

ORIGINAL ARTICLE

Isolation of Lactobacilli and Bifidobacteria Species from Human Breast Milk

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ABSTRACT

Key words:

Breast milk,
Lactobacilli,
Bifidobacteria

Background: Bacterial colonization of the infant gut is a gradual process that exerts a strong influence on the health status of the host. The source of bacterial diversity in breast fed babies remains unclear. For many decades, breast milk has been regarded as a sterile body fluid which exerts its influence on the infant's microbiota environment via presenting only some growth factors and optimal conditions for helping the growth of bacteria. However, in recent years, breast milk has been hypothesized to be a source of commensal bacteria for the infant gut. **Objective:** This study aimed at searching for bacteria in breast milk to assess the role of breast milk as their probable source. **Methodology:** Samples of breast milk were obtained from 50 lactating women and were tested for the presence of different bacteria, using specific media and specific biochemical reactions. **Results:** Culture of the 50 breast milk specimens showed growth of different species of lactobacilli in 100% of the specimens and bifidobacteria in the milk of 14 mothers (28%). **Conclusion:** breast milk can be a source of lactobacilli and bifidobacteria for the infants.

INTRODUCTION

Bacterial colonization of the infant gut is a gradual process that exerts a strong influence on the health status of the host, since the members of the gut microbiota may contribute to the barrier effect against pathogens and to the maturation of the intestinal immune system¹. Traditionally, it has been considered that facultative anaerobic bacterial groups, such as streptococci, staphylococci, enterococci and lactobacilli together with some strictly anaerobic ones, especially bifidobacteria, are among the first colonizers in breast-fed infants². These bacterial groups may play an important role in the reduction of the incidence and severity of infections in the breast-fed infant as they have the ability to inhibit the growth of a wide spectrum of pathogenic bacteria³. Some studies have suggested that infants with delayed bifidobacterial colonization

and/or decreased bifidobacterial numbers may be more susceptible to a variety of gastrointestinal or allergic conditions^{4,5}.

The source of bacterial diversity in breast fed babies remains unclear. It has been suggested that neonates acquire lactobacilli by oral contamination with vaginal strains during delivery; However, the findings confirm, at the molecular level that the bacterial composition of breast milk and infant feces is not related to the delivery method⁶. The breast skin may be claimed to be the source; however this does not agree with the strict anaerobic nature of some bacteria like bifidobacteria for example⁷.

For many decades, breast milk has been regarded as a sterile body fluid which exerts its influence on the infant's microbiota environment via presenting only some growth factors and optimum conditions for helping the growth of these important groups of bacteria⁸.

However, in recent years, breast milk has been hypothesized to be a continuous source of commensal bacteria for the infant gut^{9,10}.

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This study aimed at searching for bacteria in breast milk to assess the role of breast milk as their probable source.

METHODOLOGY

Subjects:

50 Egyptian women attending the Breast Feeding Clinic at The Center of Social and Preventive Medicine (CSPM), Cairo University Hospitals, who were breastfeeding their infants, participated in the study.

Subjects were excluded from the study if there was:

- (1) Infant and/or maternal perinatal problems,
- (2) History of antibiotic intake in the previous month or
- (3) breast inflammatory condition like mastitis or breast abscess.

The study was approved by the Ethical Committee of the Faculty of Medicine, Cairo University and prior to their enrollment in the study, the mothers were informed of the investigational character of the study and they gave their informed consent.

A. Samples :

1. Samples Collection:

Samples of breast milk were obtained from the lactating women:

- The milk samples were obtained by manual expression using sterile gloves, and collected in two sterile containers; one of which contained 0.5 ml of thioglycolate transport medium to provide an anaerobic condition. Initially, the nipples and mammary areola were cleaned with soap and water, dried with sterile gauze then wiped with 70% alcohol. The first drops (~500µl) were discarded¹¹. The two specimens including the one collected on the transport medium were subjected to culture immediately after being delivered to the laboratory.
- Skin sampling was performed as a control; the areola, after treatment with alcohol was gently rubbed using sterile cotton swabs then plated on blood agar media. It yielded no bacterial growth.

