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A Prospective Cohort Study for Characterization of Microbiome from Various Samples of Pregnant women and Neonates

임산부와 신생아의 다양한 검체에서 세균총을 분석하기 위한 전향적 코호트 연구

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서울대학교 대학원

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Abstract

A Prospective Cohort Study for Characterization of Microbiome from Various Samples of Pregnant women and Neonates

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A few researchers have reported the microbiome profiles from certain gestational products such as placenta or amniotic fluid, which had been traditionally known to be sterile, and the results received huge attention as well as controversial criticisms. In this prospective cohort study, we challenged to characterize the microbiome composition from various samples in both pregnant women and their neonates and explored the similarity or association according to the mother–neonate pairs, the compartments where the samples had been obtained, and obstetrical factors. We identified 19,597,239 bacterial sequences from the 641 samples of a total of 141 pregnant women and 178 neonates. The distribution

of microbiome tended to be grouped strongly according to the sites where the samples had been obtained, not the mother–newborn pairs. Several methods were used repeatedly to remove contamination, the critical issue, and we found out the amniotic fluid and umbilical cord blood were highly prevalent in negative controls, reflecting both compartments seem to be very close to being sterile. On the other hand, still we carefully suggest certain bacteria could be present in intrauterine environments or fetal compartments since the detection of bacteria from the meconium might reflect the premature microbial colonization. Moreover, the microbiome from neonatal gastric liquid was not completely identical to that of amniotic fluid although the fetus physiologically swallows amniotic fluid and produces urine again under normal circulation. Establishment of microbiota library from various samples that are formed only during pregnancy is essential to understand the human development and to identify microbiome pathophysiologic modification in obstetric complications.

Keywords: microbiome, pregnancy, neonate, amniotic fluid, meconium, cord blood, cesarean section, vaginal delivery

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I. Introduction

Research on microbiome has been a fascinating topic in diverse fields since this tiny world seemed to be very potential to possess the original secrets of phenomena found in human body. The human microbiome has been identified to play an important role in maintaining homeostasis in health and is associated with numerous diseases [1,2]. This trend of microbiome research naturally moved to trace the subjects such as infants or pregnant women, the origin of human development. Microbiome development is likely to start from the in-utero environment and changes in lifetime, continuously affecting the immune system and metabolism. Many studies have demonstrated that pregnancy itself modifies the microbial populations in multiple sites within the maternal body, and this alteration might influence maternal, fetal, and neonatal health conditions in the future [3]. Since pregnancy is a very unique immune condition for a human body that allows a temporary tolerance for the foreign body residency, microbiome remodeling during pregnancy to facilitate immunological and metabolic adaptations seems to be obviously necessary [4]. Some microbiome studies in pregnancy have proposed that fetal environments, including placenta and amniotic fluid, traditionally known as sterile,

contain several characteristic microbiotas not identified in routinely performed culture techniques [5,6]. However, their biomass was technically small and the criticism on the reliability of the sequencing methods or the possibility of contamination arose. In addition, the association between those microbiota and specific obstetric conditions has not yet been proven much.

The most commonly studied site of the bacteria in the female reproductive organ is the vagina. Anatomically, the vagina, connected to uterus through cervix, is the most distally placed area of the reproductive organ set and is exposed to skin. Aagaard et al. reported the vaginal microbiome differed during pregnancy by gestational age and that Lactobacillus species played a role in preventing the growth of pathogenic bacteria [7]. Pregnancy causes several changes in the vaginal microbiome, such as decreased overall diversity, increased proportion of Lactobacillus species, and higher stability [8,9]. Since preterm birth is a critical issue in obstetrics and intrauterine inflammation/infection was identified as the main cause of spontaneous preterm birth, the relationship between preterm birth and the vaginal microbiome has been explored by many groups [10–14]. Nevertheless, no significant association has been suggested specifically so far. Other sites that

had been evaluated for microbiome in pregnancy are maternal gut [15], oral cavity [16], placenta [5], amniotic fluid [17,18], and neonatal gut [19], however those previous studies were fragmentarily designed and thus, the characterization of microbiomes from samples systematically collected from a refined cohort composed of pregnant women and their newborns has not been reported yet. Unlike other fields, microbiome research in pregnancy has advanced relatively slowly because of the adamant limitations such as ethical issues on sampling procedures to pregnant women or fetuses and difficult access to the samples.

Here, we aimed to characterize the microbiome in vaginal discharge (VD) and amniotic fluid (AF) from pregnant women and then, in umbilical cord blood (CB), gastric liquid (GL), and meconium (M) from their neonates and to determine how those samples are related to each other or various obstetric conditions.

II. Materials and Methods

II-1. Study design and sample collection

A prospective study was performed on live births delivered after 35 weeks of gestation. between March 2020 and January 2021. Samples were collected from women who had delivered at Seoul National University Bundang Hospital and their newborns. Women with unstable vital signs or those requiring urgent management such as transfusion and neonates admitted to the neonatal intensive care unit (NICU) or who had unstable vital signs after birth were excluded from the study.

Samples for microbiome analysis included maternal VD, AF, CB, neonatal GL, and M. As a pregnant woman was hospitalized with expectancy of delivery, the VD sample was obtained using a polyester swab inserted into the posterior fornix of the vagina, assisted by sterile speculum examination on the day of admission. For those who had undergone cesarean section for delivery or amniocentesis for specific indications (i.e., for detection of intraamniotic inflammation/infection), approximately 10cc of AF was obtained through a syringe for the study. During delivery, both cesarean section and vaginal delivery, approximately 20cc of CB

was taken through a syringe from the vein of the umbilical cord immediately after clamping. Since removing amniotic fluid or other liquid from the newborn’s mouth and stomach after birth is a part of initial management to help the airway and to stimulate spontaneous breathing, most neonates received suctioning procedures, and the liquid collected in the suction bottle (approximately 15 ml) was carried into a conical tube for analysis of GL. The M sample, the newborn's very early stool, was carefully obtained within 24 h after birth using a polyester swab from the anus as the neonate stabilized after initial management.

We tried to collect all five different samples from each woman and neonate(s), nonetheless, a small part of samples from mother–neonate pairs were not obtained or missed for clinical circumstances. The primary outcome was the distribution and composition of the microbiome of the above samples from pregnant women and their neonates. To determine the association between the microbiome from different compartments and obstetric factors, medical records were collected and thoroughly reviewed. Data included maternal age, gestational age at delivery, delivery mode (vaginal delivery or cesarean section), the use of assisted reproductive technology (ART), other obstetric complications, and

neonatal outcomes such as sex and birth weight.

This study was performed with the informed consent of appropriate participants in compliance with the Declaration of Helsinki. The study protocol was approved by the Institutional Review Board of the Seoul National University Bundang Hospital (B-1606/350-003).

II-2. Microbial DNA isolation

Microbial deoxyribonucleic acid (DNA) was extracted from the VD, GL, AF, and CB samples with the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, CA) and the sample M using the DNeasy PowerSoil Pro Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Briefly, samples were enzymatically and mechanically lysed by bead beating, followed by washing and filtering in the provided column. Extracted DNA concentrations were measured using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). For each box of the DNA extraction kit used, no material was used as a negative control. The blanks were processed in the entire protocol and analyzed.

II-3. 16S rRNA gene amplification

The 16S ribosomal ribonucleic acid (rRNA) gene was amplified using the two-step polymerase chain reaction (PCR) protocol in the 16S Metagenomic Sequencing Library Preparation (Illumina, San Diego, CA). In the first PCR step, the V3-V4 hypervariable region of the 16S rRNA gene was amplified using 341F/785R primers and Herculase II fusion DNA polymerase (Agilent, Santa Clara, CA). In the below primer sequence, 'N' base is selected from any random base, 'W' base is A or T, 'H' base is A, C or T, and 'V' base is A, C, or G.

