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Research Article

Identification of *Lactobacillus plantarum* in Breast Milk

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Abstract

Introduction: The anti-infective effect of early colonization of infants by potentially probiotic lactic acid bacteria in human milk is a growing area of research. *Lactobacillus plantarum* colonization in early infancy may be important to health in later life. Here, we present an investigation into the presence of *L. plantarum* in breast milk from Iranian mothers.

Materials and Methods: Human breast milk samples (n = 40) were randomly collected from lactating and breastfeeding women having undergone full-term pregnancies. Information concerning personal characteristics was collected after birth. The samples were cultured in de Man, Rogosa, and Sharpe medium using the pour plate technique, and isolates were initially identified by biochemical methods. Isolates were established as belonging to the genus *Lactobacillus* based on the 16-23S rRNA region, and the species *L. plantarum* was identified using PCR and primers targeting the recA gene.

Results: In our study, 35 samples (87.5%) contained suspected lactobacilli based on phenotypic tests. Thirty of these (85.71%) were confirmed as containing bacteria of the genus *Lactobacillus* using a genotypic method (PCR), all of which were found to be *L. plantarum*.

Conclusion: Probiotic bacteria in a mother's breast milk may have positive effects on her infant's health. This insight creates new perspectives concerning the use of breast milk as a source of probiotic bacteria for bacteriotherapy.

Keywords: Breast milk, Breastfeeding, Lactobacilli, *Lactobacillus plantarum*, Probiotics

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1. Introduction

The nutritional qualities of breast milk have developed since the divergence of mammals [1]. Breast milk includes several functional nutrients that facilitate the creation of a microenvironment optimized for gut development and maturation [2, 3]. Some of these components, including regulatory cytokines and growth factors, also exert a protective effect. In addition, breast milk contains several variable components, such

as lysozyme, lactoferrin, and oligosaccharides, which contribute to the prevention of infections and support the growth of beneficial bacteria in the gut. Furthermore, breast milk represents a continuous supply of microbes, factors favoring their growth, and components that regulate host-microbe interactions. These properties emphasize one of the key functions of breastfeeding in conferring protection on newborn children during a critical period in their life, when breast milk is their principal source of nutrition and their own immune defenses, such as the integrity of the gastrointestinal tract (GIT) barrier, are immature [4–7].

It has recently been accepted that consumption of breast milk is a means by which newborns obtain microorganisms that may colonize their gut and modulate its function 8-12. Several bacteria predominate in human breast milk, including staphylococci, streptococci, micrococci, lactobacilli, enterococci, lactococci, and bifidobacteria [8, 13, 14]. These bacteria, which are present in the breast, originate from the maternal intestines and reach the mammary glands through an endogenous route, via macrophages and dendritic cells [15, 16]. Thus, breastfeeding may be a significant source of lactobacilli, along with other probiotic species, for the newborn gut. The *Lactobacillus* genus contains more than 25 varieties of gram-positive, catalase-negative, and rod-shaped bacteria, which constitute less than 1% of all intestinal bacteria in adults. However, members of this genus make up a greater proportion of the neonatal and infant intestinal microflora (between 10^5 and 10^8 CFU/g of feces) [17, 18]. The lactobacilli species isolated from breast milk to date are *L. gasseri*, *L. rhamnosus*, *L. acidophilus*, *L. plantarum*, *L. reuteri*, *L. fermentum*, *L. animalis*, *L. brevis*, *L. helveticus*, *L. oris*, *L. casei*, *L. gastricus*, *L. vaginalis*, *L. crispatus*, and *L. salivarius* [8, 14, 16, 19].

There is increasing evidence that some probiotic strains exert preventive and/or curative effects on a range of infections and inflammatory gastrointestinal infectious diseases [20–22]. The survival capacity (acid and bile salt tolerance, survival in a simulated GIT, pathogen inhibition, antibiotic susceptibility, and exopolysaccharide production) and probiotic properties (reduction of pathogen adhesion and protection of Caco-2 cells from the effect of sodium dodecyl sulfate and inflammatory stress) of a specific strain of *Lactobacillus plantarum* isolated from human breast milk have been investigated in previous work [23]. Lactobacilli constitute an essential component of the healthy human intestinal microbiota and are believed to be involved in its control and maintenance [24].