2. Isolation and identification of bacteria:

Pour plate technique was used to isolate the organisms¹². One ml of the milk was inoculated into nine ml of Man, Rogosa and Sharpe (MRS) Broth (Oxoid, United Kingdom), well shaken and then they were serially ten folds diluted. One ml aliquot of the samples and dilutions were plated into MRS medium agar (MRS; Oxoid, United Kingdom) for isolation of lactobacilli, MRS-Cys agar plates supplemented with 0.05% (wt/vol) L-cysteine hydrochloride and 50 µg

mupirocin (Delchimica, Italy) per liter of MRS for isolation of bifidobacteria¹³.

Inoculated MRS and MRS- Cys plates were incubated anaerobically (85% nitrogen, 10% hydrogen, 5% carbon dioxide) at 37°C for 48-72 h in an anaerobic jar using Oxoid anaerogen compact gas packs (Oxoid, UK)¹².

Colonies of different morphologies and sizes growing on MRS and MRS- Cys agar were chosen and transferred to MRS and MRS- Cys broth for 24 to 48 h.

Bacterial isolates were characterized on the basis of their colony morphology, microscopic appearance after Gram staining (Gram positive) and Slide Catalase Test was done to verify the bacteria as Catalase negative.

3. Preservation of isolates:

The different isolates were preserved in MRS broth medium containing glycerol and stored at -80 °C until further testing. The glycerol stocks of samples were prepared by adding 0.5 ml of active cultures to 0.5 ml MRS medium containing 40% sterile glycerol and thioglycolate plus 0.5ml reconstituted autoclaved skimmed milk⁷

Further identification of the isolated organisms:

This was done by using the API identification strips (bioMérieux, Marcy l'Etoile, France).

API[®] 50 CH in combination with 50 CHL liquid media to help in the identification of lactobacilli, API[®] 50 CH , API[®] 20A and API[®] ZYM for the identification of anaerobic bacteria following the manufacturer protocol, then, by referring to the Analytical Profile Index and by using the identification software (Apiweb), the isolates were identified to the genus and sometimes to the species level.

The statistical analysis of the results:

Data were statistically described in terms of mean ± standard deviation (±SD), median and range, or frequencies (number of cases) and percentages when appropriate.

RESULTS

Culture of the breast milk specimens showed growth of lactobacilli in the milk of 50 mothers (100% of the study population) and bifidobacteria in the milk of 14 mothers (28%).

Each genus showed growth of one to five different species per mother's milk specimen as shown in table 1.

Table 1: Number of species of each genus per breast milk sample:

Genus	Number of species per specimen						Total breast milk samples
	0	1	2	3	4	5	
<i>Lactobacillus</i>	0	26(52%)	20(40%)	2(4%)	1(2%)	1(2%)	50 (100%)
<i>Bifidobacterium</i>	36 (72%)	5 (10%)	6 (12%)	1 (2%)	2 (4%)	0	50 (100%)

The total number of bacilli revealed from breast milk were **109** as follows:

- 81 lactobacilli and 28 bifidobacteria isolates.
- Lactobacilli were detected in the milk of 100% of the mothers participating in the study. The majority of the identified lactobacilli species belonged to the

species *L. fermentum-1* (40 %) and the species *L. brevis-2* (34%). The species of 24 lactobacilli isolates could not be speciated by API.

- The distribution and types of the 81 lactobacilli isolates among the 50 milk specimens are shown in table 2.

Table 2: Distribution of lactobacilli species in mothers' milk.

Genus	Culture positive milk specimens	Species	No.	Percent
<i>Lactobacillus</i>	50	<i>L. fermentum-1</i>	20	40%
		<i>L. brevis-2</i>	17	34 %
		<i>L. acidophilus</i>	6	12%
		<i>L. fructivorans</i>	4	8 %
		<i>L. plantarum-1</i>	4	8 %
		<i>L. brevis-1</i>	2	4%
		<i>L. delbrueckii ssp delbrueckii</i>	2	4%
		<i>L. delbrueckii ssp lactis</i>	2	4%
		unidentified	24	48%

Bifidobacterium bifidum was revealed from most of the bifidobacteria culture positive specimens; 11/14 (78.57%). The species of eight bifidobacteria isolates could not be identified by API.

The distribution and types of the 28 bifidobacteria isolates among the 14 positive milk specimens are shown in table 3.

Table 3: Distribution of bifidobacteria isolates among bifidobacteria positive breast milk specimens.

