
Original Research Article

Human Milk's Microbial Population Behavior *In Vivo* and *Ex Vivo*

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ABSTRACT

The mobility of human milk microbial community the Armenian women has been studied *in vivo* and *ex vivo*. Human milk temporary microbes' quantity was varied within stages of lactation. Culture based investigations revealed the greater microbial amount in colostrum and transitory milk means 10^5 CFU/ml, then it gradually reduced and reached 10^2 in mature milk. As the predominant microbes are able to ferment cow's milk, it means that they belong to the genus of lactic acid bacteria. The size of the microbial population varied depending on the frequency of feeding, with a delay in feeding to 12 hours, it became about 1 log higher than the mean maximum. Examination of residential microbes' growth character in different intact milk samples *ex vivo* at 37°C in under anaerobic conditions have conditions revealed that the composition of human milk selectively promotes the growth of lactic acid bacteria whose titer can reach up to 10^{11} CFU / ml. The development of lactic acid bacteria successfully fermenting cow's milk does not cause visible coagulation of human milk, possibly due to the low concentration of casein proteins and the presence of anticoagulants. Since, probiotic bacteria thrive better in human milk than others; they will more effectively colonize breast feed infants intestine.

Key Words: Human milk microbiota, lactic acid bacteria, *in vivo*, *ex vivo*

INTRODUCTION

Breast milk provides a neonate not only with a unique combination of proteins, carbohydrates, lipids, minerals and vitamins but it also contains probiotic bacteria responsible for a wide range of beneficial effects on infants such as the protection against infections by colonization of gut ecosystem and the promotion of immune system maturation. [1,13] The first fluid produced by mothers after delivery is colostrum, which is distinct in volume, appearance and composition. Colostrum, produced in low quantities in the first few days postpartum, is rich in immunologic components such as secretory IgA, lactoferrin, leukocytes, as well as developmental factors such as epidermal

growth factor. Colostrums also contain relatively low concentrations of lactose, indicating its primary functions to be immunologic and trophic rather than nutritional. Transitional milk shares some of the characteristics of colostrum but represents a period of increasing milk production to support the nutritional and developmental needs of the rapidly growing infant, and typically occurs from 5 days to two weeks postpartum, after which milk is considered largely mature. By four to six weeks postpartum, human milk is considered fully mature. [14] In contrast to the dramatic shift in composition observed in the first month of life, human milk remains relatively similar in composition, although subtle changes in milk

composition do occur over the course of lactation. Human milk composition is dynamic, and varies within a feeding, diurnally, over lactation, and between mothers and populations. [3,5,6]

It was long believed that human milk was sterile, but it is now recognized that human milk harbors a microbial community, the composition of which appears to change from colostrum to late lactation, and varies within feeds, diurnally, and between mothers. [9,12,16] While many studies provide clear evidence that human milk contains indigenous bacteria, very little work has examined the microbial population behavior *in vivo* among lactating women and identified the predominant species finally passed to infants.

The aim of this study is to investigate the variability of human milk temporary microbiota's amount and composition within lactation stages and diurnally in mammary gland and in human milk *ex vivo* and identified predominant species intended to infant gut colonization.

MATERIALS AND METHODS

Media: MRS broth and agar (Himedia); Skimmed milk powder; carbohydrates; Methyl blue; Bile powder (Himedia); L-arginine hydrochloride (Sigma-Aldrich); Nessler reactive (Himedia), H₂O₂, Oxidize discs (Himedia), Gram Staining Set (Ghatran, IIR), pH Checker meter (Hanna).

For milk sampling, nipple and mammary areola of the breast was clean with soap and sterile water, and then chlorhexidine was applied. The first drops (approximately 0.5 ml) were discarded to avoid any contamination. The breast milk sample was collected in a sterile tube after manual expression using sterile-gloves. The tubes containing samples were kept in ice during transportation to the laboratory for less than two hours.

For bacterial count breast milk samples was serially diluted in skim milk and 0.1 ml were pure plated in MRS agar then all test-tubes and plates were incubated

at 37°C. The global titer of bacteria was numerated by colony counting and expressed in CFU/ml. The number of bacteria able to ferment cow milk was defined by counting test-tubes with curdled milk and expressed in logarithms. The isolates were identified according to Bergey's manual of systematic bacteriology (colony morphology, gram staining and catalase reactions, growth in 4-6.5 % NaCl and 20-40 % bile salt, at pH 8.3-9.6, formation of NH₃ from arginine, growth with 0,1 % methylene blue in milk, by growth temperatures range and utilization of carbohydrates) [10] and RAPD – PCR.

Statistical analysis of the obtained data was carried out using the Student's computer test, taking the level of $p < 0.05$ sufficient for a reliable difference in the results.

RESULTS

Human milk microbiome variations from lactation stages

Human milk bacterial community size and composition variations from colostrum to late lactation were studied. To determine total cultivating bacteria amount, serial 10 fold dilutions of breast milk samples from colostrum, transitory and mature stages were pure plated in MRS and inoculated in cow milk. Dates obtained by colony count in Petri dishes with MRS and test tube count with fermented milk are presented in Figure 1.

