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Research Article

Microbiome of the Different Body Sites in Preeclampsia Patients to Reveal the Role of Bacteria in the Multifactorial Causes

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Abstract

Aim: Preeclampsia (PE) is a pregnancy-associated disorder. Its incidence is increasing with the age. Especially for those elderly parturient women, the risk of incidence of PE is getting higher. Since human microbiota plays an important role in the maternal health, it is necessary to study the microbial differences between PE and healthy pregnant women. Microorganism dysbiosis may be detected as a biomarker for PE early diagnosis.

Methods: 24 samples were collected and categorized into 8 groups: four groups in healthy pregnant women, NF (gut), NV (vaginal fluid), NS (The second maxillary molars exudates), ND (placenta) and four groups in PE pregnant women, PF (gut), PV (vaginal fluid), PS (The second maxillary molars exudates), PD (placenta) as corresponding body sites. Microbiota from all groups was analyzed by sequencing the V4 region of the 16S rDNA gene via MiSeq.

Results: It was found that *Firmicutes* (10.84%-94.52%), *Proteobacteria* (0.27%-37.58%), *Bacteroidetes* (0.34%-48.97%), *Actinobacteria* (0.26%-28.40%) and *Fusobacteria* (0.03%-11.28%) are dominant bacteria in the pregnant women. while *Aerococcaceae* and *Peptostreptococcaceae* increase significantly ($p < 0.05$, wilcox. test) in PE women. The subgroup NF and PF have the most significant difference. *Bacteroides, Faecalibacterium, Roseburia* and *Coprococcus* were abundant in NF, while *Bifidobacterium*, *Eggerthella* and *Escherichia* were enriched in PF. Both ND and PD were detected for *Fusobacterium* and *Prevotella*, which as same as NS and PS. Overall, there are 24 level 2 KEGG Orthology groups (KOs) represented only in healthy group through PICRUSt. Membrane Transport, Carbohydrate Metabolism, Replication and Repair are the most.

Conclusions: This study suggests that differences indeed exist in the microbiota between healthy and PE women at different body parts. It indicated that Carbohydrate Metabolism and Replication and Repair are very important given the elevated inflammation that can trigger pre-eclampsia. A dedicated microbiome database of Chinese pregnant women covering larger populations and body parts is essential to reveal the underlying mechanisms.

Keywords: Pre-eclampsia; 16S rDNA; Microbiota; Placenta; Gut

Abbreviations

PE: Preeclampsia; KOs: KEGG Orthology Groups; PICRUSt: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; HDCP: Hypertensive Disorders Complicating Pregnancy; UPGMA: Unweighted Pair Group Method with Arithmetic Mean; NGS: Next Generation Sequences; CSTs: Community State Types

Introduction

Preeclampsia (PE) is a hypertensive disorder that complicates pregnancy. It is characterized by occurrence of hypertension and significant proteinuria that affect the maternal and fetal health [1,2]. Severe PE may lead to systemic endothelial dysfunction, microangiopathy, impaired liver function, thrombocytopenia and pulmonary edema [3,4]. Multiple factors, including antiphospholipid antibody syndrome, chronic hypertension, pregestational diabetes, obesity, family history and etc, may increase the risk of PE [3,5].

The primary pathology of pre-eclampsia is defective deep placentation. However, the precise cause of placental dysfunction remains uncertain [6] More recently studies [7-9] have used sequencing-based techniques (16SrRNA-based or / and metagenomic sequencing) to find that the placenta is not sterile and it is a harbor for a low-abundance but metabolically rich microbiome and that may differ between healthy and complicated pregnancies. However, the issue of bacteria presence in the placenta remains controversial. The controversy persists over the origin of microbes in the placenta, which is caused by the human placenta have a resident microbiome, the contamination of potential pathogens (labor and CS), the contamination during the experiment, or the microbial sequencing analysis. Marcus C. et al. found no evidence to support that the existence of a placental microbiome, but human placenta can contain potential pathogens [10].

