



## ORIGINAL RESEARCH

## Colonization of *Lactobacilli* among the Fecal Microflora of Healthy Iranian Breast-Fed Infants

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### Abstract

**Background:** *Lactobacilli* are important bacteria among intestinal microflora. They can prevent intestinal colonization by pathogenic bacteria, and used as probiotics.

**Aim:** The aim of this study was to investigate the rate *Lactobacillus* colonization in the fecal of healthy breast-fed infants.

**Methodology:** Fecal samples were collected from 90 healthy breast-fed infants under 18 months. *Lactobacilli* were identified by phenotypic and physiological methods. Carbohydrate fermentation patterns were determined using a miniplate method.

**Results:** Out of 90 infants' stool samples, 72 (80%) samples were positive for *Lactobacillus*. In total 238 isolates were identified as *Lactobacillus*. The dominated species were *L. casei* (55%), *L. plantarum* (20.6%) and *L. reuteri* (8.8%) respectively.

**Conclusion:** Since some *Lactobacilli* strains have health-promoting effects on infant and are use as probiotics, therefore further investigation on probiotic potential of identified species may help in finding new probiotics.

### Keywords

*Lactobacillus*, Identification, Infant, Fecal, Microflora

### Background

The gastrointestinal microflora of human contains complex community of microbial species including approximately 400 different microbial species, mostly bacteria [1]. Lactic acid bacteria and *bifidobacteria* are important group among intestinal microflora, since they have health-promoting benefits, such as the prevention of intestinal colonization by pathogens [2,3]. They are commonly used as probiotics, with effects especially against acute diarrhoea in childhood [4,5].

*Lactobacilli* species are gram positive, catalase negative, non-spore-forming and aerotolerant or anaerobic bacteria. They are found in dairy, grain, meat and fish products, beer, fruits, pickled vegetables, silage, sourdough, soil, as well as mouth, intestinal tract, and vagina of humans and many animals as normal flora [6,7]. Before the birth gastrointestinal tract of fetus is sterile, but microbial colonization immediately begins after the birth [8,9]. Several factors such as mode of delivery, preterm birth, antimicrobial agents, hygienic conditions, diet (breast or formula feed) and etc., affect microbial presence and succession in gastrointestinal tract of infants [1,10].

The rate of morbidity and mortality in breast-fed in-

infants is lower than formula-fed infants. In several studies especially in poor hygienic status countries, an inhibiting effect of breast-fed on enteritis has been shown [8]. Some studies indicated that *bifidobacteria* and *lactobacilli* are dominated microflora in the breast-fed infants, and in formula-fed infants mostly were *bacteroides*, *clostridia* and *Enterobacteriaceae* [11]. However there is a controversy about the extent of *lactobacilli* colonies the intestines of newborn infants and small children [4].

There are only limited studies about characterization of *lactobacilli* species in stool microflora of healthy, breast-fed infants. The aim of this study was to investigate the rate of *lactobacilli* in fecal microbial of Iranian breast-fed infants.

## Materials and Methods

### Sampling and culture

Fecal samples were collected from 90 healthy breast-fed infants under than 18 months, < 1 month (24 samples), 1-3 (11), 3-6 (20), 6-9 (11), 9-12 (13), and 12-18 (11). About one gram of each sample was immediately cultured in 50 ml MRS broth (Scharlau Chemie S. A, Barcelona, Spain) and incubated at 37 °C for 48-72 h under

anaerobic condition. Then, samples were sub-cultured on MRS agar (Scharlau Chemie S. A, Barcelona, Spain) plates and incubated at 37 °C for 48 under anaerobic condition. Three to four suspicious colonies of each plate were tested. Gram positive catalase negative rods regarded as *lactobacilli* and kept at -20 °C in MRS broth containing 20% glycerol. This study was approved by the Research Ethics Committee of Tehran University of Medical Sciences (TUMS), and informed parental consent was obtained.