Genus	Culture positive milk specimens	Species	No.	Percent
<i>Bifidobacterium</i>	14	<i>B. bifidum</i>	11	78.57%
		<i>B. breve</i>	5	35.7%
		<i>B. dentium</i>	4	28.57%
		unidentified	8	57.14%

DISCUSSION

Bacterial colonization of the infant gut is a gradual process that exerts a strong influence on the health status of the host, since the members of the gut microbiota may contribute to the barrier effect against pathogens and to the maturation of the intestinal immune system¹. Traditionally, it has been considered that facultative anaerobic bacterial groups, such as streptococci, staphylococci, enterococci and lactobacilli together with some strictly anaerobic ones, especially bifidobacteria, are among the first colonizers in breast-fed infants². In concert, they create the condition required for the proliferation of anaerobic bacteria, which becomes predominant after weaning¹. These bacterial groups may also play an important role in the reduction of the incidence and severity of infections in the breast-fed infant as they have the ability to inhibit the growth of a wide spectrum of pathogenic bacteria either by competitive exclusion and/or through the production of antimicrobial compounds, such as bacteriocins, organic acids, or hydrogen peroxide³. The source of bacterial diversity in breast fed babies remains

unclear. It has been suggested that neonates acquire lactobacilli by oral contamination with vaginal strains during delivery. However, findings confirm that the bacterial composition of infant faeces is not related to the delivery method⁶.

For many decades, breast milk has been regarded as a sterile body fluid which exerts its influence on the infant's microbiota environment via presenting only some growth factors and optimum conditions for helping the growth of these important groups of bacteria⁸.

However, in recent years, breast milk has been hypothesized to be a continuous source of bacteria for the infant gut^{9, 10}.

The present study aimed at searching for bacteria in breast milk, to assess the role of breast milk as their probable source to the infant.

The study was conducted on 50 lactating Egyptian women with mean age of 28 ±5.09 years (range from 19 to 37 years), who were attending the Breast Feeding Clinic at The Center of Social and Preventive Medicine (CSPM), Cairo University Hospitals, in the period from March 2011 till May 2012. They participated in this

study by providing samples of breast milk in absence of all exclusion criteria.

In the present study, culture of the 50 breast milk specimens showed growth of lactobacilli and bifidobacteria in 100% and 28% of the specimens; respectively.

This is in partial agreement with Heikkilä and Saris⁹ in Finland who recovered lactobacilli in 3/40 (7.5%) .

Also, in their study, Mehanna *et al.*¹⁴ recovered lactobacilli representing 55.2% of the total isolated bacteria from 30 breast milk specimens.

Lactobacilli were detected in the milk of 100% of the mothers participating in the study. The majority of the identified lactobacilli species belonged to the species *L. fermentum-1* (40%) and the species *L. brevis-2* (34%), followed by *L. acidophilus*, *L. fructivorans*, *L. plantarum-1*, *L. brevis-1* and *L. delbrueckii*.

Concerning the species recovered in the present study, there is a partial agreement with Martín *et al.*¹⁰, who isolated two *Lactobacillus gasseri* and one *Lactobacillus fermentum* strains from the eight breast-milk samples. A partial agreement also exists with Mehanna *et al.*¹⁴, where *L. rhamnosus* was the most frequently isolated strain from the breast milk of 30 women (20.8%); followed by *L. plantarum* (13.2%) then *L. casei* and *L. fermentum* (each, 8.8%) and in a minor percentage, the strains of *L. acidophilus*.

Contrary to our results, different lactobacilli species have been recovered from the breast milk by Heikkilä & Saris⁹ in Finland, where *L. crispatus* and *L. rhamnosus*, were the isolated lactobacilli species. They explained that *L. crispatus* is the predominant vaginal *Lactobacillus* in healthy women; accordingly, infants might have derived it from the vagina during delivery and then transmitted it to the maternal breast skin during nursing. Furthermore, their study of the *L. rhamnosus* isolates from two samples by RAPD, demonstrated a profile identical to the commercial strain *L. rhamnosus* GG, which is a commonly used probiotic strain in the milk products in Finland.

Different lactobacilli species were also recovered by Martín *et al.*¹⁵, who isolated a *L. salivarius* strain from human milk and infant feces of a mother-child pair.