341F:

5′ - TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′

785R:

5′ -GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′

PCR cycling was performed with an initial cycle at 95 ° C for 3 min, followed by 25 cycles of 95 ° C for 30 s, 55 ° C for 30 s, 72 ° C for 30 s, and a final extension cycle at 72 ° C for 5 min. The amplicons were cleaned with AMPure XP beads (Beckman Coulter, Brea, CA, USA). In the second PCR, index primers from the Nextera DNA CD Index Kit (Illumina, San Diego, CA) were added to the ends of the amplicons generated in the first PCR. PCR cycling was performed with an initial cycle at 95° C for 3 minutes, followed by ten cycles of 95° C for 30 seconds, 55° C for 30

seconds, 72 ° C for 30 seconds, and a final extension cycle at 72 ° C for 5 minutes. Each sample was cleaned with AMPure XP beads (Beckman Coulter, Brea, CA, USA) and eluted in UltraPure DNase/RNase-Free Water (Thermo Fisher Scientific, Waltham, MA). The amplified DNA was checked using a 2100 Bioanalyzer system using an Agilent DNA 1000 Kit (Agilent, Santa Clara, CA, USA). For each library production, no template was used as a negative control

II-4. 16S rRNA gene sequencing and analysis

Based on the DNA size and concentration, the amplicons were pooled in equimolar amounts and spiked with 30% PhiX (Illumina, San Diego, CA). These were then sequenced on the Illumina MiSeq platform using paired-end 250 cycle MiSeq Reagent Kit V2 (Illumina, San Diego, CA) and a 300 cycle MiSeq Reagent Kit V3 (Illumina, San Diego, CA). Negative controls from the DNA extraction and library were sequenced.

II-5. Sequencing data generation

We divided the samples into nine batches (Runs 1-9) and sequenced the V3-V4 region of the 16S rRNA gene using Illumina

MiSeq machines with a target depth of 100,000 per sample. Sequencing was performed with 250 bp paired-end reads for all of the sequencing runs except for the last one (Run 9), where sequencing was performed with 300 bp paired-end reads for practical reasons. The bcf2fastq program of Illumina was used to demultiplex raw sequencing data (BCL files) and output forward and reverse FASTQ files for each sample. Of note, some samples were sequenced more than once to assess the impact of batch effects. These included "sequencing duplicates" in which the identical NGS library of one sample was sequenced in separate runs and "library duplicates" in which multiple NGS libraries were prepared from the identical sample at different dates and then sequenced separately.

II-6. Data analysis and visualization

Unless stated otherwise, all analyses were carried out using the QIIME 2 platform, a powerful community-developed platform for microbiome bioinformatics [20]. For each sequencing run, FASTQ files were imported to QIIME 2 and the DADA2 plugin [21] to identify ASVs by trimming low-quality parts of sequence reads, denoising trimmed reads, and then merging the forward and reverse

reads. The observed ASVs from individual sequencing runs were then merged into one ASV table. To detect and remove potential contaminants, we ran the decontam program on our samples, which looked for ASVs per sequencing batch that appeared at higher frequencies in low-concentration samples and were repeatedly found in the negative control [22]. Taxonomy classification was performed using a naive Bayes classifier using the SILVA database [23]. To visualize the outputs from QIIME 2, we developed the Dokdo program (https://github.com/sbslee/dokdo), an open-source and MIT-licensed Python package for microbiome sequencing analysis using QIIME 2.

II-7. Diversity analysis

We used the QIIME 2 command "qiime diversity core-metrics-phylogenetic" to compute the alpha and beta diversity metrics of our samples. When running the command, to normalize for the difference in read depth across the samples, we used the "--p-sampling-depth" option to rarefy our samples to 5,000 sequence reads and have an equal depth of coverage. We also ensured that all samples were sequenced to a sufficient depth of coverage for diversity analysis by creating rarefaction curves. Additionally, we

used the "--i-phylogeny" option to provide a rooted phylogenetic tree of observed ASVs, which is required for performing PCoA based on the weighted UniFrac distance [24].

II-8. Statistical analysis

To assess the differential abundance of the microbiome in the context of clinical information such as preterm birth, we used the QIIME 2 command "qiime composition ancom" to perform ANCOM, which compares the centered log-ratio (CLR) of relative abundance between two or more groups of samples [25]. To determine whether groups of samples are significantly different from one another in beta diversity, we carried out PERMANOVA using the QIIME 2 command "qiime diversity adonis" which fits linear model assumptions to a distance matrix (e.g., weighted UniFrac) with the chosen variables. We performed bootstrapping hypothesis testing by building a 95% confidence interval with the "scipy.stats.t.interval" method in the scipy package to compare similarities in microbiome composition between twins and randomly chosen samples [26].

III. Results

III-1. Description of the study populations and clinical characteristics

A total of 141 women were enrolled sequentially and 178 neonates were born from the study population because 37 cases were twin pregnancies. All women were of Asian ethnicity (Korean, specifically), and the median age of the study population was 34 (interquartile range 31-37) years (Table 1). Most of the features were low-risk pregnant women. The proportion of nulliparity was slightly over a half of the population (67%), and the median values of height, weight, and body mass index (BMI) were 162 cm, 70 kg, and 27 kg/m2, respectively. About 30% were conceived by ART, including intrauterine insemination (IUI) and in vitro fertilization with embryo transfer (IVF-ET). As mentioned above, twin pregnancy was approximately one-fourth of the total population, and among them, 19% were monochorionic. The median gestational age at delivery was 37.7 weeks (interquartile range 36.9-38.6), and preterm birth before 37 weeks of gestation was 26.2% (37/141). The rate of cesarean section was 55% (77/141). Seven neonates had congenital structural anomalies (atrioventricular

septal defect, absence of corpus callosum in the brain, achondroplasia, cleft lip, polydactyly, and syndactyly), which did not directly affect the neonatal survival. The frequencies of other obstetric complications or underlying maternal diseases are described in Table 1.

III-2. Maternal and neonatal microbiome landscape during delivery

We identified 19,597,239 bacterial sequences with 22,412 unique amplicon sequence variants (ASVs) in 641 samples, including all biological samples and negative controls (amniotic fluid, n=40, gastric liquid, n=100; umbilical cord blood, n=125; meconium, n=160; and cervicovaginal discharge, n=154) and negative controls (n=62) (Figure 1). There were 85 sets that had all five types of samples, 41 sets for four types, 57 sets for three types, and 37 sets for two types. These ASVs were taxonomically annotated, but we found evidence of batch effects in our sequencing data from all sample types except VD samples, which were likely introduced during next-generation sequencing (NGS) library construction and not during the sequencing itself (Supplementary Figure S1). However, this was expected because our samples were collected from body sites with low-biomass specimens, making our samples

prone to contamination [27]. Therefore, we expected to find many false positives and applied a series of filters, as outlined in Supplementary Figure S2. Notably, we found and removed 203 ASVs that were statistically determined as contaminants because they were highly prevalent in negative controls or they showed higher frequencies in low-concentration samples or they showed higher frequencies in low-concentration samples (Supplementary Figure S3).

We measured the alpha diversity of the samples by calculating their individual Shannon indices. As shown in Figure 2, the median alpha diversity for each sample type decreased in the following order: GL, AF, M, CB, and VD. The negative control group demonstrated a slightly higher alpha diversity than that of the sample VD group, suggesting that with 16S amplicon sequencing, negative controls could have microbiome diversity as rich as real biological specimens. Next, we estimated the beta diversity of our samples by computing the weighted UniFrac distance between them. As shown in Figure 2, the samples were moderately well separated by their sample type when projected using principal coordinates analysis (PCoA).

III-3. Clinical relevance of microbiome in pregnancy

To better understand the sources of variation seen in the beta diversity of our samples, we carried out the permutational multivariate analysis of variance (PERMANOVA) using different factors, including clinical information. As shown in Table 2, when all sample types were included in the analysis, the variable "Site" explained 17.2% of variation (p-value = 0.001) and the variable "LibraryMonth," 7.4% (p-value = 0.002). This result indicates that the samples could still be separated well based on the microbiome pattern unique to their body site, despite the significant batch effects present within our dataset. When the analysis was restricted to each sample type, except for the sample VD group, the variable "LibraryMonth" was found to be significant for all sample types. The explanatory power increased to a range between 24.5% and 48.9%. These results align with the hypothesis that our samples are predominantly low-biomass specimens and prone to contamination.

