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Human milk microbiome in urban and rural populations of India

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ABSTRACT

Most of the studies examining human milk microbiota were focused on differences in bacterial taxa in relation to birthing method, gestation time and infant gender. In this study, we aimed to understand influence of different lifestyles on milk microbiota by utilizing metagenomic approach. Milk microbiota from 15 urban women living in metropolitan city and 15 rural women living in tribal village of Gujarat, India were profiled by 16S rRNA gene sequencing. The bacterial community in human milk contained over 26 phylum, 157 family and 543 different genera. Comparison of urban and rural milk microbiota revealed that they differ in both i) highly abundant taxa, including *Proteobacteria* ($P = 0.008$) and *Firmicutes* ($P = 0.046$), and ii) overall microbial diversity (Shannon index, $P < 0.001$). Despite of huge variation in microbial community structure, metabolic pathways were more stable and evenly distributed between urban and rural community. In concert, the study provided first insights into human milk microbial community of Indian population and demonstrated that different lifestyle can rapidly altered milk microbiota.

1. Introduction

Human milk is considered as a primary source of nutrition for newborns since it provides all the protective and functional nutrients required by rapidly growing infant during first 6 months of life (Fernandez et al., 2013; Latuga et al., 2014). It contain many bioactive molecules including immunoglobulins, oligosaccharides, fatty acids, polyamines, lactoferrin and lysozymes. Recent detection of bacterial DNA and live bacteria from milk has challenged the prevailing dogma that human milk is normally sterile and provided the compelling evidence that human milk contains its own unique microbiota (Hunt et al., 2011; Cabrera-Rubio et al., 2012; Jost et al., 2013; Ward et al., 2013). These bacteria may confer immunity to newborns and participate in the correct maturation of the infant immune system (Perez et al., 2007). Moreover, some of the bacteria isolated from milk also have probiotic properties (Heikkila and Saris, 2003).

Human microbiome Project (HMP) unveiled microbial diversity in healthy subjects over five different body sites (oral, skin, gut, nasal/lung and vaginal) (Human Microbiome Project, C, 2012). However, one of the key system was ignored i.e. “Human milk”. Many culture dependent and independent studies has confirmed enormous diversity of bacterial phylotypes in human milk, including *Pseudomonas, Staphylococcus, Streptococcus, Lactobacillus* and *Serratia* (Martin et al., 2003; Hunt et al., 2011; Cabrera-Rubio et al., 2012; Jost et al., 2013; Ward et al., 2013). In one study, same bacterial strain was detected from milk and infant feces, suggesting mother to infant transmission of bacteria (Martin et al., 2012). As milk received from the mother is one of the key factor determining how gut microflora will develop in the newborn baby, it is essential to study milk microbiota and its health implications on infant. Apart from this, infant diet (breastfed or formula fed) and delivery mode (vaginal or caesarian) also influence infant gut microbiota and its composition (Guaraldi and Salvatori, 2012; Dogra et al., 2015).

Despite milk bacteria has important role in infant health, we do not know the extent to which different lifestyles can change the milk microbiota. Human milk microbiome of westernized people with modern lifestyles have been studied in Finland, Switzerland, Canada and USA (Hunt et al., 2011; Cabrera-Rubio et al., 2012; Jost et al., 2013; Ward et al., 2013). But there is no information on human milk microbiota composition of Asian, especially Indian population. India offers a unique scenario for such metagenomic studies, since diverse communities (> 2000 ethnic groups) are living in India, having different religion, diet and cultural background. We believe that investigation of milk microbiome of Indian population and its' com-

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parative analysis between women living in metropolitan city (urban) and tribal area (rural) will provide insights into alteration of microbiota with different lifestyles.

The goal of the present study was to compare human milk microbiome of rural women living with traditional lifestyle with that of urban women living with modern lifestyle.

2. Materials and methods

2.1. Study population, dietary information and sample collection

A total of 30 breastfeeding women (15 from urban region and 15 from rural area of Gujarat, India) participated in the study. Women living in rural area were away from the modern lifestyles and rely on agriculture and fishing for their livelihood. All donors were healthy and donated milk between 15 and 90 days postpartum. Written informed consent was obtained from all the participants. The present study was conducted according to the ethical guidelines of 1975 Declaration of Helsinki and the procedure was approved by ethical committee of Govindbhai Jorabhai Patel Ayurveda College and Surajben Govindbhai Patel Ayurveda hospital (Approval No-IEC-3/GJPIASR/2015-16/E/3). Four to five ml of milk sample was collected in sterile falcon tube by manual expression followed by rejection of foremilk and cleaning of nipples and surrounding areola with cotton soaked in 70% ethyl alcohol. Samples were stored at 4 °C and processed immediately upon reaching the laboratory.

