 30.7±0.10 | 78.3±0.20 | 82.3±0.20 |
| *Lactococcus lactis* | 23.9±0.20 | 13.2±0,20 | 31.1±0.30 | 24.9±0.20 |
| *Lactobacillus helvaticum* | 83.3±0.25 | 63.1±0.30 | 86.4±0.10 | 89.8±0.10 |
| *Enterococcus faecium* | 31.1±0.20 | 18.4±0.20 | 33.3±0.20 | 30.2±0.10 |
| *Lactobacillus plantarum* | 33.2±0.30 | 27.5±0.20 | 30.7±0.20 | 28,8±0.14 |
| *Lactobacillus herbarum* | 64.6±0.15 | 54.7±0.20 | 68.1±0.80 | 78.9±0.30 |

Data are expressed as % of hydrophobicity and auto-aggregation measured after 24 h of incubation. The Values are means of 3 replicates ± SD.

**Table 5.** Antimicrobial activity o the isolates against five pathogens.

| Isolates | *Escherichia coli* | *Salmonella enterica* | *Staphylococcus aureus* | *Bacillus subtilis* | *Candida albican* |
|---|---|---|---|---|---|
| *Lactobacillus casei* | ++ | +++ | +++ | ++ | ++ |
| *Lactobacillus rhamnosus* | +++ | ++ | +++ | ++ | ++ |
| *Enterococcus faecium* | +++ | ++ | ++ | ++ | + |
| *Enterococcus durans* | ++ | + | ++ | + | + |
| *Leuconostoc lactis* | + | + | + | + | - |
| *Streptococcus thermophilus* | + | + | + | - | + |
| *Pediococcus pentocaseus* | ++ | ++ | +++ | ++ | + |
| *Lactococcus lactis* | + | + | + | + | - |
| *Lactobacillus helvaticum* | +++ | ++ | +++ | ++ | + |
| *Enterococcus faecium* | + | + | + | + | - |
| *Lactobacillus plantarum* | + | + | + | - | + |
| *Lactobacillus herbarum* | ++ | + | ++ | + | - |

Sign denotes the degree of inhibition in mm:(-): no inhibition; (+): weak inhibition (<7); (++): moderate inhibition (9–15); and (+++): strong inhibition (>15).

*phillu* strain had the lowest hydrophobicity, with a value of 13.2±0,20%. The adhesion percentages of the isolates for (non-polar solvent) xylene ranged from (23.9±0.20% to 87.6±0.50%). The adherence propensity to ethyl acetate, a strong basic solvent (13.2±0,20% to80.9±5.37%), and chloroform which is a strong acid range between (30.7±0.20% to 90.4±0.25%) as presented in Table 4.

The auto-aggregation range varied from (24.9±0.20% to 92.9±0.20%) after 24 h incubation (Table 4). Isolates *Lactobacillus rhamnosus* had the highest capacity of auto-aggregation after 24 h with 92.9% whilst *Streptococcus thermophillus* had the lowest capacity with 24,9 %.

### *Hemolytic and DNAse activities*

The results revealed no hemolytic(γ-haemolysis) nor DNAse activities, which was confirmed by the "no zone" in the test plates inoculated with all the isolates studied (Table 3).

### *Antagonistic activity*

The microbial activities exhibited by our isolated are presented in Table 6. Antimicrobial activity against five types of pathogens was evaluated. Results showed the maximum diameter of the inhibition zone for isolates *Lactobacillus rhamnosus*, *Enterococcus faecium*, and *Lactobacillus helvaticum* against *Eschericia coli*, and *Lactobacillus casei* against *Salmonella enteric*. Also *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Pediococcus pentocaseus*, and *Lactobacillus helvaticum* against *Staphylococcus aureus*. While the minimum zone of inhibition against *Bacillus subtilis* and *Candida albican.*.

### *Antibiotic susceptibility*

Table 6 displays the resistance spectrum of isolates to seven antibiotics. All the isolates had good resistance to kanamycin, chloramphenicol, tetracycline and gentamicin in various degrees, while the majority of them were sensitive to Amoxicillin, Ampicillin, and Cefotaxime.