### Identification of isolates

The fermentation of carbohydrates was determined according to Kandler (1986) using the miniplate method described by Oberhofer (1983) [12,13]. All isolates were investigated for the following 14 sugars (Merck): L-arabinose, D-xylose, galactose, D-glucose, mannitol, mannose, sorbitol, cellobiose, maltose, lactose, melibiose, saccharose, trehalose and raffinose. The basal medium (MRS without glucose and meat extract, but with 0.005% bromcresol purple) contain a final concentration of 0.5% (w/v) of each sugar was used. To ensure anaerobic conditions, two drops of sterile liquid paraffine (Merck) were placed in each well of miniplate (Nunc) after inoculation. Miniplates were incubated at 30 °C and

**Table 1:** Physiological and biochemical characteristics of identified species.

Identified isolates	<i>L. casei</i> , N = 131	<i>L. plantarum</i> , N = 49	<i>L. reuteri</i> , N = 21	<i>L. fermentum</i> , N = 14	<i>L. intestinalis</i> , N = 7	<i>L. curvatus</i> , N = 6	<i>L. johnsonii</i> , N = 3	<i>L. paracasei</i> , N = 3	<i>L. brevis</i> , N = 2	<i>L. acidophilus</i> , N = 2
Fermentation group	B	B	C	C	B	B	A	B	C	A
Gas from glucose	-	-	+	+	-	-	-	-	+	-
NH <sub>3</sub> from arginine	-	-	+	+	-	-	w	-	+	-
Growth at 15 °C	+	+	-	-	-	+	+	+	+	-
Growth at 45 °C	-	-	+	+	+	d	+	-	-	+
L-Arabinose	-	d	d	d	-	-	-	-	+	-
Cellobiose	+	+	-	d	-	+	+	+	-	+
Galactose	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	-	+	+	+	+
Lactose	-	+	+	+	+	d	w	+	w	+
Maltose	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	-	w	+	-	+	+	-	+
D-Mannitol	+	+	d	-	+	-	-	+	-	-
Melebiose	-	+	+	+	-	-	w	-	w	+
Raffinose	-	+	+	+	-	-	w	-	w	+
Saccharose	+	+	+	+	+	d	+	+	w	+
D-Sorbitol	+	+	-	-	-	-	-	-	-	+
D-Threhalose	+	+	d	d	+	-	-	+	-	-
Xylose	-	d	d	d	-	-	-	-	w	-

Symbols and abbreviations: A: Obligate homo-fermentative; B: Facultative homo-fermentative; C: Obligate hetero-fermentative; +: 90% or more of strains were positive; -: 90% or more were negative; d: 11-89% of strains were positive; w: Weak positive reaction

were examined after 2 and 5 days [14,15].

Hydrolysis of arginine was tested in MRS broth without glucose and meat extract but containing 0.3% arginine and 0.2% sodium citrate replacing ammonium citrate. Ammonia was detected after 3 days using Nessler's reagent (Merk) [13]. Gas (CO<sub>2</sub>) production from glucose was observed in MRS broth containing inverted Durham tubes after 5 days at 30 °C [16]. Growth at different temperatures was observed in MRS broth after incubation for 3 days at 15 °C or 45 °C. Identification was performed according to Bergey's manual of systematic bacteriology [17].

## Results

Out of 90 infants' stool samples, 72 (80%) samples were positive for *Lactobacillus*, and 238 isolates were identified as *Lactobacillus*. Physiological and biochemical characteristics of the *Lactobacilli* isolates are shown in Table 1. Isolates were classified to three physiological

groups: 1) The obligate homo-fermentative *Lactobacilli*, 2) The facultative homofermentative *Lactobacilli* and 3) The obligate hetero-fermentative *Lactobacilli*.

The most identified species from fecal microflora of infants were 131 *L. casei* (55%), 49 *L. plantarum* (20.6%) and 21 *L. reuteri* (8.8%), followed by *L. fermentum* (6%), *L. intestinalis* (3%), *L. curvatus* (2.5%), *L. johnsonii* (1.26%), *L. paracasei* (1.26%), *L. brevis* (0.84%) and *L. acidophilus* (0.84%) respectively Figure 1.

