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Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN
0976-9595
Research Article

A TEMPORARY STABILITY AND VERTICAL LACTATING MOTHER-INFANT TRANSFER OF BREAST MILK MICROBIOTA THROUGH BREASTFEEDING

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ABSTRACT

Human milk consists of a diverse microbial community i.e. human-friendly probiotics and commensalbacteria. The current study explored whether viable breast milk microbes are shared between the maternal and infant gut ecosystem via breastfeeding. Healthy lactating mother milk and corresponding neonatal faeces collected from five mothers-neonate pairs at every week's time interval were studied by culture-dependent methods (16S rRNA gene sequencing). Bythe culture-dependent method, a viable isolated strain of *Lactobacillus oris, Lysinibacillus sp., Enterococcus mundtii, Bacillus clausii, Enterococcus faecalis, Lactobacillus brevis, Lysinibacillus fusiformis,* and *Staphylococcus sp.* was revealed to be shared among both ecosystems within mother-neonate pair. This study shows that viablemicroflora may be vertically transmitted from mother to infantthrough breastfeeding. Hence, our data support the proposed hypothesis of a newpathway of mother-infant communication, in thatmother gut microflora reaches breast milk by an entero-mammary pathway to develope neonatal gut microflora and maturation of the immune system.

Keywords: Human milk, Infant faecal, Microflora, Culture-dependent method

1. INTRODUCTION

Breast milk is the best food for infants as it provides the complete nutritional supplement for their growth. It protects the newborn against intestinal diseases like diarrheal [1], reduce the risk of eczema in infants [2], respiratory diseases [3] and reduced enduring risk of obesity [4]. The protective role of human milk seems to be the consequence of a synergistic action of the wide range of health-promoting components such as carbohydrates, nucleotides, fatty acids, immunoglobulins, cytokines, immune cells, lysozyme, lactoferrin, bacteriocins and, other immunomodulatory factors [5, 6]. Breast milk has been described as a source of bacteria influencing the development of the infant gut microbiota. Bacteria that are commonly found in human milk include Staphylococci, Streptococci, Lactobacilli, Lactococci, Enterococci and Bifidobacteria [7-9]. These bacteria may play an important role in the reduction of the incidences and severity of infection to the child due to their probiotic properties using specific mechanisms i.e. probiotics are able to secrete antimicrobial substances like bacteriocin which acts as antagonists against pathogenic bacteria and their effectual antagonistic activity alone or synergistically. These antimicrobial compounds can be protein molecules and bioactive peptides. Bacteriocins are a significant antimicrobial peptide that has been demonstrated to have efficient therapeutic activity against intestinal pathogenic infection [10]. They also produce metabolites like acetic and lactic acids that decrease the pH in the intestine and making unfavourable environmental condition for the pathogen to survive [11]. Probiotics can eradicate pathogens using competitive exclusion and/or blocking the invasion of them at the infection site i.e. intestinal epithelium cells through competing for the glycoconjugate receptors [12]. Also, competition for vital nutrients is observed between probiotics and pathogens which depends on the pace of nutrient absorptions, the innate metabolic capacity, the growth rate and the secretion of specific inhibitors [13]. Although breast milk bacteria may be helpful for the infant's health, some of the pathogenic bacteria are also present in the milk which may be harmful to the infants or mother.

The human milk microbiome is established by possible mechanisms. Physiological and hormonal changes occur during and after pregnancy leading to increase gut permeability which helps in the migration of gut microflora to the mammary gland. In addition to dendritic cells and macrophages, Living bacteria also play a role in the transportation of microbes to the mammary gland [14]. Besides all above probable mechanisms, the retrograde flux, the mother's skin microbes and infant's oral microbes may contribute to the development of the human milk microbiome [15-18].

Somatic cells are mainly milk-secreting epithelial cells that have been shed from the lining of the gland and white blood cells (leukocytes) that have entered the mammary gland in response to injury or infection (Dairyman's digest, 2009). Milk somatic cells include 75% leucocytes, i.e. neutrophils, macrophages, lymphocytes, erythrocytes, and 25% epithelial cells. Erythrocytes can be found at concentrations ranging from 0 to 1.51×10^6/ml [19]. Normally, somatic cell count from the milk of a healthy mammary gland is lower than 1×10^3 cells/µl, upon bacterial infection can cause it to increase to above 1×10^4cells/µl [20].

