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# Evaluation of Probiotic Properties of *Lactobacilli* in Breast Milk and Their Inhibitory Effect on Pathogenic Bacteria of the Gastrointestinal Tracts

**Mohammad Mehdi Soltan- Dallal[1,2], Zahra Rajabi[2], Sara Sharifi Yazdi[3*], Parisa Torabi-Bonab[2], Alireza Monadi Sefidan[4], Sina Sharifi Yazdi[3]**

1. *Dept. of Food Microbiology, School of Health, Tehran University of Medical Sciences, Tehran, Iran*
2. *Food Microbiology Research Center, Tehran University of Medical Sciences, Tehran, Iran*
3. *School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*
4. *Dept. of Medical Laboratory Sciences, Faculty of Allied Medical Sciences, Tehran University of Medical Sciences, Tehran, Iran*

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**Corresponding Information:**
**Sara Sharifi Yazdi,**
School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
**E-Mail:** mksharifiyazdi@gmail.com

**ABSTRACT**

**Background & Objective:** Breast milk contains nutrients such as carbohydrates, essential fatty acids, proteins, vitamins, minerals, and a source of communal bacteria with probiotic potential that is very effective in the prevention and treatment of neonatal infections. The aim of this study was the evaluation of probiotic properties of lactobacilli in breast milk and their inhibitory effect on pathogenic bacteria of the gastrointestinal tracts.

**Materials & Methods:** In a cross-sectional descriptive study, during 10 months from January to October 2018, 100 breast milk samples were collected by referring to health centers after isolation. Lactobacilli strains were evaluated based on morphological characteristics, catalase, and hot staining tests, survival tests in acidic conditions, and bile salt tolerance to evaluate probiotic properties. Antibiotic resistance of probiotic strains and ability to inhibit pathogenic bacteria was evaluated by well method and growth inhibition zone.

**Results:** 122 lactobacilli belonging to 12 species were identified from 100 samples of breast milk by phototypical methods. The predominant species belonging to casei and other lactobacilli were Fermentum, Plantarum, and Gasseri, respectively.The highest antibiotic resistance was related to vancomycin (63.15%). The 3 isolates L4, L14 and L16 were able to strongly inhibit all the studied gastrointestinal pathogens.

**Conclusion:** Breast milk is a rich source of beneficial probiotic lactobacilli, which can be useful in breast milk for infants who are not breastfed to prevent neonatal infections.

**Keywords:** Breast milk, *Lactobacillus*, Probiotic, Inhibitory effect



## Introduction

Breast milk is the best source of nutrition for the baby. But in some cases, the use of milk powder and complementary foods is unavoidable. A variety of these foods are available in different formulations. The formulation of these products is designed to provide the nutrients needed for the optimal growth and development of the baby. One of the most important components in breast milk is its microbial flora, which contains different types of probiotic bacteria and plays a vital role in creating the baby's natural flora and strengthening the immune system. Therefore, some types of baby food formulations fall into the category of probiotic products (1, 2). Initial colonization of the baby's gut occurs during the transmission of germs from the mother or hospital. After this inoculation, the baby's gut flora changes rapidly under the influence of diet and other environmental factors. In the first study, a comparison between breastfed infants and formula-fed infants revealed that the predominant microbial flora of infants breastfed was *Bifidobacterium* (3). Subsequent studies have shown that the important factors initiating the growth of these bacteria are oligosaccharides in breast milk, which have a complex structure and exist in the form of free oligosaccharides attached to glycolipids or glycoproteins and are known as prebiotics. In the past malnutrition, respiratory infection, and diarrhea were the main causes of mortality and morbidity. Feeding with breast milk had reduced this disease (4). It has been proved that the normal flora of mother milk is able to reduce diseases (5). Approximately 4 million infants die each year, mostly in developing countries (6). One study reported that breastfeeding in the first 6 months and continuing until one year of age may reduce the deaths of 1.3 million infants annually (7). In the first 6 months, nutrition with breast milk reduces the incidence and severity of diarrhea, and the risk of celiac and *Helicobacter pylori* infection (8). Some research on pathogenic microorganisms in breast milk were performed, but in 2000 scientists began to evaluate the potential benefit of bacteria isolated from breast milk, mainly lactic acid bacteria (9). Lots of research has been carried out on the benefit of *Lactobacillus* and *Bifidobacterium* in breast milk (10). *Lactobacillus* are gram-positive, nonmotile, without spores. Mostly non-pathogen and antagonistic of the bacterial pathogen (11). Interference between normal flora and pathogens were taught to be considered in the treatment of infections followed by the use of non-pathogenic bacteria to control infections. Research shows that probiotics such as lactobacilli inhibit the growth of a wide range of human and animal pathogens. One of the beneficial potentials of probiotics is their antagonistic effect against harmful microorganisms so that these organisms can be useful in preventing and improving the treatment of gastrointestinal infections (12). In recent years the antibiotic resistance of bacteria led to alternative ways of treatment by using useful bacteria against harmful microorganisms, which is called bacteriotherapy (5, 10). Gram-negative intestinal bacteria, especially *Salmonella, Shigella*, and *Escherichia coli*, are the most important causes of foodborne illness and diarrhea in developing countries. On the other hand, drug resistance in these bacteria is increasing day by day. Therefore for the inhibition of pathogenic bacteria probiotics, including lactic acid bacteria and especially lactobacilli are used to prevent the growth of pathogenic bacteria (13). Considering the positive effects of breastfeeding and its compounds, including beneficial microbial flora, breast milk can be considered as a candidate to prove the probiotic properties. The aim of this study was to investigate the probiotic properties of lactobacilli isolated from breast milk and to determine their inhibitory effect on gastrointestinal bacterial pathogens.