The HMP [11] and MetaHIT [12] investigated the normal

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microbiome colonies at various sites to understand the synergistic interactions between the microbiome and its host. A few body sites or organs were taken into consideration for normally sterile, and the presence of microorganism was used as a marker of pathological process in the past. However, these assumptions were proven to be not correct, such as blood [13], placenta [7,8] and uterus [9]. The microbiota plays a fundamental role in immunity and inflammation of the host immune system, particularly in the systemic circulation. Differences in microbiome colonies at various sites between normal and preeclampsia patients have not been clearly clarified yet except for some limited studies. For example, Ranmalee, Amarasekara, et al., confirmed the presence of microbe in the placental tissues of PE women and demonstrated the role of microbe in the polyfactorial cause of PE [14]. Jing Li et al. [15], revealed that the gut microbiome plays a great role in cardiovascular diseases and its microbiota dysbiosis contributes to the development of hypertension. J. Liu et al suggested that there is an obvious structural shift of the microbial community in PE patients' gut and it might be associated with disease occurrence and development [2]. The oral fluids of patients with PE were detected higher levels of biomarkers related with the PE development [16]. Oral microbiota in pregnancy will be changed, especially periodontal pathogens that may be promoted to be a risk factor for the health of pregnant women [17]. Furthermore, maternal oral pathogens may enter the systemic circulation through local tissue inflammation and destruction, and may play a role in the pathogenesis of PE by affecting the placenta [18], vaginal microbial dysbiosis in pregnant women have a significant impact on PTB risk and vaginal microbiome composition vary dramatically across populations [19]. The objective of this study is to investigate the difference of microbiota from gut, vagina, placenta and oral of Chinese pregnant women with PE, and propose a new approach for PE early diagnose and precise intervention.

Material and Methods

Subjects

Three pregnant women with pre-eclampsia and three normal pregnant women were admitted to the Department of Obstetrics of Affiliated Bao'an Hospital of Shenzhen, Southern Medical University in 2016. Written informed consents were obtained from all participants. Both cases and controls were matched for their delivery way, age, BMI, region, diet, family background, education, occupation to minimize the phenotype heterogeneity (Table S1, S2). However, it is difficult to match their gestational age undergoing operation since healthy pregnant women do not experience elective cesarean section prior to term. Preeclampsia was defined according to the criteria that is the systolic blood pressure ≥140mmhg and/or diastolic blood pressure ≥ 90mmhg occurred after 20 weeks of gestation, accompanied by any of the following: urine protein ≥ 0.3g / 24h, or urine protein/creatinine ratio ≥ 0.3, or random urine protein ≥ (+) (test method when urine protein quantification is not possible); No proteinuria but with any of the following organs or systems involved: heart, lungs, liver, kidney and other important organs, or abnormal changes in the blood system, digestive system, nervous system, placenta-fetal involvement. The control group was healthy pregnant women, with no other obstetric conditions complicating pregnancy. Women with chronic or acute disease, multiple gestation, long-term steroids use, or endocrine disorders were excluded from the subjects. After collecting vaginal secretions, cervical examinations were done on the six women prior to catheterization before surgery and none had dilated cervices and nor they in labor.

Chemical and physical property analysis

Over 250 mg early morning stool were collected into sterilized sample boxes. The second maxillary molars dental plaque (12 hours after tooth brushing in the former evening) with sterile miniature subgingival scraper were collected into sterile centrifuge tube. Vaginal swabs were collected at the midpoint of the vagina using CultureSwab polyester-tipped swabs (KANGJIAN, China). After delivery of the fetus by cesarean section, the placenta was flushed with sterile saline and placed in a sterile container, and then small samples were immediately dissected from a cotyledon close to the insertion of the umbilical cord as 1x1x1cm cuboidal sections. It should be noted that all cesarean section women are given antibiotics after delivery of the placenta. These 24 samples were transferred to a -80°C freezer within 2 hours and categorized into 8 groups: four groups in healthy pregnant women, NF (gut samples), NV (vaginal fluid), NS (The second maxillary molars exudates), ND (placenta) and four groups in PE pregnant women, PF, PV, PS, PD as corresponding body sites. All frozen samples were sent to BGI-Shenzhen (Shenzhen, China) to extract the genomic DNA of the bacteria. Fecal microbial DNA was isolated by the PowerLyzer PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA, USA). The other sample DNA was isolated using the PSP Stool DNA Plus kit (STRATEC Biomedical, Berlin-Buch, Germany).