2.2. DNA extraction

DNA extraction was performed as previously described by (Bhatt et al., 2012). Briefly, milk (3–4 ml) was filtered and centrifuged at 11000 $\times g$ for 5 min to remove somatic cells; the pellet was washed 2–3 times with N-saline buffer and resuspended in 2 ml of tris EDTA pH -8, 250 μl of 10% *w/v* SDS, 10 μl of Proteinase k (20 mg/ml) and 10 μl of lysozyme (50 mg/ml). The lysate solution was then incubated for overnight at 37 °C. Following incubation, 120 μl of 5 M NaCl and 100 μl of CTAB (10% *w/v* in 0.7 M NaCl) was added to the solution. After incubation for 10 min at 65 °C in water bath, equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added. Resulting mixture was centrifuged at 12000 $\times g$ for 10 min. The supernatant was transferred to new tube and equal volume of chloroform: isoamyl alcohol (24:1) was added. The mixture was then centrifuged at 12000 × g for 10 min. The supernatant was transferred into a new tube and 1/10th volume of 7.5 M ammonium acetate and double volume of isopropanol was added to it; after that mixture was incubated at −20 °C for 2 h. Following incubation mixture was centrifuged to pellet out DNA, washed with 70% ethanol, allowed to dry and resuspended in TE buffer. Purity and concentration of DNA was quantified using Nano drop ND-1000 UV- Spectrophotometer (NanoDrop Technologies).

2.3. 16S rRNA gene amplicon sequencing

The microbial diversity was studied by sequencing V2-V3 hypervariable region of the 16S rRNA gene, amplifying a fragment of 418 bp. PCR reaction was performed by using universal primers, 101F (5′ ACTGGCGGACGGGTGAGTAA 3′) and 518R (5′ CGTATTACCGCGGCTGCTGG 3′) containing titanium Lib-L adaptor sequences, key sequence followed by 10 bp of Multiple Identifier (MID) sequences specific for the individual samples. Each PCR mixture (final volume 25, μl) contained 50 ng of template DNA, 1.25 U of Taq polymerase (Roche, USA), 2.5 μl of reaction buffer, 10 mM of 0.5 μl dNTPs, (Roche, USA) and 1 μM of each primer. The following PCR conditions were used: 96 °C for 5 min followed by 30 cycles at 95 °C for 30 Sec, 60 °C for 45 Sec and 72 °C for 60 Sec. A final extension was carried out at 72 °C for 10 min.

Resulting PCR products from the amplification of the 16S rRNA gene were purified by QIAquick gel extraction kit (QIAGEN) on 1.5% agarose gel followed by furthered purification with AMPure DNA purification beads. Purified amplicons were examined with an Agilent Bioanalyzer high sensitivity chip (Agilent, USA) and qubit fluorometer (Invitrogen, USA) to determine the concentration and to assess the size of the products. From the concentration and size of the amplicon library, the amount of DNA was calculated and finally libraries were diluted and pooled. Emulsion PCR was carried out using Ion one touch™ 400 kit (Life Technologies, USA) according to the manufacturer's instruction. Sequencing of the clonal libraries was carried out on 316 chip using the Ion Torrent Personal Genome Machine (PGM) employing Ion Sequencing 400 kit (Life Technologies, USA) according to the manufacturer's instruction.

2.4. Bioinformatic analysis and statistical methods

A total of 2,484,332 raw sequence reads were analyzed using Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010). In brief, sequence reads were filtered according to the following criteria: (i) minimum mean quality score < 20; (ii) read length shorter than 200 bp; (iii) one ambiguous base per read; (iv) no primer mismatch and maximum 1.5 error in barcode tags. Sequences were clustered de novo at 97% sequence similarity threshold and taxonomy was assigned by comparing sequences against Greengenes 13_8 reference database. Moreover, we picked OTUs by closed reference OTU picking approach for comparing Indian milk microbiota to that of available published study from Finland and Switzerland. During additional quality filtering step, we removed sequences occurring < 5 times. Potential chimeras were filtered out using Chimera Slayer (Haas et al., 2011). Microbial diversity was calculated using alpha diversity (within samples) and beta diversity (between samples) on rarefied out table. Alpha diversity was estimated using four different metrices: Observed OTUs, Shannon index, Phylogenetic diversity and Chao1. Unweighted (presence/ absence) and weighted (abundance) UniFrac PCoA was used for clustering analysis. Factor contributing to microbial compositional difference between two groups was tested by Adonis and ANOSIM test in QIIME (compare_categories.py). Mann-Whitney *U* test was used to compare alpha diversity between urban and rural populations. Statistical significant differences between urban and rural microbiota at all taxonomy level was assessed by employing Metastats software in R (White et al., 2009). LDA Size Effect (LEfSe) algorithm was used with online Galaxy interface (https://huttenhower.sph.harvard.edu) to identify significantly abundant taxa in different group (Segata et al., 2011).

2.5. Functional prediction by PICRUSt

We used PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), to predict functional metagenomes based on 16S rRNA gene data using database of reference genomes (Langille et al., 2013). For PICRUSt analysis, we performed closed reference OTU picking against Greengenes database at 97% sequence similarity. Resulting OTU table was normalized by 16S rRNA gene copy number and metagenome was predicted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) catalogue. Accuracy of PICRUSt was assessed by calculating NSTI (Nearest Sequenced Taxon Index), which calculate phylogenetic distance between 16S rRNA gene present in OTU table and 16S rRNA gene of closest sequenced reference genome. Moreover, we used HUMAnN to predict downstream pathway abundance and coverage from the KEGG Orthologous results (Abubucker et al., 2012). Two sided Welch's test in STAMP (STatistical Analysis of Metagenomic Profiles) was used to calculate most significantly abundant pathways ($P < 0.05$) (Parks et al., 2014).

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