The present study recovered *Bifidobacterium bifidum* as the predominant species in the bifidobacteria culture positive milk specimens; 11/14 (78.57%). *B. breve* and *B. dentium* were also detected. Our results are in partial agreement with Martín *et al.*¹¹, where isolates belonging to three bifidobacterial species were isolated from milk samples: *B. breve* (50%), *B. adolescentis* and *B. bifidum* (25% each). According to Martín *et al.*¹¹, isolation of some bifidobacterial species was more difficult (*B. adolescentis* and *B. bifidum*) or even impossible (*B. longum* and *B. pseudocatenulatum*) as compared to culture independent methods. This may

reflect the fastidious growth requirements of some bifidobacterial species and/or their relatively low concentrations in the samples.

Also, when sampling procedures took care of preserving samples from oxygen, isolation of bifidobacteria was achieved in a large number of samples. A finding which shows that sample preservation is of utmost importance when detection of bifidobacteria is undertaken through cultural techniques.

Most of the specimens revealed growth of a single species of the corresponding genus. However, some samples showed the growth of more than one species (up to five different species in the same specimen), making the total number of isolates retrieved from breast milk to equal to 109 (81 lactobacilli and 28 bifidobacteria).

This is in agreement with Gueimonde *et al.*⁷, who found more than one bifidobacterial species in the same breast milk sample, with a mean of 2.55 species present per sample and Grönlund *et al.*¹⁶, where the numbers of bifidobacteria species varied from one to five in breast-milk and infants' stool samples.

In Gueimonde *et al.*⁷ study, *Bifidobacterium bifidum* was detected in 46% of 20 milk samples.

In their study on *B. bifidum* isolated from breast milk, Martín *et al.*¹¹ recovered *B. bifidum* by culture from two milk samples out of eight samples positive for *B. bifidum* DNA by RT-PCR.

The elucidation of the origin of the bacteria present in breast milk has been an attractive research target.

Traditionally, it was considered that they are acquired by skin contamination during sampling of breast milk. However, since bifidobacteria belongs to a strictly anaerobic genus, this source seems very unlikely⁷. Moreover, it has been reported that lactobacilli and enterococcal isolates present in human milk are genotypically different from those isolated in the skin, within the same bacterial species and the same host¹⁷.

Another theory is that inhabitants of the oral cavity transfer from the infant's mouth to the milk ducts and consequently to breast-milk samples before analysis. However, it has been shown that breast-milk contains similar bacteria both before and after breastfeeding¹⁸.

The suggestions that the origin of the live bacteria found in breast milk could be the maternal gut and that the bacteria arrive at the mammary gland through an endogenous route (the so-called entero-mammary pathway) involving maternal dendritic cells and macrophages has been confirmed¹⁹. Dendritic cells can penetrate the gut epithelium and take up commensal bacteria directly from the gut lumen²⁰. With pregnancy, the breast-milk lymphocytes express intestinal-homing receptors as many as 20 times compared with the blood-derived lymphocytes²¹.

CONCLUSION

Human breast milk has been found to have different species of lactobacilli and bifidobacteria.

