

The influence of impact delivery mode, lactation time, infant gender, maternal age and rural or urban life on total number of *Lactobacillus* in breast milk Isfahan - Iran

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Abstract

Background: Breast milk is known as the most crucial postpartum issue in metabolic and immunologic programming of neonatal health. Human milk microbial changes over Lactation. The factors influencing the milk microbiome as well as potential impact of microbes on infant health have not yet been discovered. The objective was to identify pre- and post-natal factors that can potentially influence the bacterial communities inhabiting human milk.

Materials and Methods: Breast milk samples ($n = 40$) with all full-term breastfed infants were collected from lactating randomized. Information on personal characteristics, dietary habits, information about infants were collected after birth. The samples were plated with serial dilutions on three selective culture media man rogosa sharp and then colonies were counted. Colonies tested for catalase reaction, Gram-staining and microscopic examination.

Results: The result of this study showed that the overall incidence of positive *Lactobacillus* in mother's milk was 87.5%. The results based on (infant gender, mode of delivery, rural or urban and lactation time) rural or urban and lactation time were significant ($P < 0.05$). The results showed that all of the variables were significant in this regression model ($P < 0.001$). The median of \log_{10} *Lactobacillus* counts in rural mothers, vaginal delivery, infant male gender and Lactation time for first 3-month were meaningfully high.

Conclusions: The findings of this study about the breast milk *Lactobacillus* potential probiotic bacteria of healthy Iranian mothers, suggested that the breast milk microbiome is significantly influenced by several factors, mode of delivery, rural or urban and lactation time.

Key Words: Breast milk, delivery, infant, lactation, *Lactobacillus*

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INTRODUCTION

The presence of bacteria in human milk has been acknowledged since the seventies. For many years, microbiological analysis of human milk was only performed in case of infections and therefore the inclusion of nonpathogenic bacteria was yet unknown. Within the last decades, the application of more

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sophisticated culture-dependent and independent techniques, and the steady development of the omic approaches have been opened up a new concept of the “milk microbiome and a complex ecosystem with greater diversity than previously anticipated”.^[1]

Breast milk is the best food for rapidly-growing infant as it protects the newborn against some disease. These effects reflect that breast milk acts as a transporter for many essential substances and useful and natural microflora. It is a complex specific biological fluid not only provide the nutrients and energy required for the child’s growth and development, but also factors, which assist in microbiological protection and regulating the defense mechanisms, including the immune system and accelerating the postnatal maturation of the digestive system.^[2,3]

Breast milk and infant feces from mother-infant pairs may share the same strain(s) suggests that breastfeeding could participate to the bacterial transmitted from the mother to infant and therefore, to the infant gut colonization.^[4]

It became accepted that colostrum and breast milk are the continuous sources of beneficial bacteria to the infant gut, which is sterile at birth. Bacterial colonization occurs rapidly in the dominant bacteria based on the use of culture media include: *Staphylococci*, *Streptococci*, lactic acid bacteria (LAB), *propionibacteria* and other Gram-positive bacteria and some other Gram-negatives.^[5-11]

Results of investigation indicated that these microorganisms is establishing during the first 3-month of life in vaginally delivered breastfed full-term infants and the presence of viable *Bifidobacterium* in the corresponding breast milk samples. *Enterococcus* and *Streptococcus* have been the most frequently isolated microorganisms most frequently in on-day-old newborns, but after 10 days of age until 3-month bifidobacteria become the predominant group. In breast milk *Streptococcus* has been the most frequently isolated genus and *Lactobacillus* and *Bifidobacterium* were also obtained. Breast milk contains viable lactobacilli and bifidobacteria that might contribute to the initial establishment of the microbiota in the newborns.^[12]

The bacteria in the mammary gland secretion are not considered a contaminant in a natural way with bacterial colonization of the infant gut bacteria deemed appropriate. Their number change during lactation and consistently reduced in the time of weaning and when there is no milk in the mammary gland quickly disappears.^[4,13]

Increased lymph and blood flow and nutrient and oxytocin release to the mammary gland in a few weeks before and after delivery, cause attendance and more diversity of endogenous bacterial in this period.^[14]