DNA library construct and MiSeq sequencing

The universal bacterial primers 515f/806r (515f: 5'-GTG CCA GCM GCC GCG GTA A-3', 806r: 5' GGA CTA CHV GGG TWT CTA AT-3') were applied to amplify the 16S rDNA V4 region with a standard PCR protocol. The conditions are as follows: 94°C for 3 min (1 cycle), 94°C for 45 s/56°Cfor 45 s/72°C for 45 s (30 cycles), and final step of 72°C for10 min. Libraries and clusters were constructed using the purified PCR products. Then the libraries were sequenced using Miseq MG-302 with Paired end reads 250 bp according to protocols described by Caporaso [20].

Statistical analysis

Raw data of all samples were filtered to remove low quality sequences. Then, FLASH [21] software (v1.2.11) was used to assemble the clean reads into tags with an overlapping length no less than 15 bp and a mismatch lower than 0.1. Chimeric sequences were removed based on the UCHIME algorithm (v4.2.40) [22] with the gold database (v20110519). USEARCH (v7.0.1090) package was used to cluster tags into operational taxonomic unites (OTUs) with 97% pairwise sequence identity and assign taxonomies [23]. Representative sequences of OTU were taxonomically classified via Ribosomal Database Project Classifier v.2.2 based on Greengenes database (V201305), 0.6 confidence values as cutoff.

Unweighted Pair Group Method with Arithmetic mean was used to measure the similarity of all samples and the figure was drawn by software R (v3.1.1). Differences of microbial communities between groups were analyzed using wilcox. Test in R software (v3.0.3). PICRUSt (http://picrust.github.com) [24] was used to explore the function of samples based on the 16S rRNA abundance.

Results

In the PCR amplification of 24 samples, it was found that the PCR amplification of PS1 sample was 0, so the PS1 sample was missing. The rest 23 samples were PCR amplified successfully. We obtained in total 720,974 tags representing DNA and 31,346 ± 220 tags per sample with 152 bp length. Then, we clustered these assembled tags into 737 OTUs, ranging from 33 to 245 OTUs in samples.

Alpha-diversity analysis revealed that the intergroup diversity was not statistically significant ($p > 0.05$, t-test) among the eight subgroups and two large groups. Furthermore, the similarity of eight subgroups was measured by UPGMA, and the Bray-Curtis cluster tree indicated that three group pairs (NS: PS, ND: PD, NV: PV) had high similarity with the crossing branch, while the gut group pairs (NF: PF) had difference with the crossing branch (Figure 1A).

However, we observed that there was an obvious difference in the microbiota composition among the four body sites of healthy versus PE women.

In the phylum level, the composition analysis reveals that the most five abundant phyla in women are *Firmicutes* (10.84%-94.52%), *Proteobacteria* (0.27%-37.58%), *Bacteroidetes* (0.34%-48.97%), *Actinobacteria* (0.26%-28.40%) and *Fusobacteria* (0.03%-11.28%) (Figure 1B, Table S3). The subgroup ND and PD have the most similar microbe composition, with the most abundant two phyla *Firmicutes* and *Proteobacteria* that are higher than 98%. And the subgroup NF and PF have the most significant difference, with the phyla *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* in opposing composition (Figure 1B).

In the genus level, the composition analysis reveals that the most six abundant genus in women are *Lactobacillus* (0-86.79%), *Lactococcus* (0-42.17%), *Prevotella* (0-24.61%), *Bacteroides* (0.0095%-28.64%), Corynebacterium (0-22.37%) and *Staphylococcus* (0-33.26%) (Figure 2, Table S4).

The subgroup NF and PF have the most significant difference. *Bacteroides, Faecalibacterium, Roseburia* and *Coprococcus* were abundant in NF, while *Bifidobacterium*, *Eggerthella* and *Escherichia* were enriched in PF. The subgroup NV and PV were dominated by *Lactobacillus.* Besides, *Collinsella* and *Peptostreptococcus* were elevated in PV. The subgroup ND and PD have the most similar microbe composition. Meanwhile, it is interestingly that both ND and PD were detected for *Fusobacterium* and *Prevotella*, which as same as NS and PS. The three genus in Both NS and PS subgroups with the highest abundant were *Prevotella, Corynebacteri* and *Streptococcus,* which of them are higher than 30% (Figure 2, Table S4).