L. plantarum is a versatile lactic acid bacterium with a proven ability to survive gastric transit and colonize the intestinal tracts of humans and other mammals [25]. An increasing number of studies have; addressed the possibility of developing an ingestible living vaccine using *L. plantarum* [26]. Furthermore, work is ongoing to determine the activity of this bacterium, which has previously been found in breast milk from women in other countries, in the human intestinal tract [11, 27, 28]. The consumption of *L. plantarum* has been associated with significant health improvements in humans and other animals [29–32]. In *in vitro* and *in vivo* tests of the beneficial health effects of probiotics, an *L. plantarum* strain was found to have an adequate safety profile, a strong ability to survive in the GIT, and positive effects on hosts [33, 34]. *L.*

plantarum has also been used in human clinical trials for its promotion of the immune system and alleviation of intestinal disorders and cardiovascular diseases [35, 36]. Supplementation with probiotic *L. plantarum* IS-10506 and zinc for 90 days was found to result in a significantly increased humoral immune response, as well as improved zinc status, in young children [37]. Moreover, there is growing evidence that lactobacilli colonization at a very early age may protect infants from developing atopic allergy [38]. In addition, administration of a *Lactobacillus* strain from human milk to infants for 6 months has been shown to lead to 46%, 27%, and 30% reductions in the incidence of gastrointestinal, upper respiratory tract, and total infections, respectively [39]. The aim of the present study was to contribute to the investigation of the presence of beneficial bacteria from healthy sources, such as breast milk, in Iran.

2. Materials and Methods

2.1. Samples and data collection

Breast milk samples (n = 40) were randomly collected from lactating mothers having undergone full-term pregnancies and with breastfed infants aged 3 days to 12 months. All volunteers provided written informed consent to participate in the study, which was approved by the Ethics Committee of Isfahan University of Medical Sciences. Information regarding personal characteristics, dietary habits, and the infants (including details of delivery and early infant feeding) were collected after birth. Data concerning the duration of breastfeeding and infant feeding practices were recorded during interviews [19].

2.2. Clinical evaluation and exclusion criteria (subjects and design)

All volunteers were informed of the aim and protocol of the investigation. Subjects with fever, diabetes, infections, metabolic disease, gestational hypertension, diseases of the breast or central nervous system, malnutrition, maternal allergy, or addictions and those drinking alcohol were excluded, as were newborns with any malformation or cardiac or hemolytic disease. Diseases were diagnosed from subjects' medical histories and by physical examination. All participants were healthy and without any infant and/or maternal perinatal problems. Only healthy women who had not used antibiotics within the 2 weeks prior to the study were included. The participating mothers avoided consumption of any herbal tea and supplements containing lactic acid bacteria (LAB) for 2 weeks before sampling. There were no other restrictions to their normal diet, which may have contained naturally occurring LAB. The mothers were between 18 and 40 years of age. They refrained from breastfeeding 1 h before providing a sample, which was taken in the morning [38].

2.3. Sampling

The nipple and mammary areola were cleaned with soap and sterile water, before application of chlorhexidine (Qiagen, Hilden, Germany). The breast milk sample (10–15 ml) was collected in a sterile tube using sterile gloves. The first drops (approximately 1 ml) were discarded to avoid chlorhexidine contamination. Similarly, a swab from the nipple and mammary areola were obtained to assess the efficacy of the antiseptic treatment. The tubes containing the samples were packed in insulated boxes containing dry ice and sent to the laboratory within two hours [40].

2.4. Isolation and identification of bacteria

Aliquots (1000 µl) of the samples were plated directly for culture. In addition, serial dilutions were plated on 3 culture plates using the pour plate method with de Man, Rogosa, and Sharpe (MRS) medium (Merck, Darmstadt, Germany). This was performed in duplicate, and the medium was supplemented with 0.5% glucose (Merck) and 0.25% l-cysteine (Merck) to select for LAB and favor the growth of lactobacilli. The agar plates were incubated at 37°C under anaerobic conditions (10% H_2, 10% CO_2, and 80% N_2) in a Mac 500 chamber (Mart Microbiology, Lichtenvooorde, Netherlands) for 48–72 h. Suspected LAB colonies were purified by streaking on appropriate media. Colonies were then counted and selected for further tests. The identity of the isolates was confirmed by Gram staining, microscopic examination, and catalase and oxidase reactions. PCR amplification of the genomic DNA of the isolated bacteria was performed to determine the genus and species [41].