In 2003, the first description of the bacterial diversity of human milk from healthy women was reported which was based on *in vitro* culturing methods [8]. During the last decades, microbiological studies that focused on human milk were restricted to the identification of potentially pathogenic bacteria in stored milk or milk retrieved from maternal infected breast milk but microbes present in healthy mother breast milk were unexplored. Also, studies on human milk carried out in India were restricted to the isolation of beneficial bacteria from breast milk or studying oligosaccharides present [21]. In the present study, the effort is been made to explore the bacterial diversity in healthy mother milk and corresponding infant feces using culture-dependent method (16S rRNA gene sequencing)

2. MATERIAL AND METHODS

2.1. Samples collection from lactating mother and neonates

Healthy mothers carrying a healthy baby and planning to deliver vaginally and to exclusively breastfeed during the neonatal period were recruited for this observational clinical study at the Suruchi Hospital and Krishna Hospital (Nadiad, Gujarat). Exclusion criteria were preterm and/or cesarean delivery, any formula feeding, as well any variables known to affect the balance of the maternal and/or neonatal microbiota, such as gastrointestinal and immunological disorders, and drug administration during the neonatal period (mother and/or neonate) and at least four months prepartum. Written informed consent was obtained from all mothers.

Breast milk, maternal and neonatal fecal samples were successfully collected between 25-30 days postpartum from six mother-neonate pairs. The current study was piloted according to the ethical guidelines of 1975. Declaration of Helsinki and the procedure was approved by the ethical committee of Govindbhai Jorabhai Patel Ayurveda College and Surajben Govindbhai Patel Ayurveda hospital (Approval No- IEC-3/GJPIASR/2015- 16/E/3). Fresh feces were collected into fecal collection containers. Breast milk was collected using a sterile electrical breast pump after the rejection of the foremilk and cleaning of the breast with aseptic soap. To minimize exposure to oxygen, 5-15 ml of breast milk were injected with a sterile syringe falcon tube. Samples were transported at 4°C and processed within 4 h.

2.2. Isolation of bacteria from all collected healthy breast milk and their baby faecal samples

Breast milk and fecal aliquots of 2 ml and 0.5g respectively, were immediately subjected to culture, while further aliquots were stored at -80°C. Bacterial species were isolated from the all collected healthy breast milk and their baby fecal samples by serial dilution and agar plating method wherein the milk and their baby fecal samples were diluted from 10^{-1} to 10^{-5} dilutions, and the diluted milk samples and their baby fecal samples were spread on sterile nutrient agar (NA) plates. The inoculated plates were incubated at 37°C for 24 hours. Mixed cultures obtained after incubation were purified by quadrant streaking on sterile NA plates and also colony-forming unit (CFU) was calculated. The purity of cultures was cross-checked by gram staining procedure.

2.3. Identification of the Bacillus Strain

The isolates were scrutinized for gram natures, cell shape, and colony morphology and catalase reactions. Further isolates were more characterized by physiological and biochemical analysis [20]. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using two universal primer 8F (5′AGAGTTTGATCCTGGCTCAG3′) and 926R (5′CCGTCAATTYYTTTRAGTTT3′), where Y symbolizes for pyrimidine which is either C or T, and R

symbolizes for purine which is either G or A. Each PCR mixture (25 µl) consisted of 2.5 µl PCRTaq Buffer with $MgCl_2$, 2.5 µl 2.5mM dNTP mix, 0.3 µl 0.2 U of Taq DNA polymerase (Bangalore Genei, India), 1 µl of each forward and reverse primer, and 16.7 µl water. Thermal cycler settings included a3 mins for initial denaturation 94°C, subsequently 32 cycles of denaturation 40s at 94 °C, 45s at 54 °C, and 75s at 72 °C and a final extension at 72°C for 3 min. Amplified product (ca. 900 bp) was confirmed by electrophoresis on 1.5% agarose gel and visualized by staining with ethidium bromide under UV transilluminator. Amplified PCR products were purified with a Qiagen purification kit and sequencing of the amplicon was performed in Xcleris, Ahmedabad. GenBank database was used for searching sequences similar to the 16S rRNA gene. The analysis of alignment and homology of the partial nucleotide sequence of isolates was analyzed by the basic local alignment search tool (BLAST). Sequences were then submitted to Genbank. The multiple distance matrix obtained was then used to construct phylogenetic trees using Hasegawa, Kishino, and Yano maximum likelihood method in Mega X software. The tree topology was tested by bootstrap analysis with 1000 replicates. Further, the microbe's stability and transferring were analyzed.