In general 18 (75%) stool samples of infants under 1 month were positive for *Lactobacilli* as follow: *L. casei* (22 isolates), *L. plantarum* (14), *L. reuteri* (14), *L. fermentum* (6), *L. intestinalis* (1), *L. curvatus* (1), *L. johnsonii* (1) and *L. paracasei* respectively (1). The frequency of four commonly *Lactobacillus* species in different age groups of infants are shown in Figure 2.

Eight (72%) of stool samples of infants between 1 and 3 months were positive for *Lactobacilli*, and identi-

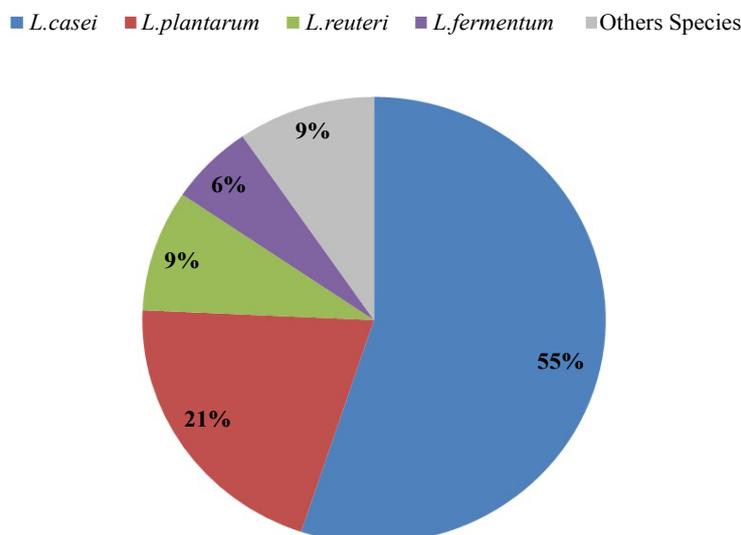


Figure 1: Frequency of isolated *Lactobacilli* species.

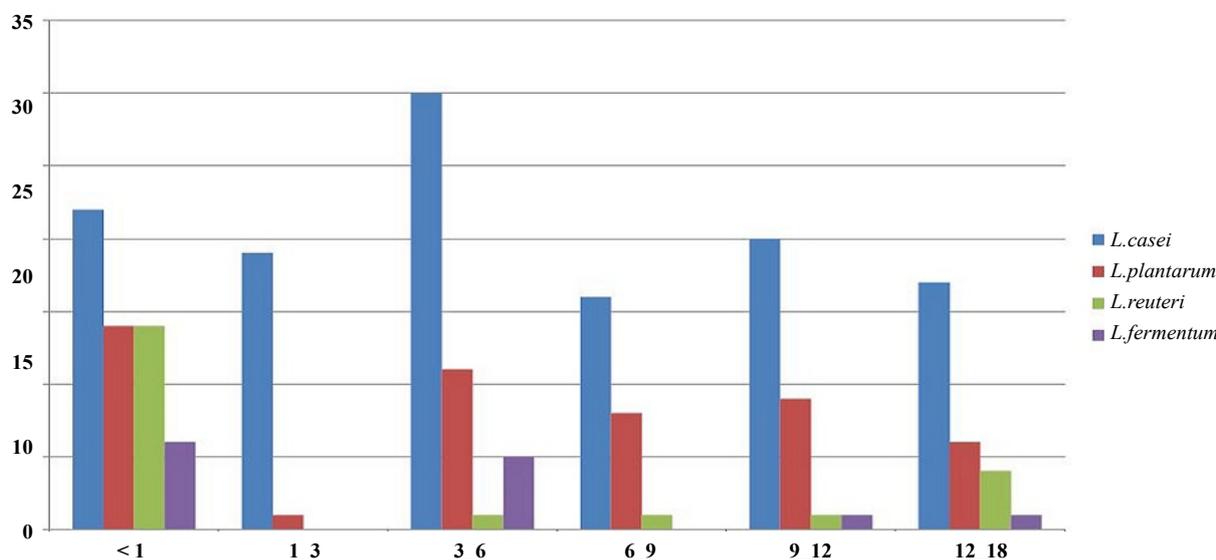


Figure 2: Frequency of four commonly *Lactobacillus* species in different age groups of infants.

fied species from this group were *L. casei* (19 isolates), *L. plantarum* (1), *L. intestinalis* (1), *L. curvatus* (1), *L. brevis* (1) and *L. paracasei* (1).