In the family level, two species *Aerococcaceae* and *Peptostreptococcaceae* have significant differences between healthy and PE women group ($p < 0.05$, wilcox. test) (Table S5). Analysis of all subgroups revealed that the level of both two species almost tripled in the PE group compared to that in the healthy group (Table S5). In the PE group, just two samples had no *Aerococcaceae.* However, in the healthy group it almost disappeared (Figure3A). Besides, all samples of the PE group also contain *Peptostreptococcaceae*. Especially, *Peptostreptococcaceae* is increased by tenfold in the PS subgroup compared to that in the healthy group (Figure3B).

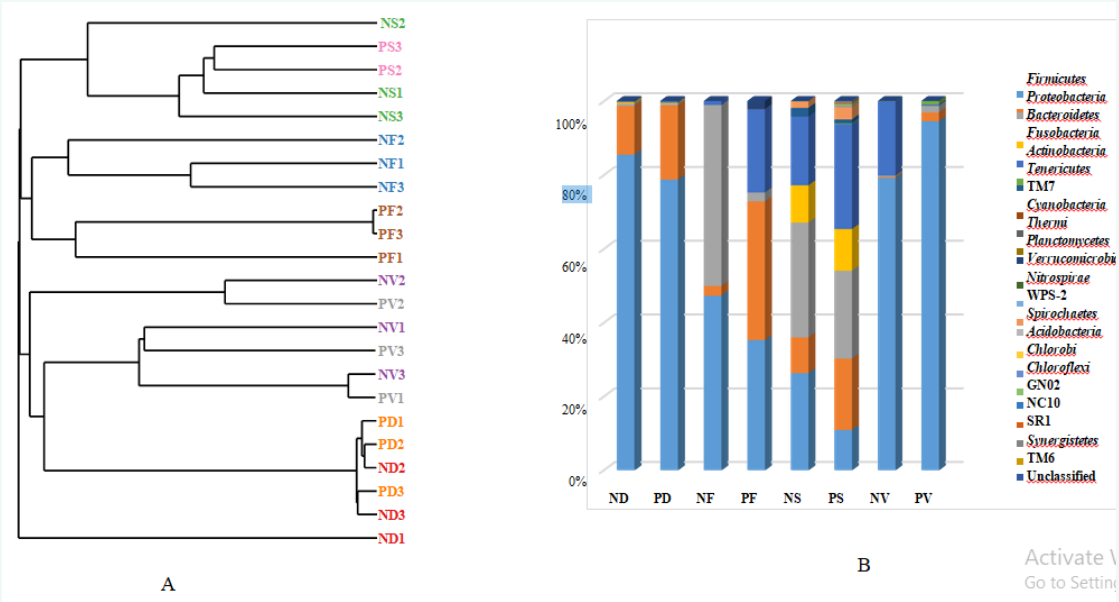

Figure 1 A: Bray-Curtis cluster tree of eight subgroups. B: The relative abundance of microbiota in the phylum level of eight subgroups.

