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Microbial architecture of pregnant women: A culture independent pilot study

Sir,

Recent understanding on human microbiome through second-generation sequencing technology postulates that organ-specific symbiotic association of 100 trillion microbes with human is crucial for immune modulation, metabolism, growth and gene regulation[1]. It has been postulated that maternal microbes may be inherited by the neonates for early life gut-flora establishment[2]. Vaginally born neonates are exposed to maternal vaginal and intestinal flora and are associated with better growth compared to those born after caesarean section[3]. Knowledge on impaired gut-flora development at early life is crucial for understanding irreversible childhood undernutrition and its morbidity particularly for countries like India where neonatal mortality is about 41 per cent of all global deaths[4]. The present study was aimed to characterize the vaginal microbial architecture of pregnant women who delivered live normal weight neonates at term.

Twelve vaginal swabs obtained through random sampling from 12 pregnant women attending an Urban Health Centre, Dibrugarh during July 2014 to June 2016 at the third trimester of their pregnancy were included in the present study after obtaining written informed consent. The study was approved by the ethics committee of ICMR- Regional Medical Research Centre, North-East Region, Dibrugarh, Assam, India. The study included the participants who had a normal pregnancy outcome; had vaginal delivery and neonates were of normal weight. Those having any chronic non-communicable diseases, acute as well as chronic infection and the complications related to pregnancy, namely, pre-eclampsia and gestational diabetes were excluded. Samples from pregnant women who had a history of urinary tract infection or vaginal discharge during the time of sampling were also excluded. Vaginal swabs were homogenized with 1× phosphate buffer saliva (PBS) and incubated with lysozyme solution (Sigma Aldrich, USA) at 37°C for 1 h. A volume of 500 µl Inhibitex buffer (Qiagen, Germany) was mixed and incubated at 70 and 90°C for 20 and five minutes, respectively. DNA was extracted using the Qiagen stool DNA Kit (Qiagen) following the manufacturer's instructions and eluted in 50 µl elution buffer provided with the kit. Amplicons were generated through 16 s universal primer for variable 4 region (V4 region: Primer: V4F 5'-GTGCCAGCMGCCGCGGTAA-3' V4R 5'-GGACTACHVGGGTWTCTAAT-3'). Amplicons were taken through a library prep using Truseq Nano HT kit (Illumina, USA) where the amplicons were ligated with indexed Illumina adapters followed by limited cycle PCR to amplify the ligated molecules. These libraries were pooled based on the data required and sequenced on HiSeq 2500 sequencer ((Illumina, USA) using Rapid SBS kit V2 (500 cycles) to generate 250 paired-end reads. The 16s sequence read files were analyzed for the 12 samples on QIIME (Version 1.9.0)[5]. Chimera filtering was performed using UCHIME (*https://www.drive5.com/usearch/manual/uchime_algo.html*). Maximum and minimum read count among the samples were (pair-end read) 3.1 and 2.7 million, respectively, with an average Q30 (phred score) of 79.8 per cent. The Green genes database (*http://greengenes.lbl.gov*) was used for taxonomy assignment. Operational taxonomic units (OTUs) picking was performed using the *pick_otus.py* command with the default UCLUST algorithm in QIIME[5]. The UCLUST algorithm uses the USEARCH algorithm to assign sequences to a cluster[5]. The most abundant read in each OTU was selected as the representative sequence; this step was performed using Pick representative set of sequences (*pick_rep_set.py*) *Assign_taxonomy.py* was used for the classification of each of the representative sequences. All the statistical analysis were done by R packages [R version 3.2.3, The R Foundation for Statistical Computing][6].

Age, nutritional and socio-economic status and weight were homogeneous among the study

Table I. Operational taxonomic units (OTUs) and alpha diversity (Shannon-Weaver, Simpson, Inverse Simpson and Pielou's Evenness) index among 12 mothers in third trimester