Additionally, the variable "DeliveryMethod" was returned as significant for the sample VD group, the variables "PretermBirth37" and "AntibioticsUse" for the sample M group, and the variable "Weight" for the sample CB group (Figure 3).

We explored the significant variables in each group using PCoA with weighted UniFrac distance. ~~The top seven bacterial taxa that led to different coordinates are shown in Figure 3.~~ Several ASVs of Lactobacillus and one ASV of Gardnerella were found in the sample VD group. In the sample M group, Staphylococcus showed a strong association with preterm birth. Lastly, the lists of bacterial taxa were connected to the weights of neonates in the sample CB group. Table 3 shows the analysis of analysis of the composition of microbiomes (ANCOM) for various clinical data to study any statistically significant relevance with bacteria in multiple sample types.

III-4. The resemblance of twin microbiome in delivery

To test the hypothesis that samples from twins, both monochorionic and dichorionic, have higher similarity in microbiome composition than randomly chosen samples, we compared the mean of weighted UniFrac distance between twin samples and randomly selected samples. More specifically, for each AF, CB, GL, and M group, we performed bootstrapping hypothesis testing by randomly sampling pairwise distances with replacement from all samples 1,000 times to build a 95% confidence interval with the means of the sampled

distances. We rejected the null hypothesis that there was no difference between the twin samples and randomly selected samples for all four sample types because the mean pairwise distance for twin samples was below the confidence interval (Figure 4 and Supplementary Figure S4). Next, we divided the twins into monochorionic and dichorionic twins and repeated hypothesis testing. We found that we could still reject the null hypothesis for all four sample types for dichorionic twins. For monochorionic twins, however, only the CB and M groups passed the test.

III-5. Characterization of the vaginal health-related microbiome

Several pathogenic and commensal vaginal microbiota have been shown to have important consequences for a woman's reproductive and general health. To establish reference ranges of vaginal microbiota with known clinical associations in generally healthy pregnant women, we searched for bacterial targets commonly tested for assessing vaginal health within VD samples. More specifically, we focused on 31 bacterial targets (15 genera and 16 species) that are tested by the "SmartJane" assay from uBiome Inc., including Lactobacillus, Sneathia, and Gardnerella [28]. Of the 31 bacterial taxa of clinical importance, 12 were identified in

our samples (Figure 5).

We observed a higher relative abundance of Lactobacillus at the genus level but lower abundances of Aerococcus, Fusobacterium, Gardnerella, Peptoniphilus, Porphyromonas, and Prevotella. Most of our patients did not have any severe pregnancy-related complications. In addition, the majority of preterm birth was ranged in the late preterm period from 34+0 weeks to 36+6 weeks. Therefore, the "SmartJane" assay did not capture almost any pathogenic microbiome. The specification level was examined and is listed in Figure 5. We found Lactobacillus iners and Lactobacillus jensenii from the assay lists, but Lactobacillus crispatus was not commonly found in the vaginal microbiome. This could be simply because the SILVA reference database we used omitted Lactobacillus crispatus. We confirmed that our data detected up to the Lactobacillus genus were linked to Lactobacillus crispatus using the National Center for Biotechnology Information (NCBI) database (data not shown).

IV. Discussion

The important implication of this study is that the microbiome composition or the possibility of colonization seemed to be completely different according to the compartments of maternal-fetal interface formed during the whole pregnancy. We designed the study to evaluate whether samples from various sites of body from pregnant women and their infants would share the similar microbiome, in other words, whether the maternal microbiome would be inherited to her fetus, however we found the samples revealed certain groups of microbiome according to the body compartments where they had been obtained, not dependent on the individuals or the pairs of mother-fetus/infants. Of all the factors including obstetric conditions, the "site" of sampling was the most powerful factor to present the similarity of microbiome.

Since the possibility of contamination is always the very critical issue in microbiome research, several up-to-dated methods were used to confirm the biomass or presence of contamination, and we could find the samples especially AF and CB were highly prevalent in negative controls. As mentioned above, there are still controversies on whether intrauterine environment is originally sterile or not, and this study supports intra-amniotic environment

and fetal circulatory compartment seem to be very closer to being sterile. A few previous reports have demonstrated the presence of the microbiome in normal amniotic fluid, umbilical cord blood, and placenta [5,6], nevertheless lack of appropriate controls for contamination and use of molecular approaches with insufficient detection limit for low biomass microbiome are to be considered. Moreover, most of the sequencing methods have to face the criticism that they do not provide evidence for the viability of bacteria themselves.

Still, based on the results of some samples, the current study discreetly suggests the possibility of microbiome formation in certain fetal compartments during intrauterine period. The detection of bacteria from the very early stool from newborns, the sample M, supports the idea of microbial colonization of the intrauterine environment during a normal pregnancy period [29,30]. Since the neonates born immediately have not eaten much and operated their gastrointestinal tract yet, their early stool must be formed when they were fetuses, swallowing only the urine (the sample AF) that they had produced, therefore the microbiome of the sample M has to be developed before birth. In addition, the microbiome from the sample GL group was similar to that of the sample AF group and it

is easily expected since the fetuses swallow AF in utero and their urine composes AF again under normal physiologic circulatory condition as already described above. Interestingly, the microbiome from the sample GL did not reveal to be completely identical to that of the sample AF, which might imply that there could be technologically unidentifiable mechanism of flora formation in the oral cavity or proximal gastrointestinal tract such as esophagus in fetal body from the intrauterine environment.

There are several studies to explore the relationship between microbiome in pregnant women and obstetric complications. One current meta-analysis review showed the significant association between preterm birth and microbiome change in vaginal discharge, which was mainly about the low proportion of Lactobacilli species [31]. Lin et al., demonstrated the maternal gut microbiome seems to change from the early period of pregnancy when combined with pregnancy-induced hypertension [32]. They revealed Eubacterium rectale and Ruminococcus bromii were more enriched in hypertensive disorder than in healthy pregnant women. Few researchers suggested the preventative effect of probiotic supplements against gestational diabetes, however Cochrane review concluded those trials failed to identify the protective effect, thus

have low certainty of evidence [33].

The most exceptional strength of this study is the nature of the study population. We excluded the extremely pathologic conditions including very early preterm births or severe underlying maternal diseased cases to explore the general pregnancy environment. The study population comprised pure Asians and represented general or low-risk pregnancies. The maternal age range was 20-45 years, which can be described as the general reproductive age. The proportions of nulliparity, cesarean section, and sex of the neonates were approximately half. The signs of fetal distress, such as low Apgar scores and the presence of meconium staining, were deficient in frequency. Extreme pathologic conditions that might influence or modify the microbiome were excluded, such as very preterm birth and preemies who needed to be treated carefully in NICU. Therefore, the microbiome analyzed in this study population is likely to reflect the features of general pregnancy. It is obviously important to establish the microbiome library of low-risk pregnant women and their normal neonates first for further comparative works with pathologic conditions to characterize the microbiome composition in responsible compartments during pregnancy.

Because of our complicated sample characteristics, including low microbial biomass and difficulty in controlling the groups like pregnant women, the ANCOM data to determine the relationship between various clinical conditions and bacteria from different samples might have many false-positive results. Regardless, the bacteria listed in Table 3 seem to indicate exciting results. *Finegoldia* and *Bifidobacterium* were previously demonstrated in a healthier pregnancy, which was also confirmed by our data [34,35]. In addition, many other taxa in the table are relevant to inflammation and pregnancy complications such as gestational diabetes mellitus, preeclampsia, and preterm birth. For example, *Campylobacter* and *Lachnospiraceae* in the sample VD group correlate with previous studies showing that these bacterial infections cause inflammation and even further preterm birth [36,37].

The comparison of the analysis with the clinical database revealed several associations from each group of samples. First, *Lactobacillus* and *Gardnerella* found to be abundant in the VD group are well-known indicators of the microbiome during pregnancy. *Lactobacillus* protects the maternal microbiome during pregnancy, whereas *Gardnerella* plays the role as a pathogen and is highly

related to preterm birth or pregnancy complications [11,13,33]. One interesting bacterium from the sample CB was *Faecalibacterium*, which demonstrated to be depleted in gestational diabetes mellitus [38] although the number of gestational diabetes mellitus was relatively small in the study population. *Staphylococcus* showed a strong association with preterm birth in the sample M group. This result coincides with the previous studies that reported infection caused by *Staphylococcus* may lead to preterm birth [39, 40].