3. RESULTS

A Somatic cell count (SCC) of all the milk samples was checked to know the presence of any infectious conditionin the mammary gland. Somatic cell count $>10^4$ cells/ µl is considered as an infectious condition in the mammary gland [20]. Thus, breast milk samples with SCC less than 10^4 cells/µl were considered for further bacterial analysis. Five healthy mother milk along with their baby fecal at an interval of one-week samples were subjected to cultural isolation. SCC of 20 healthy mother milk samples was represented in Table 1.

Table1: A somatic cell count of healthy mother milk

Sample no.	H1				H2				H3				H4				H5			
Time interval (week)	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
SCC (cells/µl)	52	66	57	63	93	110	117	104	198	142	135	108	63	96	98	121	163	201	168	186

Table 2: Biochemical analysis of isolates

Isolate no.	HM1	HM2	HM3	HM4	HM5	HM6	HM7	HM8	HM9	BF1	BF2	BF3	BF4	BF5	BF6	BF7	BF8	BF9
Gas producing from glucose	+	+	-	+	+	+	+	-	+	+	-	+	+	+	-	+	+	+
O-F test	+	+	-	-	-	+	-	+	-	+	-	-	+	-	+	-	-	+
Methyl red test	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	+	-
V-P test	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-
Indole test	-	-	-	-	-	-	+	+	-	-	-	-	-	+	+	-	-	-
Citrate utilization test	+	+	-	+	-	-	-	+	-	+	-	+	-	-	+	-	-	+
H_2S production test	-	-	-	-	+	+	+	-	+	-	-	-	+	+	-	+	+	-
Deamination test	+	-	+	-	-	+	-	+	-	-	+	-	+	-	+	-	-	+
Urea hydrolysis test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ammonia production test	+	-	+	-	+	+	-	+	-	-	+	-	+	-	+	-	+	+
Gelatine hydrolysis test	+	+	-	-	-	+	+	-	-	+	-	-	+	+	-	-	-	+
Starch hydrolysis test	+	-	-	+	-	+	-	-	-	-	-	+	+	-	-	-	-	+
Casein hydrolysis test	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+
Catalase test	+	+	+	+	-	+	-	+	-	+	+	+	+	-	+	-	-	+
Hemolysin test	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+
Triple sugar iron agar test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol salt agar test	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-

Cultivable bacterial diversity from 20 healthy mother milk and their baby fecal samples was investigated by plating different dilutions of milk on NA plates. An average of 2.0×10^4 colony-forming units per millilitre of cultivable bacteria was recovered from healthy mother milk along with their baby fecal samples. Bacterial isolates were first screened noting colony characteristics, gram natures and pigmentation. On the basis of colony morphology found in both sample (breast milk and baby fecal), 9 isolates from both the samples were comprised of discrete colony features that were nominated for further study. These 9 isolates were found in every week collected milk sample as well as they also found in baby fecal. Afterward, biochemical characterization of 18 isolates was performed and obtain results are provided in Table 2.

Antibiotic sensitivity is the susceptibility of bacteria to antibiotics. Antibiotic susceptibility testing (AST) is usually carried out to determine which antibiotic will be most successful in treating a bacterial infection *in vivo*. Testing for antibiotic sensitivity is often done by the Kirby-Bauer method. Small wafers containing antibiotics are placed onto a plate upon which bacteria are growing. If the bacteria are sensitive to the antibiotic, a clear ring, or zone of inhibition, is seen around the wafer indicating poor growth Bacterial identification and antibiotic susceptibility testing play an essential role in patient care and the control of antibiotic resistance by indicating which antibiotics are most likely to cure an infection and reducing the empirical prescription of "broad-spectrum" antibiotics, which are partly responsible for the rapid increase in antibiotic resistance.

The zone of clearance varies depending upon the antibiotic and its chemical properties. The medical effectiveness of antibiotics in patients has been correlated with a certain zone size and this is used to decide whether an antibiotic will be useful for treating test microbes.

The response of isolated microbes against different antibiotics was assessed by performing an antibiotic susceptibility test. Table 3 represents the percent sensitivity of bacteria against different antibiotics. Least sensitive antibiotics include Cefuroxime, Penicillin-G, Ampicillin, Ceflazidime and Cefoperazone. Antibiotics that give moderate sensitivity include Cefadroxil, Cefotaxime, Roxithromycin, Gentamicin, Netillin, Clarithromycin and Chloramphenicol. Antibiotics like Azithromycin, Amikacin, Co-Trimoxazole, Norfloxacin, Ciprofloxacin and Sparfloxacin proved to be the most efficient antibiotics for human microbes.