Sample ID	OTUs (n)	Trimester (wk)	*P*	Alpha diversity indices					
				Shannon-Weaver $\sum_{i=1}^{n} p_i \log_b P_i$	Simpson $\sum_{i=1}^{n} p_i^2$	Inverse Simpson $\frac{1}{\sum_{i=1}^{n} P_i^2}$	J $\frac{\sum p_i \log_b p}{\log (n)}$	Infant birth weight (kg)	Mother weight (kg)
1	110	<30	<0.001	2.55	0.86	7.55	0.54	3	48
2	97			1.69	0.67	3.04	0.37	2.4	48
3	81			1.52	0.67	3.11	0.34	3	41
4	89			1.65	0.74	3.91	0.36	2.4	47
5	32	>37		0.35	0.16	1.19	0.10	3	48
6	43			1.59	0.74	3.93	0.42	2.4	51
7	43			1.57	0.73	3.81	0.42	3	59
8	56			1.43	0.66	3.01	0.35	3	58
9	37			0.02	0.002	1.00	0.003	2.92	52
10	50			1.51	0.72	3.66	0.38	2.7	44
11	46			1.02	0.55	2.25	0.26	2.01	45
12	54			0.47	0.18	1.23	0.11	3.5	68

n=number of OTU (genus specific). p_i is the proportion of species i, and n is the number of OTUs so that $\sum_{i=1}^{n} P_i = 1$ and b is the base of the logarithm. J, Pielou's evenness index

Table II. Prevalence of *Lactobacillus* dominated vaginal microbiome across the population during pregnancy

Population/races	Prevalence of *Lactobacillus* dominant vaginal microbiome (%)	Prevalence of non-*Lactobacillus* dominant vaginal microbiome (%)
Dutch	≥70	≤30
South Asian Surinamese	≥65	≥35
African Surinamese	≥50	≥50
Ghana	≥45	≥55
Morocco	≥40	≥60
Turkish	≥40	≥60
Canada (multi ethnic Hospital based study)	≥55	≥45
Present study (n=12)	41.6	58.4

Source: Refs 7-9

participants. The study demonstrated that vaginal microbiome in the third trimester was dominated by the phyla *Firmicutes*, *Proteobacteria* and *Bacteroidetes* whereas *Actinobacteria* and *Fusobacteria* were present in lesser abundance. *Lactobacillus* [21.7% (25% percentile: 0.2% and 75% percentile 32.42%)], *Prevotella* (2.06±7.1%), *Enterococcus* (14.5±17.6%), *Escherichia* (14.08±18.4%), *Streptococcus* (13.3±26.7%), *Staphylococcus* (2.9±6.4%) and *Peptostreptococcus* (1.5±5.4%) were most abundant among the vaginal microbiota. *Sneathia* (0.2±0.7%) was also present with limited abundance. The study demonstrated that the 61.5±25.8 types OTUs (Genus-specific) were present in the third trimester among the pregnant women. During the early period of the third trimester (<30 wk) vagina harboured 94.25±12.37 types of OTUs, and it significantly reduced to 45.13±8.2 (P<0.001). Alpha diversity indices, namely, Shannon-Weaver, Simpson, inverse Simson and Pielou's evenness (J) are presented in Table I. The lactic acid bacteria (LAB) abundance was significantly high (P<0.001) among the pregnant women at gestational age 37 wk (75.95±22.95%) compared to the early period of the third trimester (<37 wk of gestation; 35.78±23.76%). Table II represents the prevalence of *Lactobacillus* dominancy in vaginal microbial architecture among the different races globally and revealed that the dominancy was highest in the Dutch population[7-9].

The present study documented a significant difference in vaginal microbial signature from early

(<37 wk of gestation) to late (37 wk gestation) third trimester and genus-specific OTUs reduced at a later stage. Further, the study demonstrated highest abundance of *Lactobacillus* compared to other bacteria. Reduced frequency of *Gardnerella*, *Prevotella* and *Sneathia* seems crucial for pregnancy outcome as bacterial vaginosis, still-birth and infection-related complication is associated with the increased abundance of the same[10]. Studies postulate that *Lactobacillus* abundance is associated with neonatal neurodevelopment, immune activation and growth[10,11]. Host hormonal switching and altered *p*H in vagina seem crucial for the increased abundance of LAB in the vagina in humans, but it is not common in other mammals[10,12]. *Lactobacillus* dominancy in the birth canal during pregnancy has been documented in other population also. Previous study reported that abundance of *Lactobacillus* in neonatal gut is higher in vaginal deliveries compared to caesarean deliveries[13].

In conclusion, our observations indicate that abundance of the LAB in the birth canal which is crucial for early life stable gut establishment and growth of infant, may be used as crucial marker for healthy birth related outcome for vaginal delivery. The limitation of the study was its small sample size. Further studies need to be done with a large sample to confirm these findings.

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