These bacteria present in the breast originate intestines of the mother; reach through an endogenous route, including macrophages and dendritic cells in the mammary gland.^[10]

It is estimated that an infant consuming approximately 800 ml/day will ingest about 1×10^5 – 1×10^7 bacteria while suckling.^[15] Among these bacteria, LAB constitutes an important part of intestinal bacteria.^[12] In fact, the bacterial composition of the infant fecal flora reflects the bacterial composition of breast milk.^[15]

Lactic acid bacteria in breast milk may play several roles like decreasing the incidence and severity of infections from the gut newly born infants by various mechanisms, including the creation of antimicrobial compounds and eliminating.^[16,17]

Various studies have confirmed that the frequency, diversity, stability and source of LAB in milk is associated with the resources available to mothers.^[18]

During the study, the incidence of *Lactobacillus reuteri* in the milk of nursing mothers living in rural or urban areas in different geographical locations has been investigated. In all, 50% of mothers from rural areas in Japan and Sweden had *L. reuteri* in their milk, while the mothers from urban areas in South Africa, Israel and Denmark had very low or nondetectable levels. Overall, 15% of mothers had detectable *L. reuteri* in their milk. There were no significant differences in the prevalence of total *Lactobacillus* or *L. reuteri* from rural and urban habitats in participating women from different countries.^[19]

A study to examine the origin of the dominant bacteria in breast milk and the method of delivery (vaginal and cesarean) at the molecular level showed that colonization of LAB is considerably not related to delivery mode.^[20]

Human milk microbial changes over lactation. *Weissella*, *Leuconostoc*, *Staphylococcus*, *Streptococcus*, and *Lactococcus* were predominant in colostrum samples, whereas in 1- and 6-mo milk samples the conventional inhabitants on the oral cavity (e.g. *Veillonella*, *Leptotrichia*, and *Prevotella*) increased significantly. Milk samples from elective, not from nonelective mothers, who underwent cesarean delivery contained another type of bacterial community than did milk samples from individuals given birth by vaginal delivery.^[21]

During a study from the provided new evidence for any link between delivery mode and infant diet as determinants with this essential microbial community during the early life results in this case observed high variability inside the profiles of fecal microbiota in many infants. The profiles were generally dominated by *Actinobacteria* (mainly the genus *Bifidobacterium*) and *Firmicutes* (with diverse representation from numerous genera). In contrast to breastfed infants, formula-fed infants had an increased richness of species, with over representation of *Clostridium difficile*. *Escherichia – Shigella* and *Bacteroides* specie were under represented in cesarean born infants. Infants born by elective cesarean delivery had particularly low bacterial richness and diversity.^[22]

According to the key role of providing a natural source of *Lactobacillus* in breast milk as a useful factor for newborn infants and the impact of different conditions on the different composition of breast milk microbial flora, this research approaches to study the population of *Lactobacillus* with the potential factors influencing its existence, which can be considered as a candidate probiotic properties used to prove the importance of breastfeeding for all infants in the first moments after birth and an improved comprehension of the hyperlink involving the milk microbiome and health benefits that can be discussed and is required to open new avenues in the field of pregnancy and lactation.

MATERIALS AND METHODS

Sample

Breast milk samples ($n = 40$) with all full-term breastfed infants (19 males/21 females) aged 3 days to 12 months were collected from lactating randomized.^[4,7,23] All volunteers gave written informed consent to the protocol, which was approved by the Ethical Committee of Hospital District of Isfahan, Iran. Information on personal characteristics, dietary habits, information about infants including the details of delivery and early infant feeding were collected after birth. Data on duration of breastfeeding, as well as infant feeding practices, were obtained by interview and were recorded.

Clinical evaluation and exclusion criteria (Subjects and design)

All volunteers were informed about the aim of the investigation and the procedure. Exclusion criteria for the subjects were fever, diabetes, infections or metabolic disease, gestational hypertension, diseases of the breast or central nervous system, malnutrition, maternal allergy, alcohol, and addiction. Other exclusion criteria were newborns with

any malformation, cardiac or hemolytic disease. The diseases were diagnosed by history, physical examination, etc., All the participants were healthy and without any infant and/or mother perinatal problem. Only healthy women who had not used antibiotics within the previous 2 weeks were included in the study. The participating mothers avoiding intake of any herbal tea and supplements containing added LAB for 2 weeks before providing the samples. No other restriction to their normal dietary intake with natural components of LAB and bacteria was made. The mothers were between 18 and 40 years of age. They were refraining from breastfeeding for 1 h before giving the sample, which was taken in the morning.