Table 3: Percent sensitivity of isolates against different antibiotics

Antibiotics	No. of sensitive isolates	% Sensitivity
Cefuroxime	2	11.11
Penicillin-G	3	16.67
Ampicillin	4	22.22
Ceflazidime	5	27.78
Cefoperazone	6	33.33
Cefaclor	8	44.44
Cefadroxil	9	50.00
Cefotaxime	9	50.00
Roxithromycin	10	55.56
Gentamicin	11	61.11
Netillin	12	66.67
Clarithromycin	13	72.22
Chloramphenicol	13	72.22
Azithromycin	15	83.33
Amikacin	15	83.33
Co-Trimoxazole	16	88.89
Norfloxacin	17	94.44
Ciprofloxacin	17	94.44
Sparfloxacin	17	94.44

Table 4: List of bacterias isolated and identified from healthy mother milk and baby fecal

Organism name	Accession no
Lactobacillus oris	MK788135
Lysinibacillus sp.	MK788138
Enterococcus mundtii	MK788136
staphylococcus sp.	MK788137
Bacillus clausii	MK788139
Enterococcus faecalis	MK788150
Lactobacillus brevis	MK788151
Lysinibacillus fusiformis	MK788152
Staphylococcus sp	MK788153

Thus, 9 bacterial isolates were subjected to 16S rDNA amplification and sequencing. A number of the bacterial isolated and identified from both sample healthy mother milk and baby fecal are represented in Table 4. The phylogenetic analysis reflected the stability and transferring of bacteria belongs in one phylum, i.e., Firmicutes. Firmicutes phylum was represented by a class of Bacilli (18/27 isolates). Bacilli class was comprised of different families like Bacillaceae (3/9) isolates), Staphylococcaceae (2/9 isolates), Lactobacillaceae (2/9 isolates), Enterococcaceae (2/18 isolates). Bacteria

belonging to the genus *Bacillus, Lactobacillus, Lycinibacillus,* and *Enterococcus* were found to be stable in every week of healthy breast milk. The phylogenetic relationship between all the isolates was drawn by using the maximum likelihood method in MEGA6 software. The phylogenetic tree represented in Fig. 1 clearly shows that bacteria have clustered according to their class.

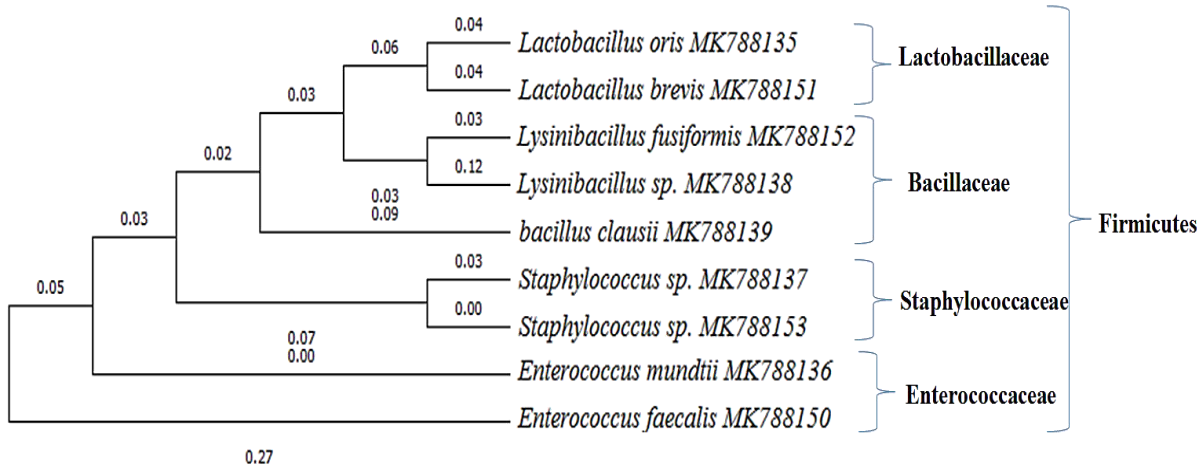

Fig. 1: Phylogenetic tree based on the maximum likelihood method reflecting the relationship ofbacteria within the respective classes.