Considering the effect of antibiotics, we sub-analyzed the relevance of antibiotic use in the sample M group, but it showed little association probably due to the small sample size. Tormo-Badia et al. reported that antibiotics altered the gut microbiota of offspring in pregnant mice [41]. As many studies suggest, if the "healthy microbiome" exists and plays an important role in maintaining the normal pregnancy, one can easily assume the possibility of adverse effects from antibiotics administration during pregnancy. Since antibiotics are not routinely used for pregnancy and are administered to pregnant women with the sign of infection/inflammation, specific diseases, or preterm premature rupture of membranes with the risk of ascending infection to the

fetus, the study to determine the effect of antibiotics to microbiome modification is practically complicating.

The sample M group showed some vaginal microbiome taxa such as *Lactobacillus, Staphylococcus*, and *Ureaplasma*, known as vaginal flora [11,42]. Vaginally delivered neonates showed microbiomes resembling their mothers' vaginal microbiota, dominated by *Lactobacillus*. In contrast, infants born by cesarean section had *Staphylococcus, Corynebacterium*, and *Propionibacterium* that are known to be dominant on the skin surfaces (of their mothers). We sought to determine the relationship between delivery modes and the microbiome from samples, however found no statistically significant composition or diversity. Dominguez-Bello et al. reported differences in bacterial communities in infants' guts according to the delivery methods [43].

The proportion of twin pregnancies in this study was approximately a quarter (37/141). Regardless of chorionicity, twins have a similar composition of microbiomes as randomly selected samples. When analyzed only in dichorionic twins, the individuals or "siblings" that have separate intrauterine compartments (placenta and amniotic cavity), but share the same mother, all four types of samples from neonates did not significantly differ in the microbiome

composition. The samples from CB and M in monochorionic twins showed a statistical difference, but the evidence seems to be weak because of the small sample size of monochronic twin. Although the evaluation for twin pregnancies in this study was proceeded as a subanalysis, this is the very first microbiome analysis performed in twin newborns in the available literatures. Since twins are different individuals sharing the same intrauterine environment and the samples from twins are relatively rare compared to singletons, future studies focusing on twins or other higher-order pregnancies are essential and the result of this subanalysis will be the basic step for the further investigation.

V. Conclusion

The exploration for microbiologic features in the compartments related to pregnancy has been historically a challenging issue for researchers to struggle for many decades although is still controversial. Of note, a part of abnormal microbial invasion to the gestational cavity such as AF or placenta definitely seems to engender serious obstetric complications including preterm birth and severe neonatal morbidities that might persist during lifetime. Despite the significance of research on microbiome in pregnancy, the advancement is relatively in a stalemate due to several limitations specified to pregnancy such as ethical vulnerability as a study subject and difficult accessibility to obtain samples. We have collected various samples in pregnant women and their neonates with a standardized protocol and established the database of microbiome which further will be used as a baseline library for samples from diverse pathologic conditions. We will continue further to understand the role of the microbiome in a normal pregnancy and the pathophysiology of microbiome modification during the development of obstetric complications and explore the significance through quantitative analysis as well. This study could be considered as the very initial challenge to build up the basic

database since it contains the samples from the general pregnant population and normal infants.

Table 1. Clinical characteristics of the study population

Characteristics	Values
Age (years)	34 (31–37)
Nulliparity	67.4% (95/141)
Height (cm)	162.4 (159.5–165.1)
Weight (kg)	69.9 (65.7–77.0)
BMI (kg/m^2)	27.0 (25.1–29.8)
Pregnancy from IVF–ET	24.1% (34/141)
Pregnancy from IUI	5.0% (7/141)
Twin pregnancy	26.2% (37/141)
… Monochorionic twins	18.9% (7/37)
Gestational age at delivery (weeks)	37.7 (36.9–38.6)
… Preterm birth before 37 weeks	26.2% (37/141)
Cesarean section	54.6% (77/141)
Birthweight (grams)[a]	2800 (2480–3124)
Male neonates[a]	50.0% (89/178)
Low Apgar score < 7 in 1 minute[a]	3.9% (5/178)
Low Apgar score < 7 in 5 minutes[a]	0.6% (1/178)
Meconium staining[a]	2.2% (4/178)
Congenital structural anomaly[a]	3.9% (7/178)
Obstetric complications and underlying diseases	
Use of tocolytics due to preterm labor	8.5% (12/141)
PPROM	3.5% (5/141)
Cerclage operation	4.3% (6/141)
Preeclampsia	12.1% (17/141)
Chronic hypertension	2.1% (3/141)
Fetal growth restriction	2.8% (4/141)
Oligohydramnios in the 3rd trimester	5.0% (7/141)

Gestational thrombocytopenia	3.5% (5/141)
Gestational diabetes	13.5% (19/141)
Pregestational diabetes	0.7% (1/141)
Placenta previa	2.1% (3/141)
Hypothyroidism	9.9% (14/141)
Hyperthyroidism	0.7% (1/141)

BMI, body mass index; IVF−ET, in−vitro fertilization and embryo transfer; IUI, intrauterine insemination; PPROM, preterm premature rupture of membranes

Values are expressed as the median (interquartile range) for continuous variables and percentage for categorical variables

[a]The denominator is the number of newborns

Table 2. Summary of the results (R^2 and p-values) from permutational multivariate analysis of variance (PERMANOVA).

Variable	All Sites	AF	CB	GL	M	VD
Site	**0.172 (0.001)**	.	.	.	.	.
LibraryMonth	**0.074 (0.002)**	**0.489 (0.002)**	**0.338 (0.008)**	**0.245 (0.016)**	**0.26 (0.001)**	0.051 (0.834)
Age	0 (0.947)	0.021 (0.506)	0.012 (0.2)	0.019 (0.248)	0.016 (0.151)	0.016 (0.211)
PretermBirth37	0.002 (0.445)	0.029 (0.317)	0.003 (0.826)	0.012 (0.469)	**0.026 (0.017)**	0.004 (0.7)
DeliveryMethod	0.003 (0.206)	0.01 (0.676)	0.011 (0.225)	0.038 (0.069)	0.008 (0.473)	**0.06 (0.005)**
HasGDM	0.002 (0.365)	0.025 (0.372)	0.015 (0.134)	0.012 (0.446)	0.018 (0.086)	0.002 (0.891)
IVFET	0.001 (0.934)	0.01 (0.845)	0.002 (0.934)	0.014 (0.364)	0.004 (0.854)	0 (0.99)
Epidural	0.003 (0.162)	0.03 (0.328)	0.018 (0.108)	0.005 (0.76)	0.006 (0.559)	0.006 (0.47)
InducedLabor	0.002 (0.373)	0.014 (0.676)	0.003 (0.839)	0.02 (0.226)	0.01 (0.378)	0.002 (0.828)
Hypertension	0.009 (0.135)	0.038 (0.573)	0.047 (0.119)	0.011 (0.875)	0.03 (0.363)	0.019 (0.434)
Weight	0.002 (0.483)	0.011 (0.776)	**0.022 (0.039)**	0.003 (0.938)	0.006 (0.59)	0.011 (0.321)
HasTwins	0.001 (0.544)	0.009 (0.802)	0.006 (0.451)	0.016 (0.338)	0.01 (0.369)	0.007 (0.463)
BabySex	0.005 (0.263)	0.024 (0.839)	0.019 (0.261)	0.031 (0.398)	0.015 (0.63)	0.022 (0.387)
AntibioticsUse	0.004 (0.086)	.	0.025 (0.06)	0.007 (0.665)	**0.031 (0.035)**	0.014 (0.23)
Residuals	0.719	0.29	0.478	0.568	0.56	0.786
Total	1	1	1	1	1	1

AF, amniotic fluid; CB, umbilical cord blood; GL, gastric liquid; M, meconium; VD, cervicovaginal discharge

Table 3. Summary of the results from analysis of composition of microbiomes (ANCOM) at the genus level.