Sampling

For sampling, nipple and mammary areola were cleaned with soap and sterile water, and then chlorhexidine was applied. The breast milk sample was collected (10–15 ml) in a sterile tube using sterile-gloves. The first drops (approximately 1 ml) were discarded to avoid chlorhexidine contamination. Parallel, a swab from the nipple and mammary areola was obtained to assess the efficacy of the antiseptic treatment. Sterile tubes containing samples were packed with dry ice in insulated boxes and sent to the laboratory within <2 h.

Isolation and identification

The culturing amount of samples (1000 μ l) was plated directly, and serial dilutions were plated on three specific culture media to the through pour plate with man rogosa sharp agar, (Merck Germany). It was done with 2 replications and for better isolation of LAB to support the growth of *Lactobacilli*, with 0.5% glucose (Merck) and 0.25% *L-cysteine*. The agar plates were incubated at 37°C under anaerobic conditions of 10% H₂, 10% CO₂ and 80% N₂) in a chamber Mac 500 (Mart System, Netherlands) for 48–72 h and then colonies were counted. Colonies with different morphologies were purified by streaking on the appropriate media and tested for catalase reaction, Gram-staining, and microscopic examination. All isolates, which were oxidase and catalase-negative and Gram-positive bacillary form [Figure 1] and then colonies were counted.

Statistical methods

Since the bacterial counts in breast milk was not normally distributed, the Mann–Whitney test was applied to test the differences between two groups of infant gender, Mod of delivery (vaginally/cesarean), area (urban and rural) and lactation time (first 3-month/after 3-month). The Poisson regression was used to assess the effects of infant gender, mode of

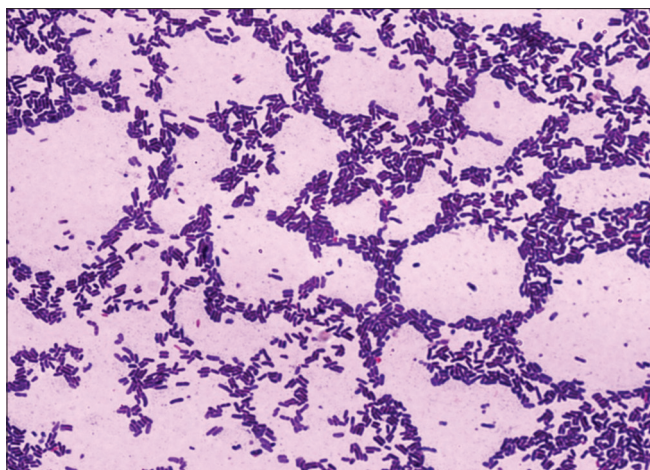


Figure 1: *Lactobacillus* Gram-positive bacilli

delivery, urban and rural and lactation time on the bacterial counts. Statistical analyses were performed using SPSS software (version 20).

RESULTS

Among 40 participated mothers in this study the overall incidence of positive *Lactobacillus* in mother's milk was obtained as 87.5%. Minimum, maximum and median of \log_{10} bacterial counts (cells/ml) of breast milk among the positive mothers was 1.55, 5.04 and 3.18, respectively. Table 1 shows characteristics of studied mothers. Table 2 denotes median, interquartile range and results of Mann–Whitney test for \log_{10} bacterial count cells/ml based on (infant gender, mode of delivery, urban and rural life and lactation time) urban and rural life and lactation time were significant ($P < 0.05$). Figure 2 shows median of \log_{10} *Lactobacillus* counts in mother's milk by mode of delivery, lactation time and urban and rural life. The median of \log_{10} *Lactobacillus* counts in rural mothers is higher than urban mothers.

