Selection of potential probiotic Lactobacillus strains from human milk
Hatice Yavuzdurmaz, Sebnem Harsa

Izmir Institute of Technology, Izmir, Turkey (haticeyavuzdurmaz@iyte.edu.tr)

ABSTRACT
Live microorganisms, have beneficial effects on their host’s health, are called as probiotics. There are various possible sources to isolate these bacteria. The most important criterion is being human origin for human applications. In this study; human milk is used as isolation source because of constructive effects on neonates’ health. It is thought that lactic acid bacteria consisted in breast milk are one of the reason of their beneficial effects. The purpose of this study is to search the human milk according to potential probiotic bacteria and investigate the probiotic properties of isolated lactic acid bacteria. 15 different human milk samples were used for isolation of bacteria. Isolates were examined according to their probiotic properties. Low pH resistance, bile salt tolerance and antimicrobial activity assays were chosen for this purpose. Isolates which had these properties were identified biochemically and genetically. Among 60 isolates 2 bacilli showed probiotic properties. These isolates showed resistance to stomach pH (pH 3.0), tolerance against 0.3% bile salt and antimicrobial activity against Salmonella thyphimurium CCM 5445, Escherichia coli O157:H7 NCTC 129000 and Escherichia coli NRRL B-3008. These isolates were biochemically identified and then characterized by using 16S rDNA sequencing. Two lactobacilli, potential probiotic strains, were identified as Lactobacillus oris and Lactobacillus fermentum from human milk origin.

Keywords: Lactobacillus fermentum; Lactobacillus oris; lactic acid bacteria; probiotic; human milk

INTRODUCTION
The term ‘probiotic’ firstly used in 1965 by Lilly and Stillwell to describe substances which stimulate the growth of other microorganisms. The meaning was improved to the closest one we use nowadays by Parker in 1974. Parker defined ‘probiotic’ as ‘substances and organisms which contribute to intestinal microbial balance’. Probiotics are suggested as food to provide for the balance of intestinal flora [1]. Probiotics have been used for long time in food ingredients for human and also to feed animals without any side effects. The excepted major criteria for being accepted as probiotics are resistance to low acidity, tolerance against bile salt and to be originated from human.

The human breast milk has been considered to be an attractive source for potential probiotic strains. After birth, breast milk becomes the best food for infants because it fulfills all required nutrients. Based on the microbiological point, human milk is really an important factor in the initiation and development and of course, for the composition of the neonatal gut microflora since it constitutes a source of microorganisms to the infant gut for several weeks after birth. Although there are limited knowledge about the commensal or probiotic bacteria in breast milk, bacteria commonly isolated from this biological fluid include staphylococci, streptococci, micrococcii, lactobacilli and enterococci [2, 3, 4]. The main scope of this study is to isolate and identify lactobacilli and search the potential probiotic properties of these isolates using breast milk as a natural source originated from human.

MATERIALS & METHODS
Samples were collected from healthy mothers in sterile carriers. Pour plate technique was used to isolate the organisms. Serial dilutions were plated onto Man, Rogosa and Sharp (MRS) agar (pH 6.2 and 5.5), TPY (Trypticase Phytone Yeast) agar (pH 6.5) and MRS-cystein agar (pH 5.5). Plates were incubated anaerobically at 37 °C for 72 h. Gram-positive and catalase-negative rods and coccioid shaped ones were randomly selected. To determine the resistance to low pH, PBS were adjusted to stomach pH which is 3.0 [5]. During incubation at 37 °C, viable organisms were enumerated as cfu (colony forming unit) at the 0, 1st, 2nd, 3rd h by pour plate technique. After determination of resistance to low pH, organisms were examined for tolerance against bile salt. Because, the mean intestinal bile concentration is believed to be 0.3 % (w/v) and the staying time of food in small intestine is suggested to be 4 h [5], analysis were carried out for this
situation. During the incubation period (4 h), viable colonies were enumerated for every hour by pour plate technique. Afterwards, the antimicrobial activity of the isolates was tested against *Salmonella typhimurium* CCM 5445, *Escherichia coli* O157:H7 NCTC 129000 and *Escherichia coli* NRRL B-3008 by spot on lawn method. In brief, after 18 h incubation, active cultures were spotted on the surface of MRS agar plates (2 strains for each plate). Then, MRS plates were incubated based on the growth requirement of cultures. The soft agar inoculated with 1% indicator organisms and overlaid on MRS plates. After the incubation, performed in the appropriate conditions for each indicator organism, inhibition zone diameters were measured. Then, isolates showed potential probiotic characteristics were characterized phenotypically. The biochemical characteristics were determined by analyzing: CO₂ production from glucose, growth at different temperatures (10, 15, 45 °C), growth at different NaCl concentrations (2%, 6.5%), hydrolysis of arginine and fermentation ability of 17 different carbohydrates. After determination of biochemical characteristics genomic DNA was isolated according to the protocol of which is modified from the protocol of Cardinal et al. [6]. Amplification of 16S rDNA region was performed with EGE1 forward primer (5’- AGAGTTTGATCCTGGCTCAG-3’) and EGE2 reverse primer (5’-CTACGGCTACCTTGTTACGA-3’) [7]. The amplification conditions were as follows: 94°C for 5 min (initial denaturation); 40 cycles of 94°C for 1 min (denaturation), 56°C for 1 min (annealing), 72°C for 1 min (elongation); 72°C for 10 min (final extension). The amplification conditions were as follows: 94°C for 5 min (initial denaturation); 40 cycles of 94°C for 1 min (denaturation), 56°C for 1 min (annealing), 72°C for 1 min (elongation); 72°C for 10 min (final extension). Then PCR products were sequenced and analyzed by using the basic local alignment search tool [BLAST, http://blast.ncbi.nlm.nih.gov/].