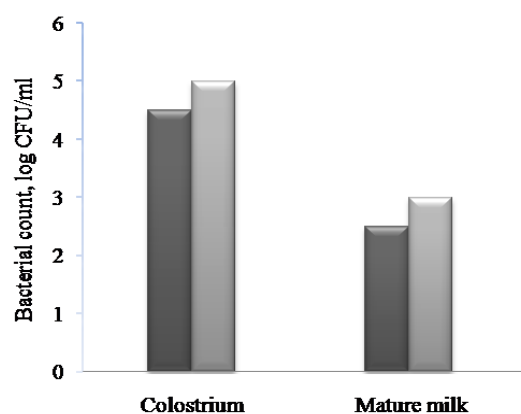


Figure 1: Bacterial count of colostrum and mature human milk obtained by MRS (■) and skimmed milk (□).

Breast milk bacterial titers obtained in both cases by colony count on MRS and number of test-tubes with fermented cow's milk do not significantly differ, which indicates that lactic acid bacteria are predominant in maternal milk. Moreover, all randomly tested colonies picked from MRS possesses the ability to ferment cow's milk of woman the amount of bacteria in colostrum and transitory milk was mean 5×10^4 CFU/ml it gradually dropped and reached in the mature milk $(2- 5) \times 10^2$ CFU/ml and not significantly changed in entire period of lactation (Fig. 1).

Influence of breastfeeding interval on amount of commensal microbes in human milk

The size of bacterial community in the breast depends on women constitution and varies from nursing cycle. Common cycle of breastfeeding is on average three hours. The variability of bacterial population in mammary glands depending on feeding interval was studied. For this, a woman in the second month of lactation was asked not to breast-feed or filter milk for 12 hours before taking the sample.

The first sample was taken just before of feeding the second half an hour after feeding and the third one after 12 hours interval from the same breast. Colony count obtained from the samples serial dilution's plating on MRS agar in presented in Figure 2.

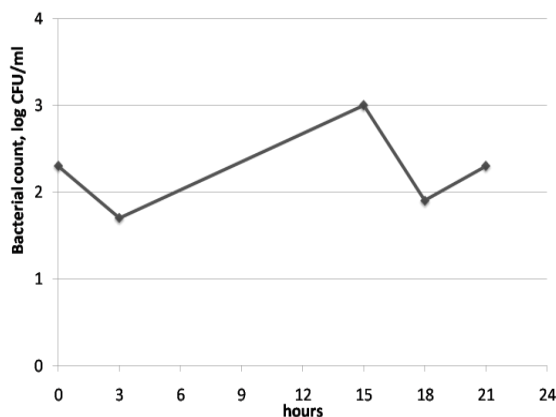


Figure 2. Bacterial count variations in human milk in vivo.

Prior to feeding, the amount of lactic acid bacteria in the mature milk was an average of

5×10^2 , after feeding, it dropped approximately 10-fold, and then at the end of the 12-hour pause in breastfeeding, the bacteria titer increased up to 10^3 CFU/ml.

Growth of residential microorganisms in human milk: Ex vivo study

The growth of resident microorganisms in human milk in thermostat at 37 °C has been studied. The bacterial count was determined every 12 h for three days by simultaneously plating of 10 fold serial dilutions on MRS agar and inoculation in cow's milk (Fig. 3).

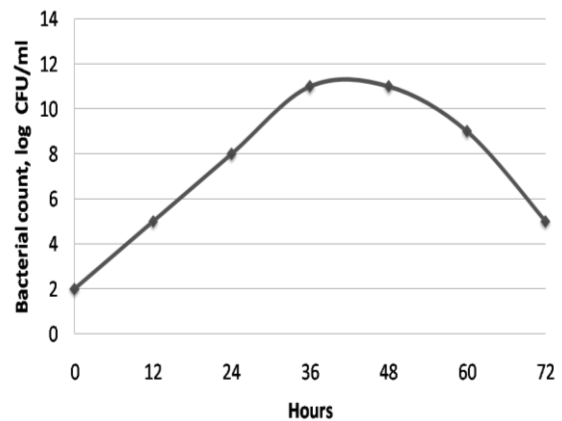


Figure 3. Growth of resident microbes in human milk ex vivo

As seen from the growth curves, the initial amount of bacteria in the mother's milk was about mean 2.5×10^2 CFU/ ml, on the next day of incubation it became 6.7×10^7 and reached maximum 3.5×10^{11} CFU/ml for 36 h, after about 12 h stationary phase the titer sharply dropped in about 7 log. It is important to note that all entire period of incubation maternal milk was not fermented despite the intensive growth of lactic acid bacteria. From comparison in vivo and ex vivo data (Fig. 2 and 3), it is evident that in the same 12 hours the number of bacteria in the breast capillaries grows by only 1 log, whereas in a test tube by 5 log. Such a significant difference can be explained by the limited space of the mammary glands capillaries and by the constant intake of freshly formed milk.