Variable	Site	Taxon	W Score	Results
DeliveryMethod	AF	d__Bacteria;p__Firmicutes;c__Clostridia;o__Peptostreptococcales-Tissierellales; f__Peptostreptococcales-Tissierellales;g__Finegoldia	88	Higher in vaginal delivery
DeliveryMethod	CB	.	.	.
DeliveryMethod	GL	d__Bacteria;p__Firmicutes;c__Bacilli;o__Mycoplasmatales; f__Mycoplasmataceae;g__Ureaplasma	49	Higher in vaginal delivery
DeliveryMethod	M	.	.	.
DeliveryMethod	VD	.	.	.
Epidural[a]	AF	N/A	N/A	N/A
Epidural	CB	.	.	.
Epidural	GL	d__Bacteria;p__Firmicutes;c__Bacilli;o__Mycoplasmatales; f__Mycoplasmataceae;g__Ureaplasma	64	Higher with epidural use
Epidural	M	.	.	.
Epidural	VD	.	.	.
PretermBirth37	AF	.	.	.
PretermBirth37	CB	Unassigned;__;__;__;__;__[b]	27	Lower in preterm birth
PretermBirth37	CB	d__Bacteria;__;__;__;__;__[c]	25	Lower in preterm birth
PretermBirth37	GL	.	.	.
PretermBirth37	M	.	.	.
PretermBirth37	VD	.	.	.
HasGDM	AF	.	.	.
HasGDM	CB	d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Frankiales; f__Nakamurellaceae;g__Nakamurella	37	Higher with GDM
HasGDM	GL	.	.	.
HasGDM	M	.	.	.
HasGDM	VD	.	.	.
InducedLabor	AF	.	.	.
InducedLabor	CB	.	.	.
InducedLabor	GL	.	.	.
InducedLabor	M	.	.	.
InducedLabor	VD	.	.	.
IVFET	AF	.	.	.
IVFET	CB	.	.	.
IVFET	GL	.	.	.
IVFET	M	.	.	.
IVFET	VD	.	.	.
Hypertension	AF	d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacterales; f__Enterobacteriaceae;g__Escherichia-Shigella	27	Higher with chronic hypertension
Hypertension	CB	d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Actinomycetales; f__Actinomycetaceae;g__Actinomyces	87	Higher with preeclampsia
Hypertension	GL	d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Bifidobacteriales; f__Bifidobacteriaceae;g__Bifidobacterium	38	Higher with preeclampsia
Hypertension	GL	d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales; f__Porphyromonadaceae;g__Porphyromonas	29	Higher with preeclampsia
Hypertension	M	d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Chitinophagales; f__Chitinophagaceae;g__Vibrionimonas	35	Higher with chronic hypertension
Hypertension	VD	d__Bacteria;p__Campilobacterota;c__Campylobacteria;o__Campylobacterales; f__Campylobacteraceae;g__Campylobacter	45	Higher with chronic hypertension
Hypertension	VD	d__Bacteria;p__Firmicutes;c__Clostridia;o__Lachnospirales; f__Lachnospiraceae;g__[Ruminococcus]_torques_group	35	Higher with chronic hypertension
Hypertension	VD	d__Bacteria;p__Actinobacteriota;c__Coriobacteriia;o__Coriobacteriales; f__Coriobacteriaceae;g__Collinsella	34	Higher with chronic hypertension
Hypertension	VD	d__Bacteria;p__Firmicutes;c__Clostridia;o__Peptostreptococcales-Tissierellales; f__Peptostreptococcales-Tissierellales;g__Fenollaria	34	Higher with chronic hypertension
AntibioticsUse	AF	.	.	.
AntibioticsUse	CB	.	.	.
AntibioticsUse	GL	d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales; f__Tannerellaceae;g__Parabacteroides	40	Higher with antibotics use
AntibioticsUse	GL	d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales; f__Burkholderiaceae;g__Cupriavidus	39	Higher with antibotics use
AntibioticsUse	M	.	.	.
AntibioticsUse[a]	VD	N/A	N/A	N/A
BabySex	AF	.	.	.
BabySex	CB	.	.	.
BabySex	GL	.	.	.
BabySex	M	.	.	.
BabySex	VD	.	.	.

AF, amniotic fluid; CB, umbilical cord blood; GL, gastric liquid; M, meconium; VD, cervicovaginal discharge

[a]Significant hits were found by ANCOM, but these results were discarded as they have a very low W score (zero in many cases)

and are likely artifacts; note that this is a known bug in ANCOM, typically caused by small sample size for a given test

[b]Amplicon sequence variants were labelled 'Unassigned' if it was not possible to classify them at the highest taxonomic level at the required confidence level

[c]These amplicon sequence variants could not be classified beyond the domain level at the required confidence

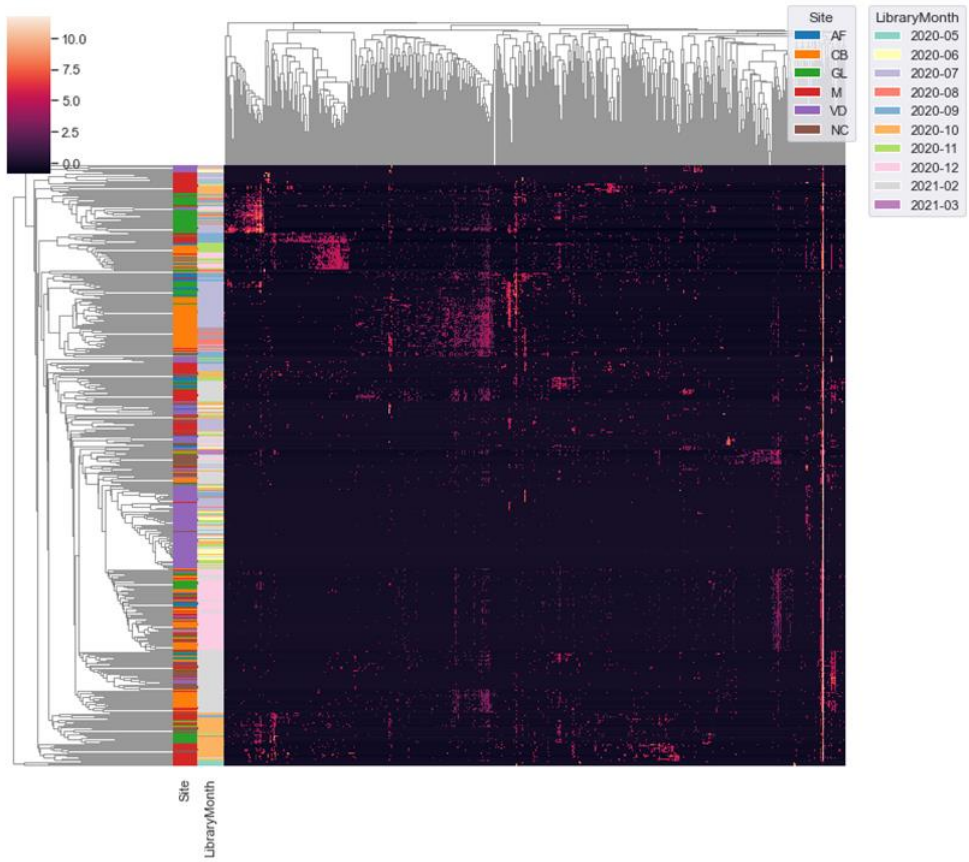
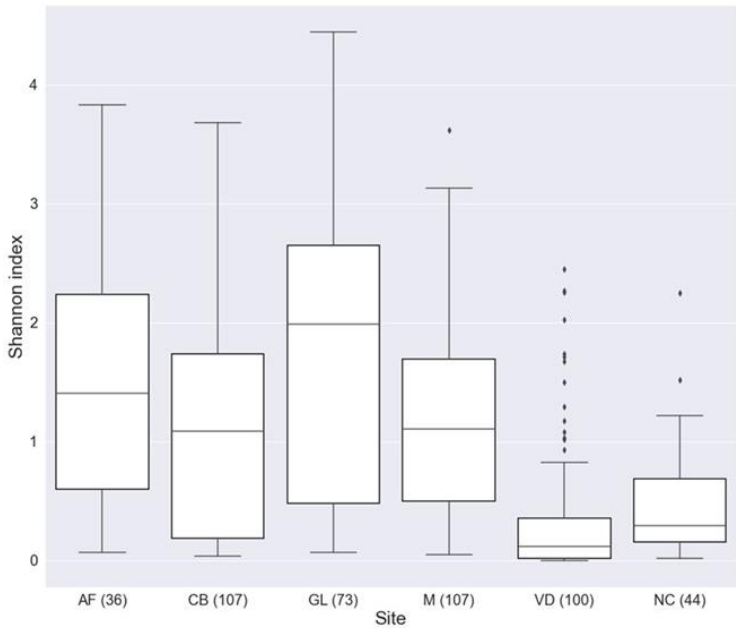
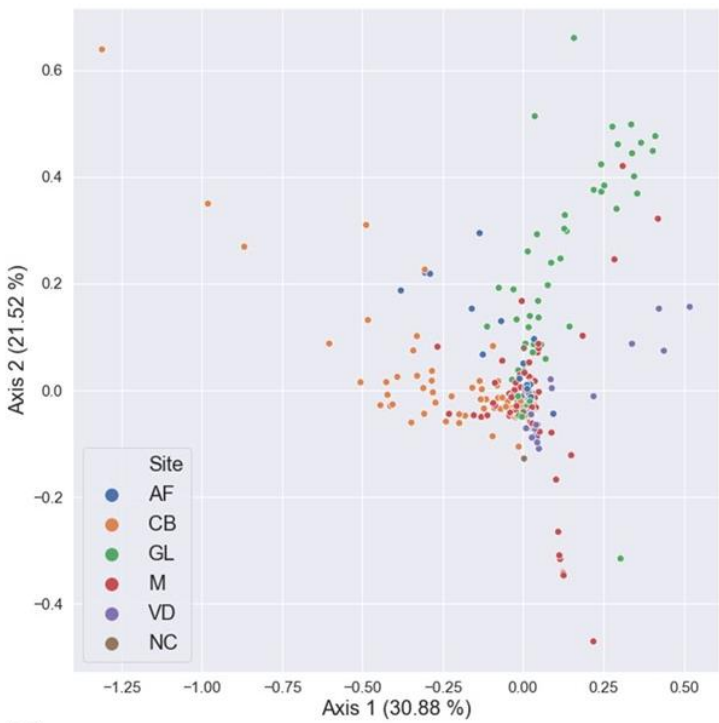