RESULTS & DISCUSSION

From 200 isolates, 60 isolates remained at the end of the isolation, purification after the loss of unstable isolates during purification and subculturing steps. All of the isolates were gram positive catalase negative rods and cocci. In order to select the most resistant strains to low pH values, PBS buffer adjusted to pH 3.0 was used. All isolates were detected whether they were resistant to pH 3.0 for 3 h, since the digestion process in the stomach completed in about 3 h. Only two bacilli isolates survived in pH 3.0 (Figure 1a) and then these isolates were screened for their ability to tolerate the bile salt. The cfu values showed that both isolates were resistant to bile at this concentration during this period (Figure 1b).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** a) Survival in pH 3.0 and b) Tolerance against 0.3% bile

Afterwards, antimicrobial activity tests were assayed towards *Salmonella typhimurium* CCM 5445, *Escherichia coli* O157:H7 NCTC 12900 and *Escherichia coli* NRRL B-3008 and the diameter of inhibition zones indicated that isolates had antibacterial effect on the indicator microorganisms (Table 1). Both isolates showed much more efficiency on *Escherichia coli* NRRL B-3008.
Table 1. Diameters of inhibition zones

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Salmonella thyphimurium CCM 5445</th>
<th>Escherichia coli NRRL B-3008</th>
<th>Escherichia coli O157:H7 NCTC 129000</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS17</td>
<td>20</td>
<td>41</td>
<td>22</td>
</tr>
<tr>
<td>AS83</td>
<td>18</td>
<td>43</td>
<td>25</td>
</tr>
</tbody>
</table>

After searching probiotic properties, isolates were characterized by physiological and biochemical methods. AS 17 was rod-shaped with rounded end while AS 83 showed coccobacilli morphology (Figure 2).

![Figure 2. Scanning electron microscopic images (a) AS17 (b) AS83](image-url)

According to the biochemical test results AS17 produced gas from glucose but did not produce ammonia from arginine. It tolerated only 2% NaCl and only grew at 45 °C. This isolate gave positive fermentation results with, xylose, ribose, arabinose, melibiose, raffinose, galactose, maltose, sucrose, fructose and lactose. AS83 produced both gas from glucose and ammonia from arginine. It was resistant to 2% salt concentration and grew at 45 °C. This isolate was positive for ribose, arabinose, trehalose, melibiose, raffinose, galactose, maltose, sucrose, fructose and lactose. When these biochemical experiment findings were compared with the literature information (Table 2), it was provided that AS17 was like to be *Lactobacillus oris*; AS83 was like to be *Lactobacillus fermentum* [8, 9].