## Materials and Methods

### Ethical issues

The research followed the tenets of the Declaration of Helsinki. Consent for operation and study had been taken. The ethical committee of Tehran University of Medical Sciences approved the research. All patients' information remained confidential. This study has the Iranian registry of clinical trials of IRCT 2015248N9.

### Sample size

Considering the prevalence of 50-65% of Lactobacillus bacteria in breast milk, a prevalence of 55% was considered to determine the sample size, which was estimated with 95% confidence level in such a way that the estimation error of a maximum of 5 %. Therefore, the required number of samples was obtained from the following formula:

$$n = \frac{Z^2 \times P(1-P)}{d^2} = \frac{1.96^2 \times 0.55 \times 0.45}{0.1^2} = 95 \sim 100$$

### Sample collection and identification

A total of 100 samples of breast milk from maternal and child health centers, in the amount of 3 ml each, were collected from healthy breastfeeding volunteer mothers aged 22 to 36 years who had not received any antibiotics for two weeks with full observance of sterile conditions. Samples were transferred to sterile containers in the food chain in the Department of Food Microbiology, School of Health, and Tehran University of Medical Sciences within two hours with cold chain. For enrichment, 1 ml of breast milk samples were added to tubes containing 9 ml of broth MRS medium and incubated for 48 hours under anesthesia at 37 ° C. Turbid samples were transferred to MRS agar medium and stored at 37 ° C for two days under anaerobic conditions until colony growth.

The initial characteristics of bacterial colonies such as diameter, color, margin, and surface were investigated (Figure 1). After purification, gram-positive and catalase-negative bacteria were selected and incubated at 37 ° C for 2 days under anaerobic conditions (11). *Lactobacillus plantarum* strain PTCC 1058 was used as a positive control for biochemical experiments.

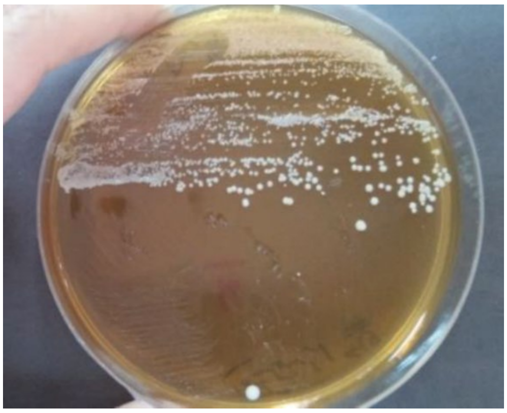

**Figure 1.** ***Lactobacillus* colony on MRS agar medium**

### Carbohydrate fermentation test by microplate method

13 sugars (Merck, Germany) were used to study the fermentation profile of carbohydrates. These sugars included L-arabinose, cellobiose, sorbitol, sucrose, raffinose, galactose, xylose, ribose, fructose, lactose, maltose, mannose, and melibiose. Modified MRS Broth medium was used as the sugar-based medium for this test.