Figure 1. Batch effect detection in 16S rRNA amplicon sequencing data. Center log-ratio transformation was used to normalize the filtered ASV table before generating a hierarchically clustered heatmap based on correlation coefficients. AF, amniotic fluid; CB, umbilical cord blood; GL, gastric liquid; M, meconium; VD, cervicovaginal discharge; NC, negative control



(A)



(B)

Figure 2. Alpha and beta diversity of the Korean maternal and neonatal microbiome. (A) Alpha diversity: The filtered ASV table

was rarefied before Shannon index was computed for each sample. The VD group exhibited the least amount of alpha diversity. AF, amniotic fluid; CB, umbilical cord blood; GL, gastric liquid; M, meconium; VD, cervicovaginal discharge; NC, negative control; (B) Beta diversity: The filtered ASV table was rarefied before the samples were projected into 2D-space with principal coordinates analysis using the weighted UniFrac distance

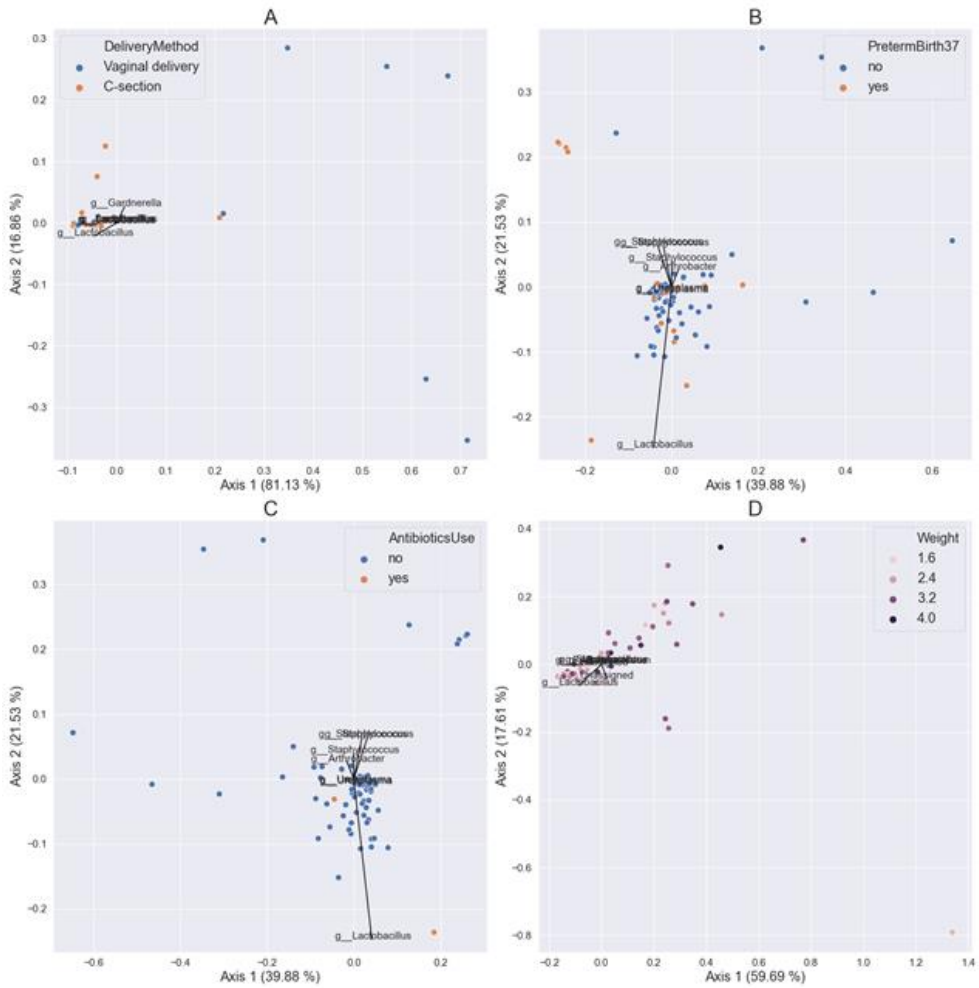


Figure 3. Beta diversity results of the PERMANOVA analysis. Principal coordinates analysis using weighted UniFrac distance is shown for A the cervicovaginal discharge samples, B and C the meconium samples, and D the umbilical cord blood samples