## Discussion

In this study, for the first time, the importance of the chosen probiotic bacteria from special Kurdish traditional fermented cheese (Zhazhi) that can withstand the human digestive system and provide health benefits is emphasized. Samples of naturally fermented Zhazhi cheese that can be used to isolate functional probiotic bacteria were gathered, and the probiotic characteristics of various mor-

**Table 6.** Antibiotic susceptibilities (diameters, in mm, of inhibition zones) of the isolated LAB strains.

| Isolates | Amoxicillin (AMX 25) | Ampicillin (AMP 10) | Cefotaxime (CTX 30) | Gentamicin (CN 10) | Chloramphenicol (C 30) | Tetracycline (TE 30) | kanamycin (K 30) |
|---|---|---|---|---|---|---|---|
| *L. casei* | R | S | S | R | R | S | R |
| *L. rhamnosus* | S | S | S | R | S | R | R |
| *Enterococcus faecium* | S | S | R | S | S | R | R |
| *Enterococcus durans* | S | S | S | R | S | S | R |
| *Leuconostoc lactis* | S | R | S | S | S | S | R |
| *Streptococcus thermophillus* | S | S | S | R | S | R | S |
| *Pediococcus pentocaseus* | S | S | S | R | S | R | R |
| *Lactococcus lactis* | S | R | S | S | R | S | S |
| *L. helvaticum* | S | S | R | R | R | S | R |
| *Enterococcus faecium* | S | S | R | S | R | S | R |
| *L. plantarum* | R | S | S | R | S | S | R |
| *L. herbarum* | S | S | S | R | S | S | R |

R resistance, S sensitive.

photypes were identified. Similar to the Greek Kopanisti cheese (17), Kurdish cheese (2, 5), and Irani Lighvan panir (9).

Out of 62 bacterial strains from cheese samples, up to the genus level, 12 isolates were identified and categorized into five distinct genera, which included Lactobacillus, Enterococcus, Streptococcus, Pediococcus and Leuconostoc. All the selected 12 LAB isolates were Gram-positive, cocci, oval and rod-a shaped, and showed negative results for catalase. Cocci isolates were presumptively identified as Enterococcus, Pediococcus and Lactococcus and oval-shaped bacteria as Leuconostoc. The remaining 6 rod-shaped isolates were presumptively identified as the Lactobacilli group as shown in (Table 1).

All the selected LAB isolates were able to survive at temperature ranges 15, 37, and 45°C but only two isolates MED17 and MED22 had not grown at 15°C and 45°C respectively. The ability to resist normal body temperature permits the probiotic to maintain an active metabolism in the gut (14). On the other hand, the capacity to withstand high temperatures reduces contamination and enables a faster rate of growth as well as a higher yield of lactic acid production during fermentation (4). Also, growth at high temperatures was used as a technological feature in industries and the packing of probiotic products.

Sodium chloride tolerance tests revealed that all LAB isolates demonstrated optimal growth as well as persistent osmotic stress at various NaCl concentrations. All strains allowed high growth in the presence of 4%and 6% (wt/vol) salt (Table 1). Moreover, the results indicated that most strains could grow in the presence of 8% (wt/vol) salt but only two isolates MED17 and MED22 strain showed no growth at 8% NaCl (Table 1). LAB is considered to be osmotolerant when it survives in 8% NaCl, allowing them to perform metabolism and produce lactic acid even in the presence of a high salt concentration in the gut. Lactic acid bacteria use various mechanisms for salt tolerance, such as the uptake or synthesis of a limited number of solutes (14). Nevertheless, our results are in line with those of the previous study.

For confirming the isolate strains up to genus level the strains were further identified by 16s rDNA sequence analysis (Genbank accession numbers in Table 2. According to the results of the sequencing, the majority of the strains belonged to the genus *Lactobacillus*, and were divided into five species: *Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus helvaticum,* and *Lactobacillus herbarum.* While the minority populations of other genera, consist of *Enterococcus* (*faecium* and *durans*), *Leuconostoc lactis, Streptococcus thermophillus* and *Pediococcus pentosaceus* indicated that these strains are compatible with the environment of this kind of cheese. The outcomes demonstrated a slight difference in LAB among traditional cheese products. The diversity of LAB species found in dairy products varies by geographic region (18).

The phylogenetic tree of traditional Kurdish cheese showing the relative position of isolated LAB having high bootstrap values, strains with identical sequences were clustered within the same group and were consequently regarded as close relatives which may belong to a novel Lactobacillus subspecies. Phylogenetic identification of the isolates is congruent with the identification outcomes of the 16S rDNA sequencing analysis.

In this study, in order to obtain probiotic LAB, a substantial effort has been devoted to selecting probiotic lactic acid bacteria from Zhazhi cheese relying upon the most essential criterion. Undoubtedly, it is worthwhile that probiotic strains isolating and identifying from fermented food since they are safe and provide a variety of health advantages.

Acid tolerance is an important selection criterion for probiotics. microorganisms used as probiotics must withstand the hostile environment of a human gastrointestinal

tract (GIT) and colonize the intestinal tract like low pH, high concentrations of bile salt, and phenol to provide the promised health benefits and o become active and viable enough through GIT without a decrease in their population, which is crucial for probiotic microbe selection (8). The capacity of putative probiotic strains to survive transit through the stomach is demonstrated by their ability to live in human gastric juice, which has a pH between 1.5 and 2.0 (10,19) In our study, we revealed that all isolates were able to survive at the pH 2, 4 and 6 (Table 3).