In order to evaluate the tolerance to acidic conditions, the strains were grown in liquid MRS at 37 ° C for 24 h. 1 ml of each bacterial culture was inoculated in 9 ml of PBS at pH 2.5 and the samples were incubated for 3 h at 37 ° C. Bacteria were counted at the moment of inoculation and at the end of 3-hour incubation in acidic PBS mineral medium. For each strain serial dilutions, up to 10 times with saline serum and the poor plate were performed. After 2-3 days of incubation at 37 ° C in the anaerobic condition the number of colonies was counted (14).

To determine the resistance of strains to bile salts, for each acid-resistant isolate, two tubes, one containing 0.3% MRS broth with 0.3% (oxgall bile salt and the other containing 9 ml MRS broth without oxgall) were considered as a control.1% of 90 µl of fresh strain was added to both tubes and the tubes were incubated in an anaerobic jar at 37 ° C. The growth rate of the isolates at 0 and 8 h after incubation was measured by a wavelength spectrophotometer. 600 nm was measured **(15)**.

Bacteria were calculated by bile salt by the relationship of inhibition coefficient (Cinh) by Gopal et al. **(13)**. Finally, acid and bile resistant strains were identified as probiotic strains.

**Evaluation of antimicrobial activity of *Lactobacillus* isolates against pathogenic bacteria**

To study the antimicrobial activity of *Lactobacillus* isolates, the well diffusion method was used, which is according to Rammelsberg and Radler. *Lactobacillus* isolates with probiotic potential (acid and bile resistance) were selected for this test. Antimicrobial activity of these isolates against *Escherichia coli O157 H7, Salmonella enteritidis* ATCC14028, *Shigella sonei* PTCC9290 and *Yersinia enterocolitica* ATCC 11010 and *Staphylococcus aureus* (M) were evaluated. At first, the pathogenic bacteria were cultured in LB Broth medium (Merck, Germany) for 24 hours at 37 ° C and their concentration was adjusted to 107 CFU / mL. Nutrient agar plates (Merck, Germany) were then prepared and a well was drilled inside them. Pathogenic bacteria were cultured on the surface of nutrient agar medium (Merck, Germany) **(16)**.

The MRS broth culture medium was then centrifuged (lactobacillus isolates grown for 20 hours in a candeljar at 37 ° C (13,000 RPM, 10 minutes). The liquid was collected on them. One hundred microliters of liquid on culture was added to each well of nutrient agar plate and the plates were incubated for 37 to 15 hours at 37 ° C. After the incubation time, the diameter of the growth inhibition zone around the wells was measured. Isolates with non-growth halo diameter <11 mm as negative, 11-16 mm as medium inhibitor (+), 17-22 mm as strong inhibitor (++) and ≥ 23 mm as very strong inhibitor (+++). Were. *Lactobacillus rhamnosus GG* was used as a positive control and MRS sterile broth was used as a negative control **(17)**.

**Evaluation of antibiotic resistance**

Lactobacillus isolates were cultured in 10 ml Müller-Hinton Broth medium (Merck, Germany) containing 10% MRS Broth and incubated in a candlejar for 48 hours. When the turbidity reached 1/2 McFarland, they were cultured by swapping on agar plates containing Müller Hinton agar (Merck, Germany) containing 10% MRS agar. Antibiotic discs (Mast, UK) were placed on the surface of the medium and the plates were incubated for 48 hours in a candle jar at 37 ° C. The diameter of the inhibitory halo of the discs was then measured. Based on the obtained results, the isolates were reported as resistant (≤ 15 mm), moderate sensitivity (20-16 mm) and sensitive (≥ 21 mm) **(18)**.

For all isolates, antibiotic susceptibility testing was performed by the disk diffusion method according to CLSI 2019 criteria (19). Antibiotic discs (Mast UK) used include chloramphenicol (30 µg), clindamycin (2 µg), erythromycin (15 µg), penicillin (10 g), rifampin (5 g), tetracycline (30 g), gentamicin (30 g).

