Probiotic Properties of a Lactobacillus fermentum Isolated from New-born Faeces

Samet Kocabay and Serap Çetinkaya

1 Department of Molecular Biology and Genetics, Faculty of Science and Art, Inonu University Malatya, TURKEY
2 Department of Molecular Biology and Genetics, Science Faculty, Sivas Cumhuriyet University, Sivas, TURKEY

Abstract: Lactic acid bacteria (LAB) have been demonstrated to have roles in many applications, ranging from lowering of cholesterol to immunological development. In this study, Lactobacillus fermentum was isolated from a new-born’s faeces and its genotypic and probiotic characterizations were performed. Our results showed that the survival rate of isolated Lactobacillus fermentum was 39.39% at pH 2 and 81.34% in the stimulated gastric juice at pH 3. It also digested bile salts. Its surface hydrophobicity was found to be 57.59% in n-hexane. These findings indicated that the isolate can be a good probiotic candidate.

Key words: faecal sample, isolate, Lactobacillus fermentum, new-born, probiotic

1 Introduction

Probiotics are described by Food and Agriculture Organization of the United Nations/World Health Organization as "living microorganisms that when consumed with adequate amounts positively contribute to the health of their host through some adjustments in the microbiota of large intestine." Lactic acid bacteria (LAB) are one of the most important group of probiotic bacteria, including Lactobacillus, Lactococcus, Carnobacterium, Enterococcus, Streptococcus, Pediococcus, Propionibacterium, and Lecanostoc. Their applications can be traced throughout the history. They have been used to produce fermented food products such as bread, yogurt, cheese. They also play a crucial role in the establishment of a healthy gut microflora by fighting the pathogens.

LABs are Gram positive, facultative-anaerobe, catalase-negative, and motile organisms. They play an important role in food preservation through the production of antimicrobial agents such as lactic acid, diacetyl, hydrogen peroxide, and bacteriocins. The marketing size of the probiotics has reached USD 46.55 billion in 2020 and is estimated to reach USD 64.02 billion by 2022. Further expansion of the market size depends on the increasing of the global health awareness on probiotic consumption. It is recommended that 100g/d of Lactobacillus acidophilus, Bifidobacterium animalis ssp. lactis, Lactobacillus casei should be consumed per day. Aging and the use of antibiotics are two fundamental factors that reduce the number of probiotics in the large intestine.

LABs also exert some therapeutic properties against some types of cancer. They have been the centre of interest due to their ability to modulate cancer cell’s proliferation and apoptosis. These features can be summed up as bacteriotherapy which has serious implications in the development of alternative therapies to replace harmful chemo- or radiotherapy. Other bacteria also seem to have therapeutic potential against cancer.

This work indicated that the Lactobacillus fermentum isolate can be a good probiotic candidate and that faeces of the breast-fed new-borns could be a rich source for the isolation of good probiotics.

2 Materials and Methods

MRS culture medium, agar (Sigma-Aldrich). Pepsin (Sigma-Aldrich), bile salts (Sigma-Aldrich), trypsin (Sigma-Aldrich), antibiotic discs (Sigma-Aldrich). The other all chemicals are analytical grade.

2.1 Isolation and identification of L. fermentum

L. fermentum was isolated from a faecal sample which had been collected for a previous research and kept at −80°C. An overnight cell culture was obtained by inoculation an aliquot of the sample into 10 mL MRS (1% peptone, 1% meat extract, 0.5% yeast extract, 2% glucose, 0.2% K2HPO4, 0.5% sodium acetate, 0.2% tri-ammonium citrate, 0.02% MgSO4·7H2O, 0.005% MnSO4·4H2O, pH 6.3), and incubated at 37°C. Single bacterial colonies were grown...
from the overnight culture by using the pour-plate method. One hundred of the colonies were then transferred onto MRS-agar plates, containing 0.5% starch. The colonies producing clear zones around them were retransferred into fresh starch-agar media until the isolates were purified. The pure cultures with amylolytic activity were stored at −80°C. For the molecular characterisation, DNA was prepared and used for the amplification of 16S rRNA gene by using the method described in Bulut et al. (2005)\(^{19}\). The sequences obtained from an automatic DNA sequencer were subjected to BLAST analysis and similarities were determined using the National Center of Biotechnology Information databases (http://www.ncbi.nlm.nih.gov)\(^ {20}\).