Table 2. Biochemical test results and literature informations [8, 9]

| Strains | Catalase | Gas from glucose | 2% NaCl | 6.5% NaCl | Growth at 10°C | Growth at 15°C | Growth at 45°C | Glucose | Xylose | Ribose | Melibiose | Mannitol | Trehalose | Melibiose | Raffinose | Galactose | Salicin | Maltose | Sucrose | Mannose | Fructose | Lactose | Rhamnose | Sorbitol |
|---------|----------|------------------|---------|-----------|---------------|---------------|---------------|---------|--------|--------|-----------|----------|-----------|-----------|----------|----------|--------|---------|---------|---------|---------|---------|--------|
| AS17    | -        | +                | +       | +         | +             | +             | +             | +       | +      | +      | +         | +        | +         | +         | +        | +        | +      | +       | +       | -       | -       | -       |
| AS83    | -        | +                | +       | +         | +             | +             | +             | +       | +      | +      | +         | +        | +         | +         | +        | +        | +      | +       | +       | -       | -       | -       |
| *Lactobacillus oris* | -        | +                | nd      | nd        | nd             | +             | +             | +       | +      | +      | +         | -        | +         | d         | +        | +        | +      | +       | +       | -       | -       | -       |
| *Lactobacillus fermentum* | +        | +                | nd      | nd        | nd             | +             | +             | +       | +      | +      | +         | +        | +         | d         | +        | +        | +      | +       | +       | -       | -       | -       |

Symbols: +: 90% or more strains are positive, -: 90% or more are negative, d: 11-89% of strains are positive, w: weak positive reaction, nd: no data available

After determination of biochemical characteristics of the isolates, these two strains were subjected to 16S rDNA sequencing and identified as *Lactobacillus oris* (AS17) and *Lactobacillus fermentum* (AS83) with 100% homology. In this study, two *Lactobacillus* strains were isolated from human milk and characterized on the basis of biochemical and genotypic characteristics. These well characterized strains were also screened for the potential probiotic properties. Survival in the gastrointestinal tract, particularly low pH and bile, is the critical prerequisite for probiotic strains. Most published studies of human milk microflora concentrate on pathogenic bacteria which cause infectious diseases [2]. Furthermore, the importance of the beneficial bacteria
for infants’ health normal flora and the have received little attention. We searched for the lactobacilli strains which might have potential probiotic properties. There are only limited studies about the probiotic potential of lactic acid bacteria isolated from human milk. Martin et al. [4] showed that the natural microbiota of human milk contributes to prevent newborn infections. Bacterial groups commonly isolated from this liquid include streptococci, micrococci, lactobacilli, enterococci and staphylococci. However, the knowledge of being a potential source of probiotic bacteria is very limited [4]. Preciously, Martin et al. [3] investigated the human milk as a source of potentially probiotic lactic acid bacteria. They isolated and identified *Lactobacillus fermentum*, predominately *Lactobacillus gasseri* and *Enterococcus faecium* among the lactic acid bacteria. These species were in use of commercial probiotic products and they were considered as probiotic bacteria [3]. The lactobacilli strains showed variation in the study performed by Heikillä and Saris [2]. 12.5% samples contained lactic acid bacteria namely; *Lactobacillus rhamnosus*, *Lactobacillus crispatus*, *Lactococcus lactis* and *Leuconostoc mesenteroides*. They focused on the inhibitory effects of these isolates on *Staphylococcus aureus* and expressed that all of them were effective against *Staphylococcus aureus*. One of *Lactococcus lactis* strain was also produced nisin, a bacteriocin used as protective agent against bacterial pathogens in the food production [2]. Besides lactic acid bacteria, there are some searches that showed the presence of bifidobacteria known as potential probiotics that may promote healthy microflora, in human milk. Isolated bifidobacteria strains from human breast milk were mostly *Bifidobacterium longum* and the others were *Bifidobacterium animalis*, *Bifidobacterium bifidum*, *Bifidobacterium catenulatum*, *Bifidobacterium breve* and *Bifidobacterium adolescentis* [10, 11].

**CONCLUSION**

The results of this study indicate that human milk may be used as a potential natural source to isolate probiotic lactic acid bacteria. The presence of lactic acid bacteria most probably provides the beneficial effects of this liquid on the newborns’ health. The strains, that were isolated and identified as *Lactobacillus fermentum* (AS83) and *Lactobacillus oris* (AS17) in this study, fulfill the requirement of being used as potential probiotics. They both survive at low pH, resistant to bile salt and have a considerable antimicrobial effect against the tested bacteria. Our results showed similarity on the base of isolation of *Lactobacillus fermentum* from human breast milk with the other isolated strains from this liquid. However this is the first study for the isolation of *Lactobacillus oris* from human breast milk.

**REFERENCES**