## Results

A total of 122 *Lactobacillus* strains were isolated from breast milk samples. To identify *Lactobacillus* species, carbohydrate fermentation, and growth test at 15 and 45 ° C, arginine hydrolysis, and glucose gas production were used. Among 122 isolates, 111 *Lactobacillus* species (90.98%) were identified by biochemical tests, and 11 isolates (9.02%) were not identified.

A total of 12 different species of *Lactobacillus* were identified in breast milk samples that had probiotic properties. These species included: *Lactobacillus casei, Fermentum, Plantarum, Gasseri, Corvatus, Paracasia, Acidophilus, Ruteri, Bruis, Pentosus, Rhamnosus and Johnsonium*. The most prevalent species of *Lactobacillus casei* was 43 isolates with a frequency of 35.24%. *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Gasseri* were identified as the most abundant species with 14.75, 9.83 and 8.19%, respectively.

**Evaluation of probiotic potency of isolates**

**Resistance acid and bile salt**

Out of 122 *Lactobacillus* isolates, 22 *Lactobacillus* were resistant to acidic conditions with a pH of 2. In the next step, these 22 acid-resistant strains were examined for resistance to 0.3% oxgall bile salt. Of these isolates, 19 strains were finally resistant to both acidic and bile salt conditions, and these strains were confirmed as strains with probiotic potential.

**Evaluation of antimicrobial effects of probiotic lactobacilli against gastrointestinal pathogens**

The antimicrobial activity of a supernatant culture of 19 acid and bile-resistant lactobacilli isolates against a number of gastrointestinal pathogens was investigated. Antimicrobial activity of a supernatant culture of all 19 lactobacilli isolates was performed without applying temperature and enzymatic treatments. Among 19 lactobacilli isolates resistant to acid and bile, 10 strains (52.63%) were able to inhibit the growth of *E. coli O157 H7*, of which 6 strains had a strong inhibitory effect and 4 strains had a moderate inhibitory effect and 9 isolates had no inhibitory effect. The results showed that 12 isolates of probiotic lactobacilli (65.15%) were able to inhibit the growth of *Salmonella*, of which 9 isolates strongly or strongly inhibited *Salmonella*. 3 isolates had a moderate inhibitory effect and 7 isolates had no inhibitory effect. All 19 probiotic isolates (100%) were able to inhibit *Shigella* growth, of which 9 strains had a strong effect, 6 strains had a very strong effect and 4 strains had a moderate inhibitory effect.7 probiotic strains (36.84%) had an inhibitory effect on *Staphylococcus aureus*, of which 5 strains had a strong inhibitory effect and 2 strains had a moderate inhibitory effect. The other 12 strains of lactobacilli had no inhibitory effect on staphylococcal growth. Eleven strains of probiotic lactobacilli isolates (57.89%) had a strong inhibitory effect on *Yersinia* growth and 8 of them had no inhibitory effect on growth. Among 19 probiotic lactobacilli strains, 3 L4, L14, and L16 strains were able to strongly or very strongly inhibit all gastrointestinal pathogens tested (Table 1).In this study isolates with growth inhibition zone diameter <11 mm considered as negative (no inhibitory effect), 16-16 mm as a moderate inhibitor (+), 22-17 mm as strong inhibitor (++) and > 23 mm were classified as a very strong inhibitor (+++). Sterile broth MRS was used as negative control and *Lactobacillus GG* as a positive control.

**Table 1. Antimicrobial activity of probiotic lactobacilli against pathogens**

| Number of species | *Staphylococcus* | *Yersinia* | *E. coli O157:H7* | *Shigella* | *Salmonella* |
|---|---|---|---|---|---|
| **L1** | - | ++ | ++ | +++ | ++ |
| **L2** | - | - | - | + | - |
| **L3** | ++ | - | - | + | + |
| **L4** | ++ | ++ | ++ | +++ | ++ |
| **L5** | - | - | + | ++ | ++ |
| **L6** | - | ++ | ++ | ++ | ++ |
| **L7** | - | ++ | - | +++ | ++ |
| **L8** | - | ++ | + | +++ | ++ |
| **L9** | - | - | - | + | - |
| **L10** | - | - | - | ++ | - |
| **L11** | + | ++ | ++ | +++ | ++ |
| **L12** | - | ++ | + | ++ | - |
| **L13** | ++ | ++ | + | ++ | - |
| **L14** | ++ | ++ | ++ | ++ | ++ |
| **L15** | - | ++ | - | ++ | + |
| **L16** | ++ | ++ | ++ | +++ | ++ |
| **L17** | - | - | - | ++ | + |
| **L18** | + | - | - | ++ | - |
| **L19** | - | - | - | ++ | - |