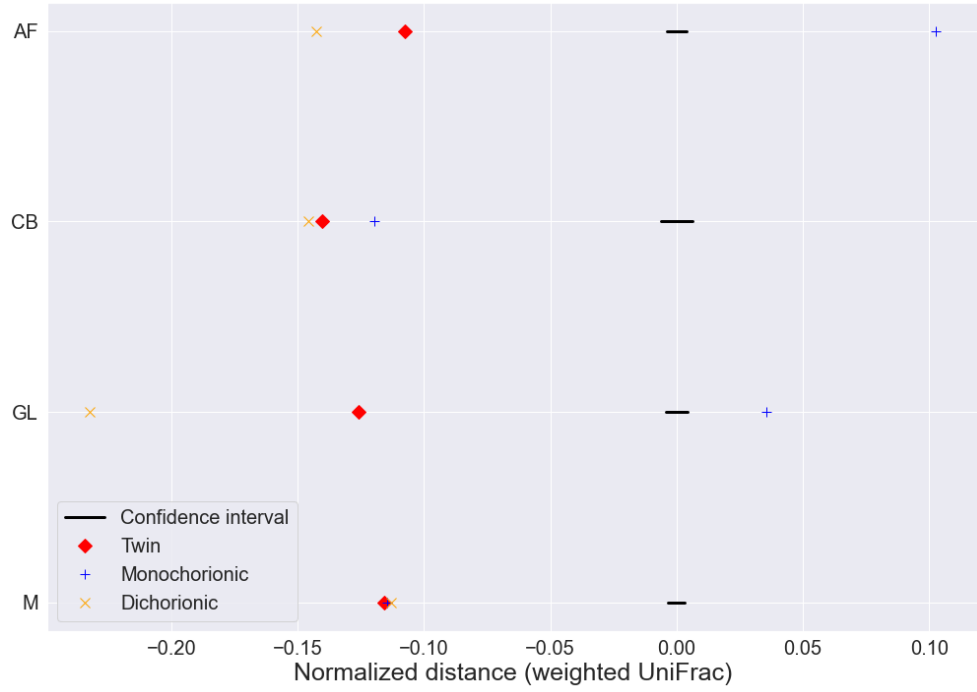


Figure 4. Higher similarity of microbiome composition in twin samples than in randomly chosen samples. For each sample type, the means of weighted UniFrac distances are shown for the twin samples. A 95% confidence interval was constructed by randomly sampling pairwise distances with replacement from the samples for 1,000 times

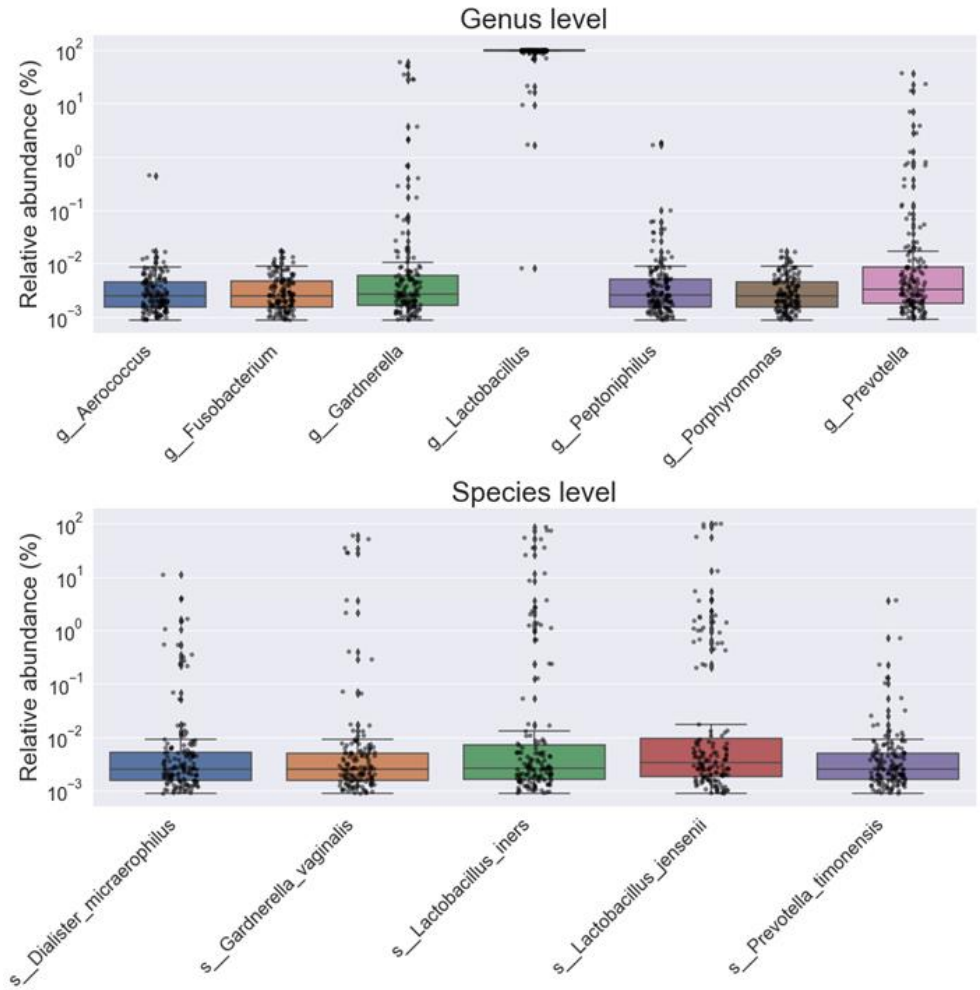
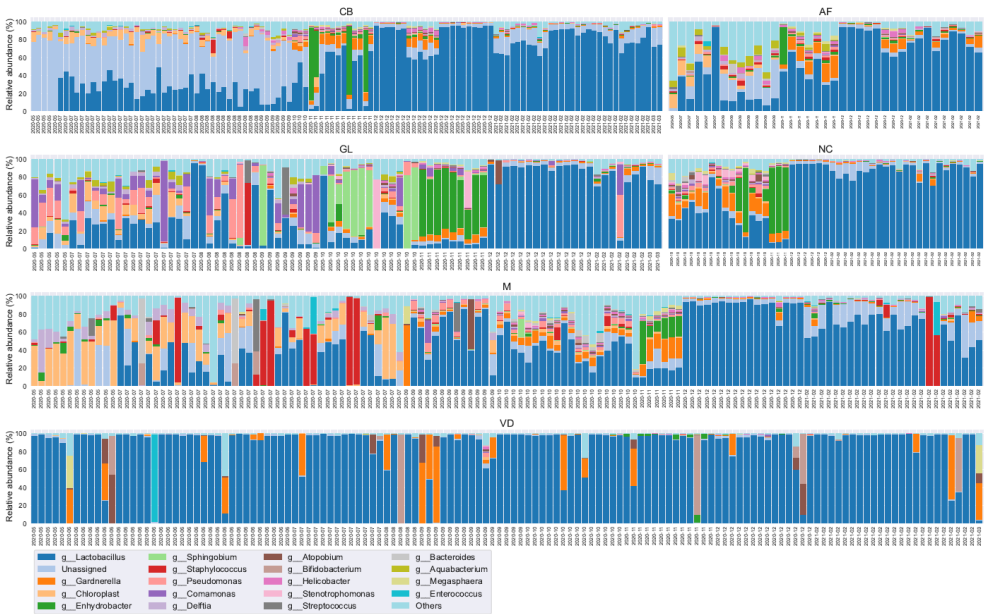
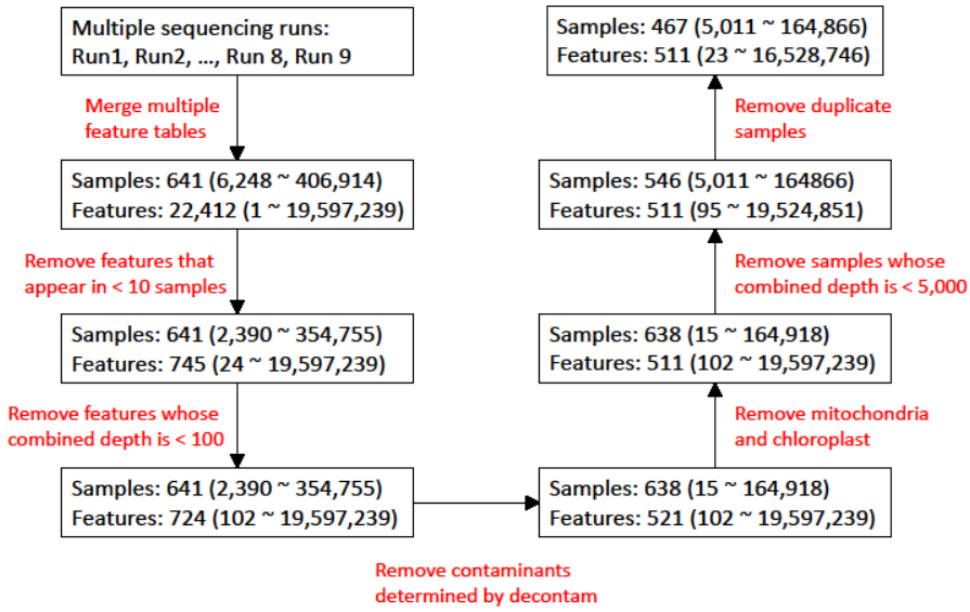


