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# Isolation, identification of probiotic Lactobacilli from traditional yogurts produced in rural areas of Mazandaran province (Northern Iran)

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## Abstract

Probiotic microorganisms are naturally present in milk and fermented milk products such as different kinds of yogurt, cheese, butter, etc. Probiotic bacteria are microbial food supplements with beneficial properties on human health. The objective of this study was the isolation of potentially probiotic *Lactobacilli* from traditional yogurt produced in rural areas of Mazandaran province (Northern Iran). In total, 40 samples of traditional yogurt were collected randomly from rural areas of Mazandaran province. The samples were aseptically weighed and homogenized. From each sample, a 1:10 dilution was subsequently made using peptone water followed by making a 10 fold serial dilution. The 0.1 ml from each dilution was then cultured, on MRS agar medium. *Lactobacillus* sp. was isolated from traditional yogurt samples, identified and characterized on the basis of their morphological and biochemical characteristics at genus level. The pure isolated *Lactobacilli* were assessed for various probiotic properties such as tolerance to acidic pH, bile salt, antibacterial activity. Out of the 40 traditional yogurt samples, 15 gram-positive, non-spore-bearing bacilli were identified at the first stage. At the next stages, 5 isolates of acid-stable and bile-stable lactobacilli were identified, including; *Lactobacillus acidophilus*, *Lactobacillus rhamnosus, Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus brevis.*

Key words : Lactobacillus, Antimicrobial activity, Probiotics, Traditional yogurt

## INTRODUCTION

Probiotics are bacterial cultures or living microorganisms upon ingestion in certain quantity promote and enhance health benefits[1]. Probiotic has been recently defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" [2]. Two genera, *Lactobacillus* and *Bifidobacterium*, have been found to be excellent potential sources of bacterial probiotics [3]. Bacteria in this study belong to *Lactobacillus* genus that is highly important in food industry. Their functional properties include, inducing boosting immune system, decreasing gastrointestinal infections, decreasing cholesterol level, cancer and diarrhea. These bacteria could inhibit pathogenic bacteria by producing compounds such as fatty acids, peptidic compounds such as bacteriocin and various types of acids [4, 5]. Probiotics are usually administered orally and are available in various forms, including food products (dairy food), sachets, capsules or tablets. The choice of the format of probiotics has much to do with personal preference, product availability or individual needs. An advantage of food sources of probiotics, such as dairy products, is that they may additionally provide nutrients, such as calcium and protein. However, the shelf life of tablets, capsules and sachets is generally longer than that of dairy food [6]. Investigation on survival of probiotic bacteria in gastrointestinal tract is one of the important factors in selection of probiotic strains. Factors such as tolerance against bile salts, tolerance under acidic conditions and antagonistic activity against pathogenic bacteria were used during this study in order to identify the probiotic potential of the isolated strains [7]. Recently, increased focus has been given to food as potential vehicles of microorganisms with probiotic properties. The nutritious and therapeutic benefits of probiotic microorganisms have been most extensively investigated in dairy products such as milk cheese and yogurt [3]. The aim of this study was the isolation of potentially probiotic Lactobacilli from traditional yogurt produced in rural areas of Mazandaran province (Northern Iran).

## MATERIALS AND METHODS

### Isolation, Identification and Characterization of *Lactobacillus* Sp. from yogurts

A total of 40 different traditional yogurts collected from Mazandaran province. All samples were transferred to the laboratory under refrigeration and stored at 4°C until their analysis. The samples were aseptically weighed and homogenized. From each sample, a 1:10 dilution was subsequently made using peptone water followed by making a 10 fold serial dilution. The 0.1 ml from each dilution was then cultured, on MRS agar medium and incubated in anaerobic conditions at 37°C for 48 h. The characterization *Lactobacillus* sp. was carried out by gram reaction, spore formation, growth at different temperatures and catalase activity. Gram positive, non-sporforming and catalase negative rods were sub-cultured on MRS agar medium at 37°C for 48 h.