RERERENCES

1. Newburg DS and Walker WA. Protection of the neonate by the innate immune system of developing gut and of human milk. *Pediatr. Res.* 2007; 61: 2-8
2. Favier CF, De Vos WM and Akkermans AD . Development of bacterial and bifidobacterial communities in feces of newborn babies. *Anaerobe* 2003; 9: 219-229
3. Medellín-Peña MJ and Griffiths MW. Effect of Molecules Secreted by Lactobacillus acidophilus Strain La-5 on Escherichia coli O157:H7 Colonization. *Appl. Environ. Microbiol.* 2009; 75: 1165–1172
4. Arvola T, Ruuska T, Keränen J, Hyöty H, Salminen S and Isolauri E Rectal bleeding in infancy: clinical, allergological, and microbiological examination. *Pediatrics* 2006; 117: 760-768
5. Mah KW, Björkstén B, Lee BW, van Bever HP, Shek LP, Tan TN, Lee YK and Chua KY. Distinct pattern of commensal gut microbiota in toddlers with eczema. *Int. Arch. Allergy Immunol.* 2006; 140: 157-16
6. Martín R, Heilig HG, Zoetendal EG, Smidt H and Rodríguez JM. Diversity of the *Lactobacillus* group in breast milk and vagina of healthy women and potential role in the colonization of the infant gut. *J. Appl. Microbiol.* 2007; 103: 2638-2644
7. Gueimonde M, Laitinen K, Salminen S and Isolauri E. Breast-milk: a source of bifidobacteria for infant gut development and maturation? *Neonatology* 2007; 92: 64–6
8. Lawrence RM. Host resistance factors and immunologic significance of human milk. In Lawrence RA & Lawrence RM (eds.): *Breastfeeding: A Guide for the Medical Profession*. 6th edition. St Louis, Mosby; 171-214, 2005
9. Heikkilä MP and Saris PEJ. Inhibition of *Staphylococcus aureus* by the commensal bacteria of human milk. *J. Appl. Microbiol.* 2003; 95: 471-478
10. Martín R, Langa S, Reviriego C, Jiménez E, Marín ML, Xaus J, Fernández L and Rodríguez JM. Human milk is a source of lactic acid bacteria for the infant gut. *J. Pediatr.* 2003; 143: 754-758
11. Martín R, Jiménez E, Heilig H, Fernández L, Marín ML, Zoetendal EG and Rodríguez JM. Isolation of Bifidobacteria from Breast Milk and Assessment of the Bifidobacterial Population by PCR-Denaturing Gradient Gel Electrophoresis and Quantitative Real-Time PCR. *Appl. Environ. Microbiol.* 2009; 75: 965–969
12. Turrone F, Foroni E, Pizzetti P, Giubellini V, Ribbera A, Merusi P, Cagnasso P, Bizzarri B, Luigi de'Angelis G, Shanahan F, van Sinderen D and Ventura M. Exploring the Diversity of the Bifidobacterial Population in the Human Intestinal Tract. *Applied and Environmental Microbiology* 2009; 75: 1534-1545
13. Simpson PJ, Fitzgerald GF, Stanton C and Ross RP. The evaluation of a mupirocin-based selective medium for the enumeration of bifidobacteria from probiotic animal feed. *J. Microbiol. Methods* 2004; 57: 9-16
14. Mehanna NSH, Tawfik NF, Salem MME, Effat BAM and Gad El-Rab DA. Assessment of Potential Probiotic Bacteria Isolated from Breast Milk. *Middle-East Journal of Scientific Research* 2013; 14: 354-360
15. Martín R, Jiménez E, Olivares M, Marín ML, Fernández L, Xaus J and Rodríguez JM. Lactobacillus salivarius CECT 5713, a potential probiotic strain isolated from infant feces and breast milk of a mother-child pair. *Int. J. Food Microbiol.* 2006; 112: 35-43
16. Grönlund MM, Gueimonde M, Laitinen K, Kociubinskiz G, Grönroos T, Salminen S and Isolauri E. Maternal breast-milk and intestinal bifidobacteria guide the compositional development of the Bifidobacterium microbiota in infants at risk of allergic disease. *Clinical and Experimental Allergy* 2007; 37: 1764–1772
17. Lei V. *Probiotic potential of African fermented millet*. PhD Thesis. Department of Food Science, Food Microbiology, Royal Veterinary and Agricultural University, Denmark, 2006
18. Martín R, Langa S, Reviriego C, Jiménez E, Marín ML, Olivares M, Boza J, Jiménez J, Fernández L, Xaus J and Rodríguez JM. The commensal microflora of human milk: new perspectives for food bacteriotherapy and probiotics. *Trends Food Sci. Technol.* 2004; 15: 121-127
19. Perez PF, Doré J, Leclerc M, Levenez F, Benyacoub J, Serrant P, Segura-Roggero I, Schiffrin EJ and Donnet-Hughes A. Bacterial Imprinting of the Neonatal Immune System: Lessons From Maternal Cells? *Pediatrics* 2007; 119 (3): 724-732
20. Rescigno M, Urbano M, Valzasina B Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl JP and Ricciardi-Castagnoli P. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol.* 2001; 2: 361–367
21. Sabbaj S, Ghosh MK, Edwards BH, Leeth R, Decker WD, Goepfert PA and Aldrovandi GM. Breast milk-derived antigen-specific CD8(1) T cells: an extralymphoid effector memory cell population in humans. *J. Immunol.* 2005; 174: 2951–295