In infants between 3-6 months, 16 (80%) stool samples were positive and the identified species were *L. casei* (30 isolates), *L. plantarum* (11), *L. fermentum* (5), *L. intestinalis* (5), *L. curvatus* (2) and *L. reuteri* (1).

In infants between 6-9 months, 9 (82%) stool samples were positive and the identified species were *L. casei* (16 isolates), *L. plantarum* (8), *L. acidophilus* (1) and *L. reuteri* (1).

At the age between 9-12 months, 11 (84%) stool samples were positive and identified species from this group were *L. casei* (20 isolates), *L. plantarum* (9), *L. curvatus* (2), *L. reuteri* (1), *L. fermentum* (1), *L. johnsonii* (1) and *L. paracasei* (1).

And finally in age of between 12 to 18 months 10 (91%) stool samples were positive for *Lactobacilli* and identified species were *L. casei* (17 isolates), *L. plantarum* (6), *L. reuteri* (4), *L. fermentum* (1), *L. johnsonii* (1), *L. acidophilus* (1) and *L. brevis* (1).

## Discussion

Few studies have examined *Lactobacilli* diversity at the species level in fecal microflora of infants and children. The human milk has some ingredients and properties such as low content of iron and phosphorus, various oligosaccharides, and numerous humoral and cellular mediators. These factors seem to increase the development of an infant gut microflora dominated by *bifidobacteria* and *Lactobacilli* [11].

The frequency of *Lactobacillus*-positive stool samples was higher than the study of Ahrné, et al. (17-45%) [4]. Ahrné, et al. detected that *L. rhamnosus* and *L. gasseri* were the most commonly isolated species from faeces of infants under 2 month age. Also at 6 month of age *L. rhamnosus* (21%) and *L. paracasei* (15%), at 12 month of age *L. paracasei* (7%) and *L. fermentum* (4%), and at 18 month of age *L. paracasei* (22%) and *L. fermentum* were the most commonly isolated species [4].

However, our results were not in accordance with the findings of Ahrné, et al. [4]. In our study the *L. casei* (36.6%), *L. plantarum* (23.3%) and *L. reuteri* (23.3%) were the predominant species in the faeces of infants under 1 month age. In addition *L. casei* was the most prevalent species isolated from breast-fed infants between 1-18 months, followed by *L. plantarum*.

Ihekweazu, et al. showed that *L. rhamnosus*, *L. johnsonii* and *L. paracasei* were the predominant species in neonatal gut microflora up to the first month of life [8]. In a study by Arici, et al., *L. rhamnosus* (33.3%) was the most identified species from the faeces of infants and children under 2 years of age [18]. In a study by Mirlohi, et al. in the Isfahan Province of Iran, the *L. acidophilus*

and *L. plantarum* were the most recovered species from the faeces of healthy infants between 1 to 19 months [19]. In most above studies, *L. rhamnosus* was the predominant species in the faeces of healthy infants. Our results, like those of Mirlohi, et al. in Iran, did not show the predominance of *L. rhamnosus* in the faeces of infants. Variations in *Lactobacilli* microflora may be due to differences in feeding (breast or formula) and the geographical zone [8,19] or in identification error. Also variations in methodology may account for the differences, since *Lactobacilli* are notoriously difficult to identify by traditional biochemical methods [4].

In conclusion, our results showed the predominance of *L. casei* and *L. plantarum* species in stool microflora of breast-fed infants. Some *Lactobacilli* strains have health-promoting effects in the developing infant and used as probiotics. Therefore, further investigation about the probiotic potential of identified species may help in finding of new probiotics.

## Acknowledgements

This work was supported by the Vice-Chancellor for Research grant (no. 21491) of Tehran University of Medical Sciences (Tehran, Iran).

## Authors' Contribution

All authors contributed equally.

## Financial Disclosure

The authors declare no conflict of interest.

## Funding/Support

This work was supported by the Vice-Chancellor of Tehran University of Medical Sciences (Tehran, Iran).

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