The lactic acid bacteria isolated from the breast milks in *ex vivo* experiments were identified by Bergey's manual and RAPT-PCR as: *L. fermentum*, *L. reuteri*, *L. casei*, *L. salivarius*, *L. delbrueckii*, *L. lactis*, *Str. thermophiles*, *L. raffinolactis* and *L. cremoris*. In some of *ex vivo* cultures, probably due to symbiosis and synergism, more than one species become predominant.

DISCUSSION

Examination of breast milk from hundreds of women revealed the presence of significant probiotics, including bifidobacteria, *L. reuteri* and other lactobacilli, [19] but the populations of probiotic bacteria in the women's milk varied among countries and between urban and rural subjects. [7] In some regions, only 20 percent of the milk samples showed colonies of these bacteria, while in others it was up to 100 percent. One of the reasons for the lack or minorities of probiotic bacteria in breast milk may be the formula feeding of the mother in childhood and/or low level of consumption of fermented dairy products. Thus, intestinal microflora transmitted from generation to generation through breast milk and once provides interrupted there remains little chance for further restoration. One of the ways to fix the situation is intensive consumption of special probiotics isolated from healthy women's milks in last trimester of pregnancy. [11,17] The high concentration of LABs in milk from healthy mothers strongly suggests that they may play an important biological role during the first months of life.

Molecular-biological studies distort the biological meaning and ancestral purpose of the microflora of breast milk. Because the culture-based analysis only considers viable cells, whereas molecular analysis includes all live, dead, destroyed microorganisms and even DNA fragments. [4] The core milk microbiome existed among lactating women on the way to the infant's intestine passes through several control points: 1) it is suggest that certain bacteria

from the maternal gastrointestinal tract should translocate through a mechanism involving mononuclear immune cells, migrate to the mammary glands via an endogenous cellular route (the bacterial entero-mammary pathway); [18] 2) Breast milk contains antibacterial compounds such as lysozyme, lactoferrin and other scavengers of pathogenic microorganisms and parts of therein; [15] 3) Infant's gastric acidity average pH 4.0 is hostile for non-friendly bacteria but not for LABs and 4) acid-resistant LABs due to acidification of the environment and production of different antimicrobial substances eliminate other microorganisms and become predominant in infant intestine. [8]

The multistage hurdles through which the breast milk predominant bacterial species pass can be one of the main reasons why the intestinal microbiota of infants consists of a relatively narrow spectrum of Gram-positive bacterial species and why a more diverse microbiota develops only after the progressive withdrawal of breast milk. Once in the infant gut, such bacteria may contribute to the protective effect that breastfeeding confers against infectious diseases. The commensal probiotic bacteria can express antibacterial activity even in the breast milk. In addition, the presence of lactobacilli in breast milk could also explain, at least partially, the existence of prebiotic oligosaccharides in this fluid since the synthesis of such compounds by fructosyl and glucosyl transferases from human lactobacilli has been demonstrated recently. The human milk oligosaccharides influence intestinal colonization and may also effect influences the bacterial community composition of milk. [2]

Most Gram-negative bacteria are not susceptible to the action of lysozyme alone because their outer membrane prevents access of the enzyme to the peptidoglycan layer. However, this barrier is overcome in the innate immune systems by the production of lactoferrin which permeabilizes the outer membrane. It was also found, that Gram-positive lactic acid bacteria isolated

from human origin possesses high resistance to lysozyme. Two mechanisms are proposed to explaining this phenomenon: 1) production of high specific bacterial proteinaceous lysozyme inhibitors and/or 2) modification of cell wall peptidoglycan the target of lysozyme.

It is known that lactobacilli coagulating cow's milk by producing lactic acid and when acidity reaches pH 5.5, casein micelles formed and by incorporation of water molecules form solid gels. Concentrations of the different proteins in milk are important for the outcome of coagulation processes. Several factors could be reasons for the lack of the gel in human milk. The relatively low concentration of casein proteins (0.4%), while in cow's milk - 2.5%. Beside these, cow's milk per se is more acidic (pH 6.8) than breast milk (pH 7.3 ± 0.19) and also titrable acidity is much higher 18-20 °T vs 5-6 °T. Breast milk has also poor (0.3) buffering capacity, compared with formula milk (1.2), and this leads to market differences in the pH of the colon of breast and formula - fed infants (5.1 and 6.5), respectively. This low pH promotes the growth of several *Bifidobacterium spp.* and lactobacilli, but it inhibitory to many other bacteria.

CONCLUSIONS

In human milk the highest amount of LABs as predominant species obtained in colostrum and in transitory milk mean 10^5 gradually reduced to mean 10^2 CFU/ml in mature milk. The resident bacteria from all investigated human milk samples of 10^{-4-2} dilutions were able to ferment cow's milk but not human one. *Ex vivo* incubation at 37°C promotes the rapid growth of resident bacteria exclusively LABs up to 10^{11} CFU/ml where one, rare two of them become predominant.

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