The phylogenetic tree was concluded using Hasegawa, Kishino, and Yano maximum likelihood method (1000 bootstraps) in MEGA X software. The bar indicates a 5 % sequence divergence

4. DISCUSSION

In the current study, we explored whether viable gut-associated microbes are shared between the maternal and infants gut microflora via breastfeeding. Hence, the microbial diversity in healthy mother milk and corresponding infants feces collected from five mother-infant pairs was investigated at every week's time interval for one month during the neonatal period using the culture-dependent method.Using the culture-dependent method, microbes in the fecal samples of the current study were ~10^7 CFU/g and whereas microbes in breast milk were measured ≤ 10^4CFU/ml. Taxonomic classification at the genus level revealed that isolates belonging to facultative anaerobic and aerobic genera, especially *Bacillus, Lactobacillus, Lycinibacillus,* and *Enterococcus* were shared by breast milk and corresponding their infants fecal.

The occurrence of microflora in the various human body parts is made the dynamic and interconnected networks [22]. Hence, the probability that the neonate's mouth or mother skin may deliver few bacteria to the breast milk but it is not only responsible for the developments of breast milk and infant's gut microflora. Physiological and hormonal alteration occurring during and after pregnancy increased gut permeability which in turn helps in the transfer of gut microflora to the mammary gland. Dendritic cells and macrophages also play an important role in the migration of microbes to the mammary gland [14]. These bacteria are transferred from the maternal community to breast milk *via* the entero-mammary pathway. Along with the above apparent mechanisms, the retrograde flux between the mother's skin microbes and infant's oral microbes may also help in the development of the human milk microbiome [15, 17]. Some microbiota of the newborn's oral cavity might contaminate breast milk at the time of breastfeeding due to milk flow back again into the milk ducts of the breast [23]. Still, this retrograde flux does not clarify why colostrum consists of the microflora which characterizes breast milk [24]. Though the human salivary microbiota is still fully explored, *Streptococcus* species present dominantly in both adults [25, 26] and in infants [27, 28]. *Streptococci* are also predominantly found in breast milk [16] which that salivary microbiota was significantly affect the breast milk microbiome.

Some of the common skin microflora like *Corynebacterium, Staphylococcus*, and *Propionibacterium* [29], is also found in human milk. But, it should be highlighted that the prevalence of this group of microbiota also occurs in the mucosal layer of the genitourinary tract and gastrointestinal tracts. *Streptococci* and *Staphylococci* have gained attention about their role of initial colonization in the infant gastrointestinal tract [18].

Remarkably, the studies reveal that abundance of *Staphylococcus epidermidis* was significantly different between the feces of healthy breast-fed newborns to formula-fed newborns [30, 31] which reveals that such microbes are already present in mammary glands and development in the mammary environment at the time of lactation. Regardless of the sharing of a few phyla, the prevalence of microbiota in breast milk and breast skin microbiome significantly different from each other [32]. For example, bacterial belongs to the Bifidobacterium genus are strictly anaerobic so they can't able to grow on the breast skin. As an example, *Bifidobacterium longum* DNA was shared by human milk, maternal and neonatal feces inside the same mother-infant [33].

It is reported that anaerobic genera, like *Bacteroides*, members of the *Clostridia* class *Bifidobacterium* and *Parabacteroides* was shared among human milk, maternal and neonatal feces using a pyrose quencing approach [34]. A disadvantage of metagenomics studies is that it is not given data in regards to the viability of the identified microbes and also strain-level identification that is essential for demonstrative the presence of the same microbial strain in mother and neonate. Therefore, without confirming the occurrence of these microbes by the culture-dependent method, it remains indistinct whether breast milk is a source of viable gut-incorporated anaerobes or dead cells [34]. However, transmission of *lactobacilli*, *bifidobacteria* and other bacterial strain from the mother gut to the infant gut [35], from the mother gut to breast milk [36], from breast milk to the infant gut [18] has also been confirmed using bacterial strain-specific study. Such studies support the hypothesis which stated that microbes may be vertically transmitted from lactating mother to infant thru breastfeeding. Thus, in current study using culture, isolation and partial 16S rRNA gene sequencing, viable strains of *Lactobacillus oris, Lysinibacillus sp., Enterococcus mundtii, staphylococcus sp., Bacillus clausii, Enterococcus faecalis, Lactobacillus brevis, Lysinibacillus fusiformis,* and *Staphylococcus sp.* were isolated from fecal and breast milk samples, in regards of mother-neonate pair, they were isolated from. In the shade of present study fact, strains potentially transferred within mother-neonate pairs, these isolates denote the best indicator for representing vertical transfer.

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