### Bile tolerance test

Firstly, the screening for bile tolerance was carried out by growing the isolated *Lactobacilli* in MRS broth containing 0.3% of bile salt for 24 h under anaerobic conditions at 37°C. Culture broths with turbidity more than 0.5 units at 600 nm were classified as bile tolerant strains. These strains were selected for exposure to broths containing higher concentrations of 0.5 and 1.0% (w/v) of bile salt. The survival rate of each strain was expressed as the percentage of viable cells in the presence of bile salt compared to that without bile salt. The experiment was performed in triplicate and the mean values were calculated.

### Acid tolerance test

For the determination of optimal growth and pH of

*lactobacilli*, 1% (v/v) fresh over night culture of *lactobacilli* were inoculated into MRS broth with varying pH ranging from 2.5-8.5. The pH were adjusted with concentrated acetic acid (99%) and 5 N NaOH. The inoculated broths were incubated in anaerobic condition 24 h at 37°C. After 24 h of incubation growth of the bacteria were measured using a spectrophometer, reading the optical density at 560 nm (OD) against the un-inoculated broth. The isolated *Lactobacilli* were subjected to primary screening for acid tolerance in MRS broth adjusted to pH 2.5 with 1N HCl for 90 min at 37°C. The determination of survival was performed by single streaking on MRS agar plates, and the growth was observed after 24-48 h after anaerobic incubation at 37°C. Isolates which were growing on the agar were considered to be acid tolerant strains. These strains were selected and cultivated in MRS broth under anaerobic atmosphere at 37°C. Cultures ($10^7$$10^8$ cfu/ml) were inoculated in 10 ml of 0.05 M sodium phosphate buffer adjusted to pH 2.0, 3.0, and 7.0 with 1 N HCl. Samples were incubated at 37°C for 2 h.

**Antimicrobial activity**

Antimicrobial activity of all isolated lactobacilli species against indicator bacteria was determined by the agar diffusion method, *Staphylococcus aureus* PTCC 1431, *Salmonella enterica* PTCC 1231, *Shigella dysenteriae* PTCC 1188 and *Escherichia coli* PTCC 1399, were used as indicator bacteria. Supernatants of lactobacilli species were monitored for antibacterial activity against indicator bacteria inoculated on nutrient agar. A volume of 100 µl of cell free supernatants was filled in 8-mm diameter sealed wells cut in the nutrient agar. The inoculated plates were incubated for 24 h at 37°C, and the diameter of the inhibition zone was measured with calipers in millimeters.

## RESULTS

In the present study, the isolated lactobacilli were tested for resistance to bile salt, acidic pH, as well as their ability to inhibit pathogens and survival in different storage conditions. All isolates were catalase-negative, gram positive and oxidase negative rods producing no gas from glucose. Based on survival rate of the isolates under acidic conditions, 4 strains were designated as susceptible, 6 as moderarate tolerant and the 5 remaining strains as good tolerant. Based on tolerance of species to bile salts they were classified as four groups including tolerant strains (equal delay growth or less than 15 minutes), highly tolerant strains (delay growth between 15 to 40 minutes), poorly tolerant strains (delay growth between 40 to 60 minutes) and susceptible strains (delay growth more than 60 minutes) [8]. So,

**Table 1.** Identification of Lactobacillus sp. based on biochemical and morphological tests

| Test parameters | Results | | | | |
|---|---|---|---|---|---|
| | *L. plantarum* | *L. rhamnosus* | *L. casei* | *L. acidophilus* | *L. brevis* |
| Gram Stain | + | + | + | + | + |
| Catalase | - | - | - | - | - |
| Oxidase | - | - | - | - | - |
| Growth at 15°C | + | - | - | - | + |
| Growth at 45°C | - | - | + | + | - |
| Growth at 15 and 45 °C | - | + | - | - | - |
| Motility | - | - | - | - | - |
| NH3 from arginine | - | - | - | - | + |
| Mannose fermentation | + | + | - | + | - |
| Fructose fermentation | + | + | + | + | + |
| Trehalose fermentation | + | + | - | - | - |
| Lactose fermentation | + | + | + | + | + |
| Arabinose fermentation | - | + | - | - | + |
| Sucrose fermentation | + | + | + | + | + |
| Xylose fermentation | + | - | - | - | + |
| Galactose fermentation | + | + | - | + | + |