Table 3 shows the results of Poisson regression. All of the variables (mode of delivery, urban and rural life, lactation time, infant gender and maternal age) were significant in this regression model ($P < 0.001$). The impact of mode of delivery on mean of *Lactobacillus* counts in milk for a mother with vaginal delivery to a mother with cesarean delivery is an increase by factor $\exp(0.303) = 1.35$. The mean of *Lactobacillus* counts in rural mother's milk to urban mother's increase by factor $\exp(0.735) = 2.08$. The impact of lactation time on the mean of *Lactobacillus* counts of mother's milk is an increase by $\exp(1.517) = 4.56$ for the first 3-month to after 3-month. The mean of *Lactobacillus* counts in mother's milk with female infants to male infant was decreased by factor $\exp(-0.656) = 0.518$. With 1-year increase in mother's age, the mean of *Lactobacillus* counts in her milk decrease by factor $\exp(-0.068) = 0.93$.

Table 1: Characteristics of studied mothers

Continuous variables	Mean	SE	Categorical variables	Frequency	Percentage
Maternal age (years)	27.05	0.63	Gender of infants (female)	21	52.50
			Mode of delivery (vaginal)	16	40
			Lactation time (first 3-month)	22	55
			Area (rural)	22	55

SE: Standard error

Table 2: The completion of *Lactobacillus* counts based on various variable

Variables	Median	IQR	P
Infant gender			
Female	2.87	2.32–3.68	0.464
Male	3.01	2.03–4.51	
Mode of delivery			
Vaginal	2.99	2.43–4.43	0.490
Cesarean	2.81	2.15–3.76	
Area			
Rural	3.56	2.9–4.48	0.002
Urban	2.41	1.08–2.86	
Lactation time			
First 3-month	3.64	2.45–4.48	0.015
After 3-month	2.64	1.77–3.22	

Values given in \log_{10} bacterial cells/ml. IQR: Interquartile range

Table 3: Poisson regression results

Parameter	Coefficients	SE	P
Intercept	9.863	0.0089	<0.001
Mode of delivery (vaginal)	0.303	0.0026	<0.001
Area (rural)	0.735	0.0043	<0.001
Lactation time (first 3-month)	1.517	0.0047	<0.001
Gender (female)	-0.656	0.0029	<0.001
Maternal age (year)	-0.068	0.0003	<0.001

SE: Standard error

All of the above results were based on adjusting of other variables in the Poisson regression model.

Poisson regression model equation is as follows:

$$\text{Log}(\mu) = 9.863 + 0.303 \text{ Mod of delivery} + 0.735 \text{ urban and rural life} + 1.517 \text{ lactation time} - 0.656 \text{ gender} - 0.068 \text{ maternal age}$$

That μ , is the mean bacteria counts in breast milk.

DISCUSSION

The results of the current study provide new insight into the analyzing the *Lactobacillus* of breast milk samples obtained from healthy women showed the depending status of the lactating women with type of location and method of delivery and infant gender

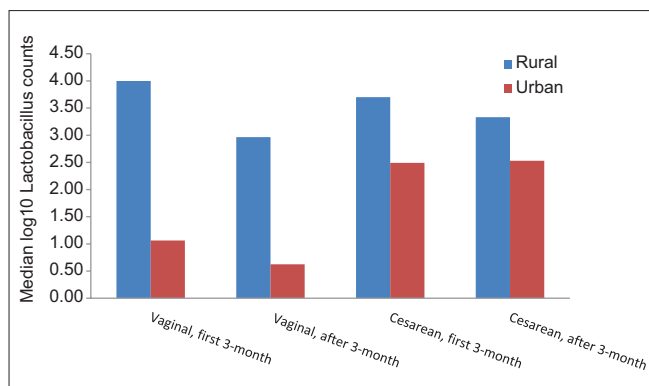


Figure 2: Median log₁₀ *Lactobacillus* counts in breast milk samples by mode of delivery, lactation time and rural or urban