2.5. Genomic DNA preparation

Genomic DNA was obtained as follows: a 10-ml overnight culture of bacteria was pelleted by centrifugation (3023 ×*g* for 10 min at 4°C), washed twice in phosphate-buffered saline (pH 7.2), and suspended in 400 µl of lysis buffer (2.5 mg.ml^{-1} lysozyme, 12% polyethylene glycol 20,000, and 10 mM Tris at pH 8). After incubation for 2 h at 37°C, the cells were harvested by centrifugation (as above), and resuspended in 400 µl of 20 mM Tris (pH 8). Cells were lysed by the addition of 40 µl of 10% sodium dodecyl sulfate and incubation for 30 min at room temperature. After adding 55 µl of 5 M NaCl, phenol-chloroform extractions were performed. Chromosomal DNA was precipitated by isopropanol, washed with 70% ethanol, and resuspended in 30 µl of TE (10 mM Tris-HCl and 1 mM ethylenediaminetetraacetic acid) containing 25 µg·ml^{-1} RNase. The extracted DNA samples were stored at -20°C as 1:20 dilutions.

One microliter of diluted DNA was used as a template for PCR. The positive controls comprised DNA extracted from *L. plantarum* subsp. *plantarum* PTCC1745 (prepared by the Persian Type Culture Collection). The negative controls consisted of *Escherichia coli* DNA extracts (prepared by the Department of Microbiology, Faculty of Medicine, Isfahan University of Medical Sciences). DNA extraction was performed as described above [42].

3. PCR Analysis

3.1. PCR amplification of the 16S-23S rRNA region and flanking 23S rRNA gene

To confirm that isolates belonged to the genus *Lactobacillus*, they were selected from MRS medium and subjected to 16S rRNA gene sequence analysis using PCR. Amplification of the 16S rRNA gene was performed using the following primer pair: forward, 5′-GCT GGA TCA CCT CCT TTC-3′; and reverse, 5′-CCT TTC CCT CAC GGT ACT G-3′. The 25-µl reaction mixture contained 2.5 µl of 10× PCR buffer, 0.5 µl deoxynucleoside triphosphate mixture (10 mM), 0.75 µl of $MgCl_2$ (50 mM), 1 µl of each primer (100 pmol.µl^{-1}), 2 µl of DNA, and 0.25 µl of *Taq* DNA polymerase (5 U.µl^{-1})) Fermentas, Sankt Leon-Rot, Germany). The reaction conditions were as follows: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, followed by 72°C for 30 s and 4°C until needed. The PCR products were analyzed by electrophoresis on a 1.2% agarose gel (Invitrogen, Karlsruhe, Germany(containing the nucleic acid stain called Green Viewer (Qiagen). *Lactobacillus* isolates were identified by an amplicon size of 700–800 bp. After confirming the isolates as belonging to the genus *Lactobacillus*, we identified the species using specific primers in a further PCR-based analysis [43].

3.2. Identification of *L. plantarum*

To identify *L. plantarum* among the *Lactobacillus* isolates, the extracted DNA was subjected to PCR using forward (5′-CCG TTT ATG CGG AAC ACC TA-3′) and reverse (5′-TCG GGA TTA CCA AAC ATC AC-3′) primers targeting the *recA* gene, yielding an amplicon of approximately 319 bp. A 25-µl reaction mixture was used, containing 2.5 µl of 10× PCR buffer, 0.5 µl deoxynucleoside triphosphate mixture (10 mM), 0.75 µl of $MgCl_2$ (50 mM), 1 µl of each primer (100 pmol.µl^{-1}), 2 µl of DNA, and 0.25 µl of *Taq* DNA polymerase (5 U.µl^{-1}). The cycling conditions were as follows: 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 80 s, followed by 72°C for 10 min, and 4°C until needed. The PCR products were then analyzed on a 1.2% agarose gel containing the nucleic acid stain called Green Viewer [44].

4. Results

4.1. Culture and PCR-based detection of specific genes

Of the 40 mothers that participated in this study, 35 (87.5%) had lactobacilli in their breast milk. The minimum, maximum, and median log10 bacterial counts in the breast milk of the positive mothers were 1.55, 5.04, and 3.18 cells·ml^{-1}, respectively, considering all oxidase- and catalase-negative, gram-positive, and rod-shaped bacteria. Table 1 shows the characteristics of the studied mothers.

Amplification of the 16S rRNA gene using genus-specific primers and PCR (yielding an amplicon of 700–800 bp) (Figure 1), followed by homology analysis with the

Table 1: Characteristics of the study population (mothers and their infants).