### 2.2 Survivals of bacterial strain at in low pH

According to our previously published articles\(^ {21, 22}\), the *Lactobacillus* isolates were grown overnight in 10 mL sterile MRS broth (1% peptone, 1% meat extract, 0.5% yeast extract, 2% glucose, 0.2% K\(_2\)HPO\(_4\), 0.5% sodium aceate, 0.2% tri-ammonium citrate, 0.02% MgSO\(_4\cdot7\)H\(_2\)O, 0.005% MnSO\(_4\cdot4\)H\(_2\)O, pH 6.3) with shaking at 110 rpm at 37°C. Ten milliliters of the bacteria were centrifuged for 10 min at 4000 rpm at +4°C. The pellet was suspended in fresh MRS broth (pH 6.3, pH 4, pH 3, pH 2). Bacterial survival at pH 6.3, pH 4, pH 3, pH 2 was determined. Sequential dilution (10\(^{-2}\)-fold) was performed in sterile 4.5 mL NaCl (0.85%). One hundred microliters of the last two dilutions were inoculated by the pour-plate method. The colonies grown were then incubated overnight at 37°C. The colonies growth were counted and CFU/mL was counted and plotted. Survival rate was calculated by using the following formula:

\[
\text{Survival rate %} = \left( \frac{\log \text{CFU N}_1}{\log \text{CFU N}_0} \right) \times 100%
\]

\((\text{N}_1 = \text{Total number of the cells survived after each of the pH treatments}, \text{N}_0 = \text{Total number of alive cells before the treatment})\).

### 2.3 Tolerance to stimulated gastric acid juice

The isolate was treated with stimulated gastric juice\(^ {23}\). Fresh stimulated gastric juice solutions were prepared, including 3 g/L pepsin in 1x PBS at differing pH points (pH 2, 3, and 4). The solutions were filter-sterilized. Cell pellets of a 10 mL bacterial culture were suspended in each of these solutions. Treatment lasted for 5 h. After this, 0.5 mL of the samples were spread on agar plates after a serial dilution step, and cell survival rate was estimated by the number of colonies, which were obtained the following day:

\[
\text{Survival rate %} = \left( \frac{\log \text{CFU N}_1}{\log \text{CFU N}_0} \right) \times 100%
\]

\((\text{N}_1 = \text{Total bacterial number in stimulated gastric juice}, \text{N}_0 = \text{Total bacterial number at first time in stimulated gastric juice})\).

### 2.4 Tolerance to stimulated intestinal juice

The isolate was were examined in stimulated intestinal juice\(^ {24}\). Fresh stimulated intestinal juice was prepared 1x PBS, pH 8, and supplemented with 1 g/L trypsin. The solution was sterilized using the 0.20 nm filters. Bacterial survival rate was monitored for 24 h with 5 h intervals. Survival rate was estimated by using the formula below:

\[
\text{Survival rate %} = \left( \frac{\log \text{CFU N}_1}{\log \text{CFU N}_0} \right) \times 100%
\]

\((\text{N}_1 = \text{Total bacterial number after 24 h in stimulated intestinal juice}, \text{N}_0 = \text{Total bacterial number after 5 h in stimulated gastric juice})\).

### 2.5 Tolerance to bile salts

The bile tolerance of the isolate was assessed as described\(^ {25}\). Ox-bile salt solutions (0.5%, 1%, and 2%) were prepared in MRS broth, pH 6.3. The survival rate was monitored for 4 h with 1 h intervals, and assessed as described above.

### 2.6 Antibiotic sensitivity

Disc diffusion method was used\(^ {26}\). Fresh agar (1.5%) medium was prepared and 100 µL bacterial culture was taken and spread on the agar plates. Antibiotic discs included kanamycin K 30, ampicillin AM 10, streptomycin S 10, tetracycline TE 30, gentamicin CN 30, chloramphenicol C 30, penicillin P 2 units, erythromycin E 15, rifampin RA 5, neomycin N 30, vancomycin VA 30, and a negative control 00. The antibiotic discs were placed on the agar surface. After overnight incubation under the optimum growth conditions, diameters of inhibition zones were measured with a ruler and the measurements were recorded.

### 2.7 Hydrophobicity

Surface hydrophobicity was investigated as described\(^ {27}\). The isolate was grown overnight under the optimum conditions and the culture was divided into 3 mL aliquots in sterile falcon tubes. Cells were pelleted by centrifugation for 10 min at 4000 rpm at +4°C. The supernatant was removed and 5 mL of phosphate-urea-magnesium sulphate buffer, pH 6.5, were added. The pellet was re-suspended and the centrifugation step was repeated for 3 times. The initial cell densities were set to 1 at 450 nm and then 0.6 mL of n-hexadecane, n-hexane, xylene were gently added onto the cell suspensions (3 mL). The mixed solution was placed in a water bath at 37°C and incubated for 15 min. The samples were then vortexed for 2 min and left on the bench for 25 min or until a hydrocarbon layer formed. Absorbance values were recorded at 450 nm. Percent hydrophobicity was calculated by using the formula below:

\[
\text{Hydrophobicity %} = \left( \frac{\text{OD}_{450\text{nm}} \text{N}_0 - \text{OD}_{450\text{nm}} \text{N}_1}{\text{OD}_{450\text{nm}} \text{N}_0} \right) \times 100%
\]

\((\text{OD}_{450\text{nm}} \text{N}_1): \text{the absorbance value for final bacteria con-}

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centration, after the experiment, OD_{initial} N_0: the absorbance value for initial bacteria concentration before the experiment).