**Table 2.** Inhibition of tested bacteria by Lactobacillus sp. isolates by agar diffusion method.

| Strains | Isolate no. | Zone diameter (mm) | | | |
|---|---|---|---|---|---|
| | | *S. aureus* PTCC 1431 | *S. enterica* PTCC 1231 | *E. coli* PTCC 1399 | *Sh.dysenteriae PTCC 1188* |
| *L. plantarum* | R3 | 11 | 12 | 13 | 12 |
| *L. rhamnosus* | R6 | 9 | 10 | 11 | 10 |
| *L. casei* | R8 | 8 | 9 | 11 | 11 |
| *L. acidophilus* | R11 | 10 | 11 | 12 | 12 |
| *L. brevis* | R13 | 8 | 10 | 10 | 9 |
| Mean±SD | - | 9.2±1.16 | 10.4±1.01 | 11.4±1.01 | 10.8±1.16 |

based on this classification 2 strains were designated as tolerant, 3 as highly tolerant, 6 as poorly tolerant and 4 as susceptible. Finally, only 5 out of 15 strains with a potentially good capacity of resisting acidic conditions and tolerating bile salts were selected for final identification. These isolates were identified as *Lactobacillus acidophilus*, *Lactobacillus rhamnosus, Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus brevis* by observing their colony morphology, physiological and as well as some biochemical characteristics. Their characteristics have shown in Table 1. A total of 5 *Lactobacillus* sp. were tested for their antimicrobial activity against *Staphylococcus aureus* PTCC 1431, *Salmonella enterica* PTCC 1231, *Shigella dysenteriae* PTCC 1188 and *Escherichia coli* PTCC 1399. The antimicrobial activity of *Lactobacillus* sp. are given in Table 2. All of *them* exhibited antimicrobial activity against tested bacteria. Moreover, *L. plantarum* had highest antimicrobial activity against to tested microorganisms. In general, bacteria with an average inhibition power of 10.45 mm produced a good capacity for inhibiting of pathogenic bacteria.

## DISCUSSION

Produced yoghurts in traditional way, are carriers of potentially probiotic bacteria, which their addition to industrial products increase their features and enhance marketability. These bacteria play an important role in human health by improving gastrointestinal system and boosting immune system. Lactobacilli are a part of normal flora, contained antimicrobial substance that has inhibitory effect on growth of pathogens. The MRS medium used is selective for the isolation of lactobacillus strains since they are extremely fastidious [14]. During this study, 5 strains tolerant to acid and bile salts were isolated from 40 samples of traditional yogurts. In Italy, 63 strains of *Lactobacillus* were isolated from one type of traditional cheese, of which only 3 strains produced a high tolerance against acidic conditions and bile salts. In another study, 6 out of 88 *Lactobacillus* strains isolated from un-pasteurized milk and cheese were tolerant against acidic conditions and bile salts [9, 10]. In the study of Chowdhury et al, four isolates were identified as *Lactobacillus plantarum* based on their growth and biochemical characteristics. The Isolates were resistant to NaCl (1-9%) and bile-salt (0.05-0.3%) and showed good growth in the acidic condition, while maximum growth was observed at pH around 6.0. The isolates were examined for their antibacterial activity against nine different test pathogens and found all pathogens are inhibited their growth to some extent but maximum zone of inhibition was observed against *Bacillus cereus* (53.20 mm) and minimum was against *Staphylococcus aureus* (19 mm) after 72 hour incubation [11]. In the study of Osuntoki et al, *Lactobacillus* spp. isolated from fermented dairy products showed antibacterial activity against some clinically important pathogens such as Enterotoxigenic *E. coli* (4.2 mm), *Salmonella typhimurium* (4.3 mm) and *Listeria monocytogenes* (5.0 mm) [12]. Alvarado *et al*. showed that only 25 out of 94 isolated LAB strains from traditional Mexican foods are able to show inhibition against at least one pathogenic indicator microorganism [13].

## CONCLUSION

According to this study, strains of *Lactobacillus* isolated from traditional yogurts, which were tolerant against acidic conditions and bile salts and had good antimicrobial effects, could be used widely in production of industrial products and native probiotic strains, so contribute to enhance health in the society. At presence, with increasing of the antibiotic resistance and side effects of chemical drugs, it seems, we need to use alternative remedies. Probiotics and their produced metabolites can have therapeutic application in future.

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