Bile tolerance is the second selection criterion for potential probiotics. probiotic-potential LAB isolates should also overcome the stress of bile salts found in bile juice generated by the gallbladder in order to thrive and proliferate the small bowel of the host (18). For probiotic strains, bile salt tolerance is another essential selection criterion to maintain the lipid bilayer integrity of bacterial cell membranes, preserve metabolic activity, and promote colonization of the human GIT (20). The usual concentration of bile salts in the small intestine is roughly 0.2–0.3% but it can rise up to 2% (w/v), based on the host's physiological as well as the kind and amount of food consumed. The probiotic bacteria must be able to tolerate at least 0.3 percent bile (20). As evident from Table 3, all 12 isolates were able to survive at 0.5% and 1.0% bile salt, except isolate *Streptococcus thermophillus* not able to grow at 2% bile salt (Table 3). These findings indicate that most of the isolated LABs from cheese samples under study are bile salt resistant. Despite this, considerable intra-species variability has been observed, indicating that bacterial isolation may affect a species' tolerance to biliary salts (21).

Phenol tolerance is the other selection criterion for potential probiotics. Gut bacteria that de-aminate a variety of aromatic amino acids from the food or endogenous proteins create phenols in the intestines. These phenolic substances can stop probiotic LAB development. As a result, their tolerance for phenol is important to their ability to survive in the digestive system (19). As can be seen from Table 3, all 12 were able to survive at 0.1% and 0.3% of phenol except isolate *Streptococcus thermophillus*, *Lactococcus lactis* and *Enterococcus faecium* not able to grow at 0.4% phenol (Table 3).

Lactic acid bacteria promotes health through a variety of methods, the tendency to adhere to and penetrate intestinal epithelial cells, preventing pathogens from proliferating the body by forming several types of interaction, and this phenomenon maintains the health of the gut environment (22). Several workers have reported that hydrophobicity and aggregation ability refer to phenotypic properties that have been directly related to the adhesion capacity of bacteria (1,5). In this regard, The degree of hydrophobicity and autoaggregation capability of LAB isolates have been evaluated.

The adhesion of microbes to epithelial mucosa is connected to hydrophobic properties of the cell surface that allow for a robust interaction between bacteria and host gut mucosa (4). The non-specific contact between host and bacterial cells is called cell surface hydrophobicity. This property is measured with solvents like xylene, chloroform, n-octane, n-hexadecane, and ethyl acetate (8). This property is thought to have a significant role in influencing the capacity of LAB to attach to intestinal cells and, as a result, their proliferation. Thus, increased hydrophobic qualities imply a greater potential for adhesion and colonization, as well as the capability to compete with other dangerous microbes through competitive exclusion (20).

In our study, three hydrocarbons namely xylene, ethyl acetate, and chloroform —were used to examine various bacterial cell surface features. percentages of hydrophobicity of the LAB isolates tested were greatly variable ranging from (13% to 90%) based on the bacterial cell. *Lactobacillus rhamnosus* had the highest hydrophobicity out of all tested isolates, with a value of 90.4±0.25%, while the *Streptococcus thermophillu* strain had the lowest hydrophobicity, with a value of 13.2±0,20%. The adhesion percentages of the isolates for (non-polar solvent) xylene ranged from (23.9±0.20% to 87.6±0.50%). The adherence propensity to ethyl acetate, a strong basic solvent (13.2±0,20% to80.9±5.37%), and chloroform which is a strong acid range between (30.7±0.20% to 90.4±0.25%) as presented in Table 4. A similar observation was recorded by (1). The majority of the isolates showed high hydrophobicity levels and they could be ideal candidates for probiotic applications.

Probiotic bacteria may form clumps and aggregates, giving them a competitive advantage over pathogenic microorganisms and allowing them to bypass adhesion sites in the human GIT (23). The auto-aggregation test is crucial for determining whether probiotic candidates can adhere to mucosal surfaces or epithelial cells, colonization, and creation of biofilm. The greater auto-aggregation percent indicates a greater capability for cell adhesion in the human gut epithelium (5). In our investigation, the auto-aggregation range varied from (24.9±0.20% to 92.9±0.20%) after 24 h incubation (Table 4). These findings demonstrated that amongst all the isolates, *Lactobacillus rhamnosus* had the highest capacity of auto-aggregation after 24 h with 92.9% whilst *Streptococcus thermophillus* had the lowest capacity with 24, 9 %. The autoaggregation rate of each putative probiotic strain should be greater than 40%. The percentage of our autoaggregation came from the *Ent. faecium* isolates were comparable to (8,23). Also, They found that autoaggregation traits are strain-specific and can vary within taxonomic groupings.