and lactation time could be different in the presence of this probiotic bacteria based on cultures express. The results of the current study showed that breast milk as a source of live *Lactobacillus* to the infant gut. They were found in 87.5% of our breast milk samples; other study groups have reported that bacteria belonging to such genera can be isolated from fresh breast milk of healthy women from distant countries suggesting that their presence in this substrate is a common event.^[7,19,23-26] The total count of *Lactobacillus* in the first 3-month of lactation was the most, especially in colostrum and transitional milk. So that the number of women having babies within after 3-month of lactation were reduced. Our study found and corresponded to a lot of research in relation to the number of bacteria have shown that this development starts during the last third trimester of pregnancy, reaches the highest complexity and number at the end of 3rd month of lactation period, remains quite constant throughout lactation, declines sharply at weaning, and rapidly disappears when there is no milk in the mammary gland.^[4,6,13,16] In a recent study by Khodayar-Pardo *et al.* higher bacterial concentrations in colostrum and transitional milk were found.^[27] The total bacterial counts in fecal samples were at their peak by 3-month, thereafter decreasing with age.^[28]

Milk from colostrum compared with 6-month milk sample showed different patterns of bacterial diversity, points to several hundred species in human breast milk, with colostrum having higher diversity than transition and mature milk.^[21] Colostrum greater variation of bacteria may be related to nutrient contents in this substrate. Our data showed that breast milk from mothers residing in rural areas tended to possess higher median counts of total lactobacilli inside the breast milk in comparison with those living in urban areas. Hence that in this study the levels of *Lactobacillus* observed in breast milk from urban and rural habitats ranged from 2.0×10^4 to 5.0×10^4 and 1.0×10^1 to 6.0×10^4 cfu/ml, respectively.^[16] In

accordance with other studies women living in rural areas tended to have higher median counts of total lactobacilli in the breast milk compared with those living in urban areas (1.3×10^3 vs. 3.0×10^2 cfu/ml). The highest counts were found in the breast milk from mothers in Israel, South Africa, Japan and South Korea.^[19] Japanese rural women seem to have a higher prevalence of *L. reuteri* colonization than women from the other countries in the study. This may be related to the wide use of functional foods, probiotics and various fermented foods as an important part of the Japanese diet.^[19,29] In this study, the diet of lactating mothers was using a questionnaire that in rural maternal local bread, local cheeses fermented and pickles fermented and fruit consumption was higher suggesting that the incorporation of probiotics in the mother's diet before delivery and in the infant diet during breastfeeding may positively influence the maturation process of gut immunity.^[30]

We also considered other factors such as delivery mode in *Lactobacillus* involvement, who showed mothers with vaginal delivery compared with cesarean delivery were on average of more bacteria. It has also been reported in earlier studies that vaginally delivered children have been discovered to own acquired bacterial communities more resembling that of their own mothers contrary to those delivered by cesarean section. The differences in microbiota colonization could be evidenced at 7 years old.^[31] Microbiota profiles are themselves influenced by mode of delivery that play significant roles in the nutritional, immunological and microbiological programming of infant health.^[28] Vertical transmission of vaginal and fecal bacteria from the mother to the newborn is held being important to the formation from the intestinal microbiota in infants. However, modern delivery practices and the top standards of hygiene currently witnessed in neonatal care could possibly have reduced this vertical transmission of commensal bacteria.^[27,32] The bacterial composition of breast milk and infant feces is not related to the delivery method.^[33,34] As the results, we will determine the effectiveness of the two groups of bacteria in breast milk, vaginal delivery compared with cesarean delivery was not significantly different. Milk samples from elective however, not from nonelective mothers who underwent cesarean delivery contained another type of bacterial community than did milk samples from individuals vaginally born suggesting that it is not the operation *per se* but the lack of physiological stress or hormonal signals can influence the microbial transmission process to milk.^[21] Observed high variability from the profiles of fecal microbiota of many Infants born by elective cesarean delivery had

particularly low bacterial richness and diversity.^[22] As the present research showed, maternal age causes a reduction in the number of *Lactobacillus* in milk and the average number of *Lactobacillus* in breast milk compared with female infants to male infant were declining. More research in relation to these two factors is required.

CONCLUSION

The findings of this study about the breast milk *Lactobacillus* potential probiotic bacteria of healthy Iranian mothers suggested that the breast milk microbiome is significantly influenced by several factors. Because bacteria present in breast milk are among the very first microbes entering the human body, our data emphasize the necessity of understanding the biological role that the milk microbiome could potentially play for health later in life.

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