	Maternal age (years)	Mode of delivery		Sex of infant		Area		Baby age	
		Vaginal	Cesarean	Female	Male	Urban	Rural	Below 3 months	Above 3 months
Total 40 cases	18–35[a] 27.5[b]	24[c] 60%[d]	16[c] 40%[d]	21[c] 52.5%[d]	19[c] 46. 3%[d]	19[c] 46.3%[d]	22[c] 53.7%[d]	22[c] 53.7%[d]	19[c] 46.3%[d]

[a]**Mothers' age range;** [b]**mean mother age;** [c]**sample count;** [d]**sample percentage.**

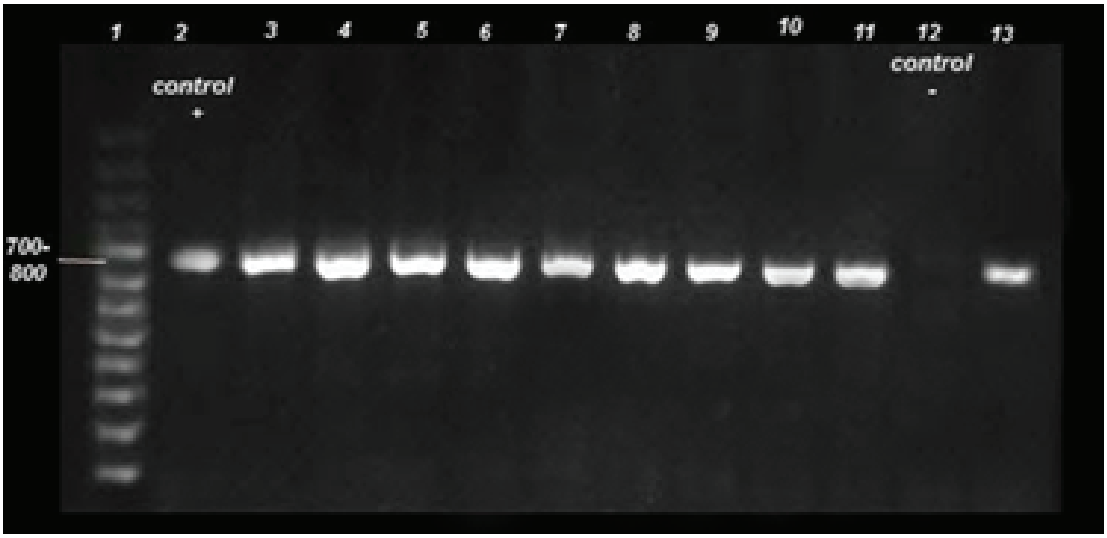

Figure 1: Results of electrophoresis of 16S rRNA gene PCR products. Lane 1: 100-bp ladder. Lane 2: positive control. Lanes 3–11 and 13: lactobacilli-positive samples. Lane 12: negative control.

Basic Local Alignment Search Tool (BLAST), revealed that 30 (85.71%) of the isolates belonged to the genus *Lactobacillus*. PCR amplification of the *recA* gene using species-specific primers (resulting in a 319-bp product) (Figure 2), followed by a BLAST homology search, revealed all of the *Lactobacillus* isolates to be *L. plantarum*.

5. Discussion

This study represents the first attempt to determine the presence of *L. plantarum* in human breast milk in Iran. Breast milk is not sterile, even when collected in an aseptic manner; therefore, it likely harbors a natural bacterial inoculum that influences colonization of the neonatal GIT. Breast milk not only provides a variety of substrates for bacterial growth [45], but is also a significant source of LAB that appear to be of endogenous origin rather than contaminants from breast skin 8-10. The presence of lactobacilli in breast milk has been associated with that of prebiotic oligosaccharides in this same fluid [46–48]. In the present study, even though no supplements or foods containing probiotics or prebiotics were given to the participants, the breast milk collected contained strains of *L. plantarum*, the first time such bacteria have been found in breast milk in Iran.