2.8 Degradation of Sodium Salts

The isolate was grown on MRS agar overnight at 37°C. Sodium salts, 0.005 g/mL, (sodium glycocholate hydrate, sodium taurodeoxycholate, sodium taurocholic acid) were prepared in MRS agar, pH 6.3. The colonies formed were immersed in the sodium salt solution and left for overnight incubation at 37°C. Salt break-down was checked and the results were recorded.

3 Results

3.1 Molecular identification

A phylogenetic analysis based on 16S ribosomal RNA (rRNA) gene sequence comparison showed that the isolate belonged to Lactobacillus fermentum. The sequence was submitted to GenBank (Accession Number: MT796490) (Fig. 1).

3.2 Survival rate at low pH

Survival rate up to pH 3 was the same as that of at pH 6.3. However, it decreased dramatically at pH 2 in a time dependent manner (Fig. 2).

3.3 Survival rate in artificial gastric juice

Survival rate was 16.57 log(CFU/mL) at pH 2. After the 3rd hour, it decreased to 5.55 log(CFU/mL). In stimulated intestinal juices the survival rate was 1.6 log(CFU/mL) after 24 h at pH 8 (Fig. 3).

3.4 Bile tolerance

The survival rate was 21.9 % at 0.5% bile concentration after 4 h. Higher concentrations, especially 2% led to the death of all the cells within one hour (Fig. 4).

3.5 Antibiotic sensitivity

The isolate showed the relatively high susceptibility to

![Fig. 1 Dendrogram produced by 16S rRNA gene sequence homology (http://www.ncbi.nlm.nih.gov).](image1)

![Fig. 2 Cell survival at low pH points.](image2)

![Fig. 3 Graphical representations of the survival rates in gastric juice.](image3)

![Fig. 4 Survival rates in oxbile salts.](image4)
chloramphenicol (C30), erythromycin (E15), rifampin (RA5), vancomycin (VA30), and tetracycline (30) (Fig. 5).

3.6 Surface hydrophobicity
The isolate displayed 23.96%, 57.59%, and 63.94% in n-hexadecane, n-hexane, and in xylene, respectively (Table 1).

3.7 Sodium salt degradation
The isolate decomposed three of the four sodium salts (Table 2).

4 Discussion
The isolate 14 showed good survival rates at pH 6.3, pH 4, and pH 3. Similar results could be found in the literature. These data suggested that LABs could be influenced negatively by high hydrogen concentration. The presence of abundant protons might interfere with the glucose fermentation and thus with the ATP synthesis.

As probiotics are generally consumed orally, they must be resistant low pH in order to survive through the stomach. Therefore, high survival rates have generally been accepted as the primary criterion. The survival rates in intestinal environment were 28.82%, 58.14%, 30.91% at pH 2, pH 3, and pH 4, respectively. These results were also compatible with those of the literature. The isolate displayed 21.9% survival rate at 0.5% bile concentration after 4 h. In the literature only 0.1% bile concentration have been used and this made the comparison difficult.

Antibiotic sensitivity is another important feature of probiotics as resistance is the negative aspects of all the microorganisms. Horizontal transfer of the resistance genes in the gut flora to the pathogenic bacteria is possible. The tested microorganism showed differing amounts of resistance to the antibiotics used. Lactic acid bacteria are generally known to be sensitive to chloramphenicol and to vancomycin.

Surface hydrophobicity is another important property which determines the capacity for cellular attachment between bacteria and to the mucosal cells of the intestine. Attachment to the epitel cells of the gastrointestinal track renders a survival advantage to the microorganisms. The highest hydrophobicity percentage was obtained in xylene. In other studies, hydrophobicity values have varied greatly, ranging from 16.90 to 96.62%.

Degradation of sodium salts by probiotics is one of the key ways of controlling the cholesterol levels in the host organism. The tested isolate degraded sodium glycocholate hydrate, sodium taurodeoxycholate, and sodium taurocholic acid salts. These data were also compatible with those of the literature.

5 Conclusion
The tested isolate of *Lactobacillus fermentum* appeared the possess many of the desired characteristics of a good probiotic strain. This isolate might deserve *in vivo* studies with laboratory animals in the near future.

### Table 1
<table>
<thead>
<tr>
<th>Surface Hydrophobicity of <em>Lactobacillus fermentum</em></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonpolar solvents</td>
<td>n-Hexadecane</td>
<td>n-Hexane</td>
<td>Xylene</td>
</tr>
<tr>
<td>Hydrophobicity (%)</td>
<td>23.96</td>
<td>57.59</td>
<td>63.94</td>
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</tbody>
</table>

### Table 2
<table>
<thead>
<tr>
<th>Degradation of Sodium Salts by <em>Lactobacillus fermentum</em></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Salts</td>
<td>Sodium glycocholate hydrate</td>
<td>Sodium taurodeoxycholate</td>
<td>Sodium taurocholic acid</td>
</tr>
<tr>
<td>Isolate 14</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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