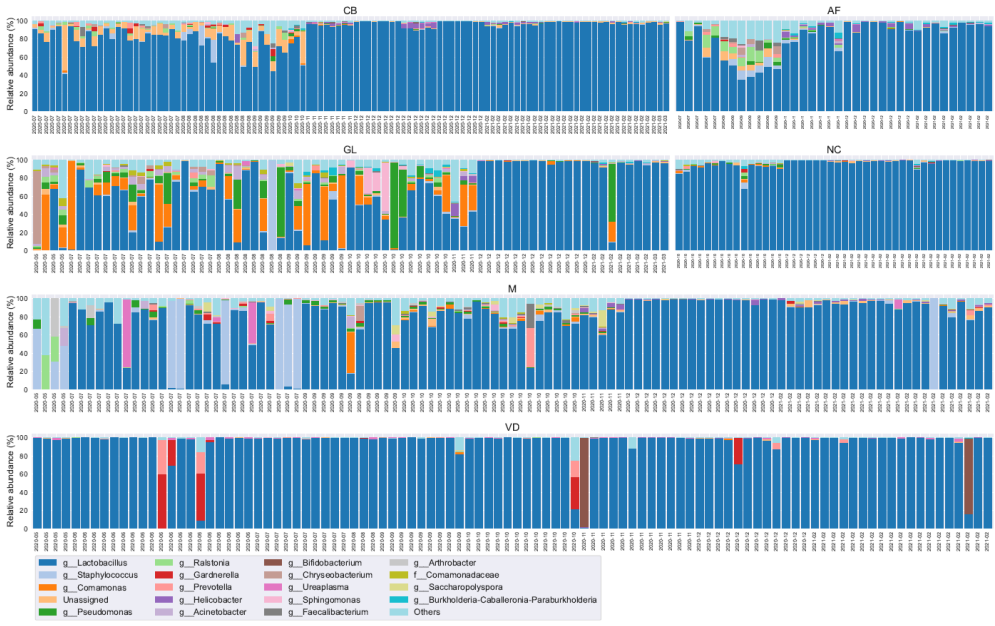
Figure 5. Relative abundance of bacteria associated with vaginal health. Only bacterial targets in uBiome's SmartJane assay that are also present in the vaginal discharge samples are shown



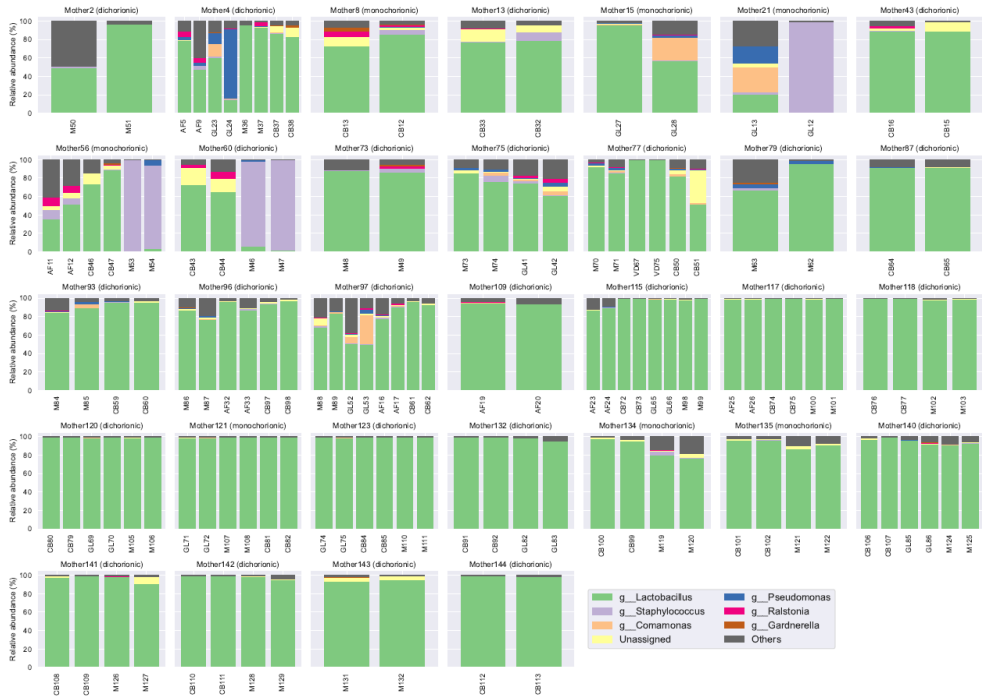
Supplementary Figure S1. Relative microbiome abundance at the genus level without removal of contaminants. The 19 most abundant genera are labelled. Abbreviations: AF, amniotic fluid; CB, cord blood; GL, gastric liquid; NC, negative control; M, meconium; VD, vaginal discharge.



Supplementary Figure S2. Filtering scheme for contaminant removal



Supplementary Figure S3. Relative microbiome abundance at the genus level after removal of contaminants. The 19 most abundant genera are labelled. Abbreviations: AF, amniotic fluid; CB, cord blood; GL, gastric liquid; NC, negative control; M, meconium; VD, vaginal discharge.



Supplementary Figure S4. Taxonomic bar plots for twins showing relative abundance of the microbiome with removal of contaminants. The filtered ASV table was used to generate taxonomic bar charts with relative abundance of microbiome at the genus level. The top 6 most abundance genera are labelled.

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요약(국문초록)

최근 일부 연구자들이 태반이나 양수에서 세균총을 발견하여 분석하고 보고하였는데 이는 기존에 이러한 임신산물은 무균상태라고 알려진 것에 반하는 결과이다. 본 연구는 전향적 코호트 연구를 통하여 임산부와 그들의 신생아에서 얻을 수 있는 다양한 검체의 세균총을 확인해보고 모체-신생아 간의 연관성이나 각종 임신과 관련한 인자에 따른 차이를 탐색해보고자 하였다.

총 141명의 임산부와 178명의 신생아에서 641개의 검체(질분비물, 양수, 제대혈, 신생아 위액, 태변)를 얻어 19,597,239 세균 염기서열을 파악하였다. 본 연구의 세균 염기서열 분석에는 차세대 염기서열 분석기법이 사용되었으며, 검체의 오염을 제거하기 위하여 다양한 기법이 사용되었다. 질분비물의 경우 오염의 가능성이 가장 높은 검체였으며, 잘 알려진 락토바실러스 균이 다수를 형성하고 있었다. 세균총은 모체-신생아 간의 연관성 보다는 검체가 획득된 구획에 따라 높은 유사성을 보였다. 양수와 제대혈의 경우 검출된 세균의 양이 분석에서 대조를 위하여 사용한 무균 처리된 생리식염수와 거의 비슷한 정도였다. 반면에 태변에서는 유사성이 있는 세균총 군집이 관찰되었는데, 신생아의 장내세균이 형성되는 과정과 관련이 있을 수 있다. 신생아 위액의 경우 그 세균총이 양수와 완전히 동일하지 않다는 점에서 태아가 양수를 삼키고 다시 소변을 배출하여 양수를 형성하는 과정에서 이미 자궁내에서 태아의 위장관계가 고유 세균총의 상재화를

시작하는 과정이 존재할 수 있다는 추측도 가능하다.

요약하면, 임산부와 신생아에서 채취한 검체의 세균총은 각 구획에 따라 유사한 군집이 형성되었고, 일부 자궁내 환경을 구성하는 검체의 경우 무균 상태에 매우 근접한 결과를 보였다. 본 연구를 통하여 임산부와 신생아의 다양한 검체에서 세균총의 데이터베이스를 형성할 수 있었으며, 이와 같은 자료의 축적은 향후 임신에서 발생하는 합병증이나 주산기의 부정적 결과를 초래하는 각종 위험 인자와 연관된 세균총 탐색의 기초로 사용될 수 있다는 점에서 의의가 있다.

주요어 : 세균총, 임신, 신생아, 양수, 태변, 제대혈, 제왕절개술, 질식분만

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