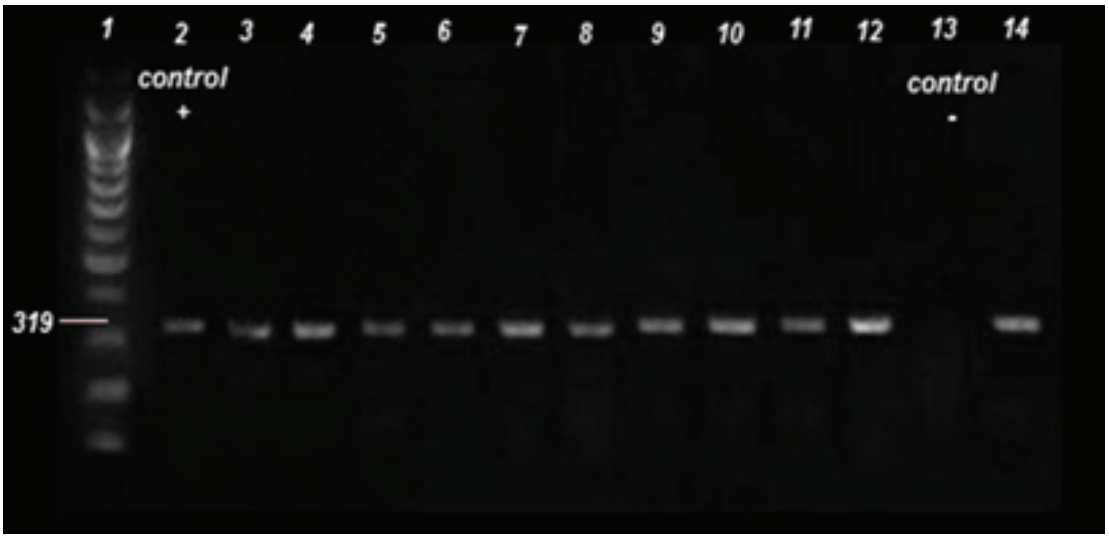

Figure 2: Results of electrophoresis of *recA* gene PCR products indicating the presence of *Lactobacillus plantarum*. Lane 1: 100-bp ladder. Lane 2: positive control. Lanes 3–12 and 14: *L. plantarum*-positive samples. Lane 13: negative control.

Consistent with our findings, many investigations performed in other countries have also isolated this species from breast milk samples [16, 27, 49, 50]. Martín et al. (2006) found *L. plantarum* in only 1 of 20 breast milk samples 11, whereas Mehanna et al. (2013) reported that 4 of the 30 samples that they examined contained this bacterium. Jara et al. (2011) tested the microbiota of 48 breast milk samples, finding most of the isolates (52%) to be *L. acidophilus*, with *L. plantarum* (30%), *L. paracasei* (7%), *L. salivarius* (7%), and *L. curvatus* (4%) also being recovered [49, 50]. In addition, Martín et al. (2007) isolated *L. fermentum*, *L. rhamnosus*, and *L. plantarum* from Spanish mothers' breast milk [51]. Based on this information, breast milk has been proposed as a good source of potentially probiotic LAB. Moreover, it can be considered a natural symbiont-containing food harboring a combination of probiotics and prebiotics that may ultimately foster a specific "healthy" microbiota in the infant gut [52, 53].

Lactobacilli strains in human milk contribute to infant digestion through the breakdown of sugars and proteins and are metabolically active in the infant gut, resulting in increased production of functional metabolites such as butyrate, the main energy source of colonocytes and a compound considered relevant to the modulation of intestinal function. Importantly, such bacteria also improve intestinal habits, increasing fecal moisture content and stool frequency and volume [54, 55]. They also contribute to reducing the incidence and severity of infections in breastfed infants by several mechanisms, such as competitive exclusion, production of antimicrobial compounds, and improvement of intestinal barrier function by raising mucin production and reducing intestinal permeability [9, 10, 13, 56]. As a result, and as the rate of infectious diseases is significantly lower among breastfed infants than formula-fed infants, anti-infective properties have been attributed to breast milk [57].

Thus, lactobacilli from breast milk are excellent candidates for the development of infant probiotic products [56]. The isolation of probiotic bacteria from human milk could clearly be employed to benefit the intestinal microbiota and immune development of

infants who, for various reasons, cannot be breastfed [57]. Indeed, there is evidence indicating that some strains isolated from breast milk demonstrate good probiotic potential, favoring their inclusion in products targeted at infants [49]. Modulation of the maternal intestinal microbiota can have a direct effect on an infant's health, offering new perspectives on bacteriotherapy, which may be used as an effective alternative to antibiotics. Work is in progress to elucidate the mechanisms responsible for such effects [38]. The potential role of the microbiome in human milk appears to have implications for not only short- and long-term infant health, but also mammary health. A better understanding of the link between the milk microbiome and health, as well as other potential factors influencing this association, will open new avenues in the study of pregnancy and lactation [58, 59]. Further research is needed to better understand the associations between health status and actual microbial communities, as well as their possible beneficial impacts on both mothers and their infants. Human milk could be a good and safe source of probiotic bacteria capable of improving the infant intestinal microflora.

6. Conclusion

L. plantarum is present in the human milk. It is found in a minority of nursing mothers but in geographically distant countries. Further studies are needed to provide a better understanding of the significance of this organism and other LAB in breast milk and their relevance to infant and maternal health. This study supports the use of isolated breast milk probiotics in the development of new pharmaceutical preparations, as supplements in the food industry, and in functional foods to benefit public health. Their inclusion in formula milk would be advantageous for infants not receiving breast milk.

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Conflicts of Interest Statement

The authors whose names are listed immediately below certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; or expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this manuscript.

This statement is signed by all the authors to indicate agreement that the above information is true and correct.

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