**Evaluation of antibiotic resistance**

All lactobacilli isolates with probiotic potential were tested for resistance to 8 different antibiotics (Table 2). Among these isolates, the highest antibiotic resistance was seen to vancomycin (7.73%), followed by clindamycin (1.42%), gentamicin (3.26%) rifampin (3.5%), chloramphenicol (0%) and penicillin (0%) respectively. The lowest resistance to the studied antibiotics was observed in chloramphenicol (0%) penicillin (0%).In this study, the isolates were divided into resistant (≤ 15 mm), medium sensitivity (20-16 mm), and sensitive (≥ 21 mm) based on the diameter of the growth inhibition zone.

**Table 2. Distribution of antibiotic resistance pattern of *Lactobacillus* strains isolated from breast milk**

| Numbe r of species | *vancomyci n* | *gentamici n* | *tetracyclin e* | *rifampi n* | *penicilli n* | *erythromyci n* | *clindamyci n* | *chloramphenic ol* |
|---|---|---|---|---|---|---|---|---|
| **L1** | R | S | R | S | S | S | S | S |

| Number of species | *vancomycin* | *gentamicin* | *tetracycline* | *rifampin* | *penicillin* | *erythromycin* | *clindamycin* | *chloramphenicol* |
|---|---|---|---|---|---|---|---|---|
| **L2** | R | I | S | S | S | R | R | S |
| **L3** | R | R | I | S | S | S | S | I |
| **L4** | R | I | S | S | S | S | S | S |
| **L5** | I | S | S | S | I | S | I | S |
| **L6** | S | I | S | S | I | S | R | I |
| **L7** | R | I | I | I | S | S | R | S |
| **L8** | R | I | S | S | S | S | I | S |
| **L9** | R | R | I | S | S | I | R | S |
| **L10** | I | R | R | I | S | I | R | S |
| **L11** | R | I | S | S | S | S | S | S |
| **L12** | I | I | S | S | S | S | R | S |
| **L13** | R | I | S | S | S | S | S | S |
| **L14** | I | R | R | I | S | R | R | S |
| **L15** | R | I | S | S | S | S | S | S |
| **L16** | R | I | I | R | S | R | R | I |
| **L17** | R | I | S | I | I | R | I | I |
| **L18** | R | R | S | S | S | S | I | S |
| **L19** | R | I | S | S | S | S | S | S |
| **Number of species** | *vancomycin* | *gentamicin* | *tetracycline* | *rifampin* | *penicillin* | *erythromycin* | *clindamycin* | *chloramphenicol* |
| | | | | | | | | |
| **L1** | R | S | R | S | S | S | S | S |
| **L2** | R | I | S | S | S | R | R | S |
| **L3** | R | R | I | S | S | S | S | I |
| **L4** | R | I | S | S | S | S | S | S |
| **L5** | I | S | S | S | I | S | I | S |
| **L6** | S | I | S | S | I | S | R | I |
| **L7** | R | I | I | I | S | S | R | S |
| **L8** | R | I | S | S | S | S | I | S |
| **L9** | R | R | I | S | S | I | R | S |
| **L10** | I | R | R | I | S | I | R | S |
| **L11** | R | I | S | S | S | S | S | S |
| **L12** | I | I | S | S | S | S | R | S |
| **L13** | R | I | S | S | S | S | S | S |
| **L14** | I | R | R | I | S | R | R | S |
| **L15** | R | I | S | S | S | S | S | S |
| **L16** | R | I | I | R | S | R | R | I |
| **L17** | R | I | S | I | I | R | I | I |

| Number of species | *vancomycin* | *gentamicin* | *tetracycline* | *rifampin* | *penicillin* | *erythromycin* | *clindamycin* | *chloramphenicol* |
|---|---|---|---|---|---|---|---|---|
| **L18** | R | R | S | S | S | S | I | S |
| **L19** | R | I | S | S | S | S | S | S |

## Discussion

The presence of lactic acid bacteria, especially lactobacilli and Bifidobacterium, is very important ,because of the balance of intestinal flora**(3)**.due to their importance in creating a proper balance in intestinal microorganisms as well as beneficial properties on neonatal health and immune system promotion (especially cellular immunity**(3)**. The lactobacilli are the normal intestine flora **(20)**, a heterogeneous group of lactic acid bacteria and are present in the intestinal flora of healthy individuals **(20)** .The baby's gastrointestinal tract is sterile until birth, and microbial colonization begins gradually after birth. The feeding influence by different factors such as normal birth and use of antibiotic by mother **(4, 9)**.