For the selection of probiotics, additional important characteristics need to be progressively screened which are the lack of unfavourable features (virulence factors and transmissible antibiotic resistances) and the safety aspect of probiotic isolates. In this regard, the hemolytic and DNase activity, antimicrobial activity and antibiotic resistance of the isolates were assessed. These activities demonstrate that the test probiotic isolates are not pathogenic and are frequently used to evaluate potential probiotic strains.

In order to be regarded as meeting the standards of international authorities α and β haemolytic activities are thought to be unfavourable to probiotic potential (16). According to our findings, none of the examined isolates after incubation in blood agar plates revealed α or β haemolytic activity, thereby, all the isolates were proven to be no haemolytic (γ-hemolytic) *in vitro*. Similarly, a DNaes activity experiment revealed the absence of DNase enzyme, resulting in DNA hydrolysis was approved which was confirmed by the “no zone” in the test plates inoculated with all the studied isolates **(**Table 3**)**, highlighting the putative safety in agreement with prior research (16,19).

Both probiotic criteria and food safety considerations rely on the antimicrobial activity of isolated strains. By preventing the growth of pathogenic bacteria in their im-

mediate environment, probiotic bacteria provide the host with immunological benefits. Additionally, it has been reported that the antimicrobial activity of LAB may provide an alternative to antibiotic treatments (21).

Antimicrobial substances produced by LAB, such as metabolites, short fatty acids, hydrogen peroxide, carbon dioxide, organic acids, and bacteriocins are attributed to the antimicrobial activity of LAB strains. Bacteriocins typically prevent the growth of Gram-positive infections, whereas organic acids, hydroxyl fatty acids, and hydrogen peroxide are more effective against Gram-negative pathogens (4). It is interesting to note that the acidic environment in the stomach can increase the antibacterial activity of these compounds, thereby competitively eliminating harmful bacterial species from the GIT (3).

In our study, the LAB strains displayed varying degrees of antimicrobial activities against the chosen indicator pathogens ranging from weak (less than 7) to strong (more than 15) as presented in Table 5. The highest inhibitory effects were shown against *Staph. aureus*, followed by *Escherichia coli*, *Salmonella enteric*, *Bacillus subtilis* and *Candida albican* amongst indicator pathogens. These results are consistent with the results of previously published studies (1,5,8). These investigations also demonstrated that antibacterial activity varied by species and strain.

The antibiotic resistance profile of the selected LAB isolates was tested against seven different antibiotics. In this study, most of the isolates were sensitive to Amoxicillin, ampicillin and Cefotaxime, chloramphenicol, and tetracycline in various degrees, while for kanamycin, and gentamicin all isolates were resistant (Table 6**).** Our findings are nearly identical to those of (16). The resistance against a particular antibiotic may be due to the absence of the target site of that particular antibiotic in the LAB cell (24). However, minor deviations from our findings may be related to strain and species variations. Antibiotic resistance or sensitivity alone will not pose a problem in probiotic LAB candidates. The capability to transmit antibiotic resistance-producing genes is the true source of danger. Additionally, It provides information regarding probiotic uses and how the isolate will react to antibiotics.

These LAB isolates can indeed be advised for their positive impact in enhancing gut health, particularly when combined with antibiotics, which can avoid infection produced by other bacteria. Several studies have proven that probiotics with certain antibiotic resistances can aid in the preservation of the microbiota structure by stimulating the immune system, protecting the intestinal barrier, or preventing pathogen colonization (21, 24). Additional investigations are necessary to identify the minimal inhibitory concentration of the evaluated antibiotics and study the molecular characterization of the antimicrobial resistance genes to estimate the possibility to be transmitted. Understanding the mechanism behind antibiotic resistance in new strains may help determine whether antibiotic resistance was acquired or intrinsic.

This study presented in this paper is the first to examine the ability of Kurdish cheese to contain possible probiotic LAB. The results concur with those of other studies. The findings of this study revealed that the isolated five LAB strains comprised *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Enterococcus faecium*, *Pediococcus pentocaseus* and *Lactobacillus helvaticum* were safe with potential properties, including bile salt, acid and phenol tolerance along with outstanding autoaggregation and hydrophobicity abilities, antimicrobial and antibiotic activities that are crucial criterion for them to be considered probiotics. After all these experiments, their significant results demonstrated that these isolates are suitable for use in food and medical industries for the benefit of humans and animals.

These strains may be critical in the formation of special Kurdish cheese taste and flavor. Therefore the strains can be further assessed for possible benefits *in vivo* and used for the development of dairy products exerting multiple health benefits.

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**Interest conflict**
The authors declare that they have no conflict of interest.

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