Probiotic strains of human origin have a significant advantage over strains that are isolated from other sources and the reason for this is the greater adaptation of these strains to the body and gastrointestinal tract **(21)**.

The breast milk is important in for the development and production of microorganisms in the baby's intestine **(22)**.In this study, the number of lactobacilli isolated from 100 samples of breast milk was reported to be 122, among which *Lactobacillus casei* with 35.2% was the most abundant species. Out of 122 strains, 11 strains (9%) could not be identified by phenotypic methods of fermentation of carbohydrates. This may be due to genes mutation related to intermediate enzymes in the fermentation of sugars that have mutated or deleted during mutation **(23, 24)**.A study by Vega-Bautista et al, showed that 67% of lactobacilli isolated from neonatal feces belong to the group that is exclusively breastfed. It also showed an increase in the number as well as the predominance of lactobacilli in the gastrointestinal tract of breastfed infants **(25)**. The results of the questionnaire information provided in this study showed that the incidence of diarrhea in breastfed infants was only 3%. It has been proven that the normal flora of breast milk is effective in preventing infants from infectious diseases and diarrhea **(5)**. The highest rate of isolation was *Lactobacillus casei*, which is consistent with the results of the study carried out by Davoodabadi et al. They identified *Lactobacillus casei* as the predominant fecal flora of infants who were exclusively breastfed **(13)**. This correlation can clearly confirm the relationship between breast milk flora and fecal flora of Iranian infants who are exclusively breastfed. Similar results were obtained in a study by Martin et al. They showed the probiotic potential of *Rhamnosus Lc* 705 and *Lactobacillus* strains, which were isolated from both breast milk and infant feces **(26)**. In the study of Martin et al., the rate of isolation of lactobacilli from eight groups of mothers from their milk and skin flora were 178 strains, compared to our study, the rate of isolation of lactobacilli was 122 strains out of 100 mothers. This difference in the rate of separation can be affected by the type of nutrition, which varies according to geographical conditions **(27)**. Soto et al were examined 160 samples of breast milk for the presence of lactobacilli and bifidobacteria. They isolated lactobacilli from 91.4% of the samples. The predominant species were *Lactobacillus salivarius, fermentum* and *Gasseri*, respectively. They noted that lactobacilli and Bifidobacterium was significantly lower in mothers treated with antibiotics compared to healthy mothers. The isolation rate of lactobacilli from breast milk samples was 97%. In order to isolate maximum lactobacilli in breast milk flora, mothers who were on antibiotic treatment for two weeks before sampling were excluded from the study **(28)**. In study carreid out by Martin and Gomez on the isolation of lactic acid family, only *Plantarum* species were isolated from lactobacilli, compared to our study, 12 species of *Lactobacillus* were isolated. This difference might be due to regional such as genetics, health status, maternal nutrition and geographical **(29, 30)**.

The results of the study of antibiotic resistance pattern of lactobacilli isolated from breast milk in this study indicate the sensitivity of this strain to a large number of antibiotics and, conversely, resistance to vancomycin. The vancomycin resistance is an intrinsic feature of lactobacilli **(31)**. Kumar et al. evaluated 80 breast milk samples from four different geographic regions, in Europe, South Africa, and China. Their results showed that breast milk in different regions were completely different for the presence of microbial flora **(32)**. Also, the results of studies performed on 133 breastfeeding mothers from different regions of Taiwan and China, showed that microbiome diversity and the composition of the microbial flora of breast milk was different **(33)**.

## Conclusion

The results of this study showed the diversity of species of *Lactobacillus* in the milk of healthy mothers. This might be due to the type of nutrition, diet, lifestyle, age, climatic and geographical conditions.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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