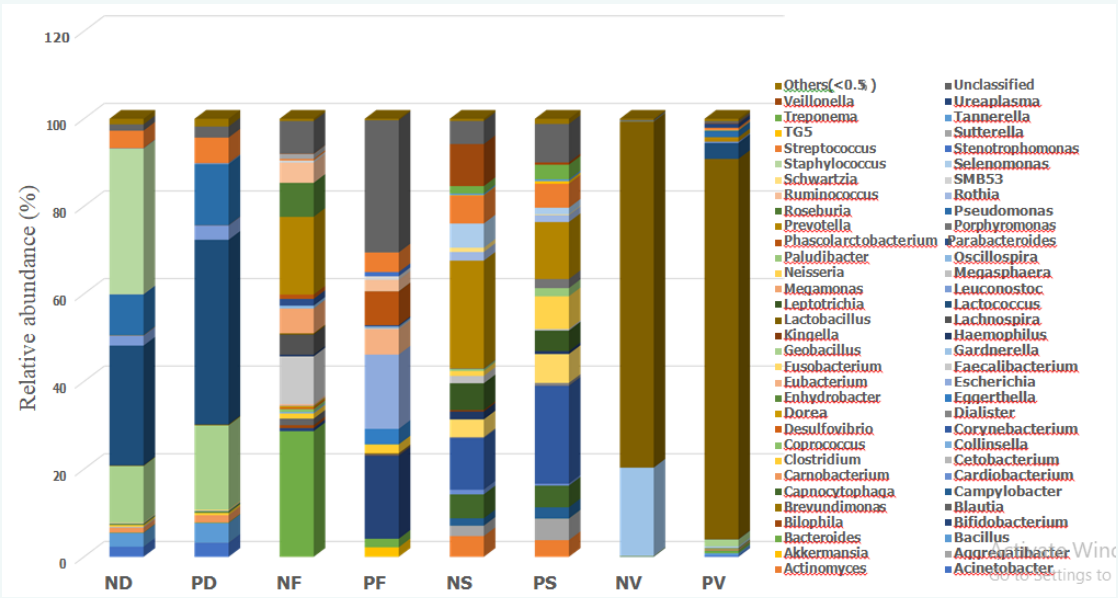

Figure 2 The relative abundance of microbiota in the genus level of eight subgroups.

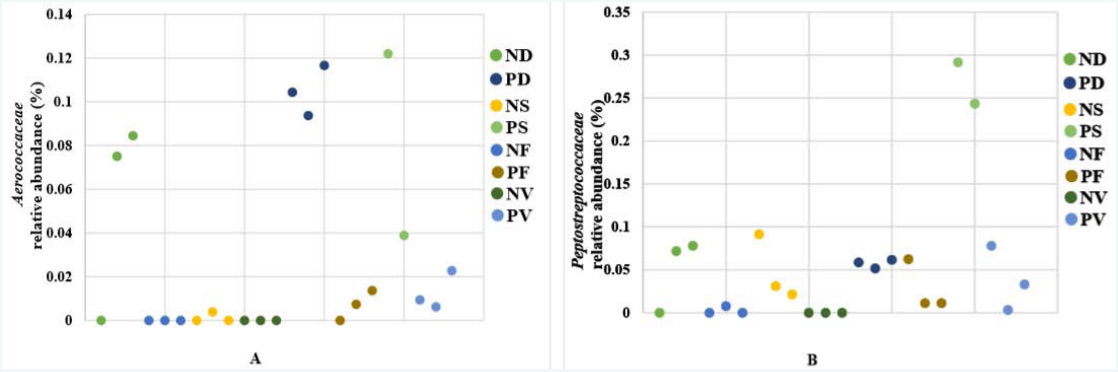

Figure 3 A: The comparison of relative abundance of *Aerococcaceae* at the class level in eight subgroups. B: The comparison of relative abundance of *Peptostreptococcaceae* at the class level in eight subgroups.

Taking PICRUSt as a predictive tool, we found that there are in total 24 level 2 KEGG Orthology groups (KOs) that exist only in the healthy group (Figure 4, Table S6). The most three abundant KOs are related to Membrane Transport, Carbohydrate Metabolism, as well as Replication and Repair, which are involved in environmental information processing, metabolism and genetic information processing. However, only the groups related to Carbohydrate Metabolism and Replication and Repair are significant different (t-tests, P-value < 0.05) as indicated in (Table S6). Besides, seven KOs show great differences (t-tests, P-value < 0.05) among three subgroups.

Discussion

Humans are inhabited by trillions of microbes, residing in different body sites, including skin, gastrointestinal tract, oral cavity, urogenital tract, and airways [25]. These microbes developed a symbiotic relationship with human during evolution, which establishes a dynamic balance system that functions in metabolism, immunity and nutrition absorption [26-28]. However, the microbial composition varies among different body sites and population. Here our results showed that there are great differences in the microbiota composition between healthy and PE women at different parts of the body.

Studies demonstrated that *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* are the most abundant phyla existing in human gut, which is, followed by *Bacteroidetes*. These four phyla in total comprise 90% of all bacteria in the human gut [29, 32].

The first trimester gut microbiota is similar with the composition to that of healthy nonpregnant women. From first to third trimesters, gut microbiota takes dramatic remodeling in diversity between pregnant mothers, with an increase of Proteobacteria and Actinobacteria, while a decrease in

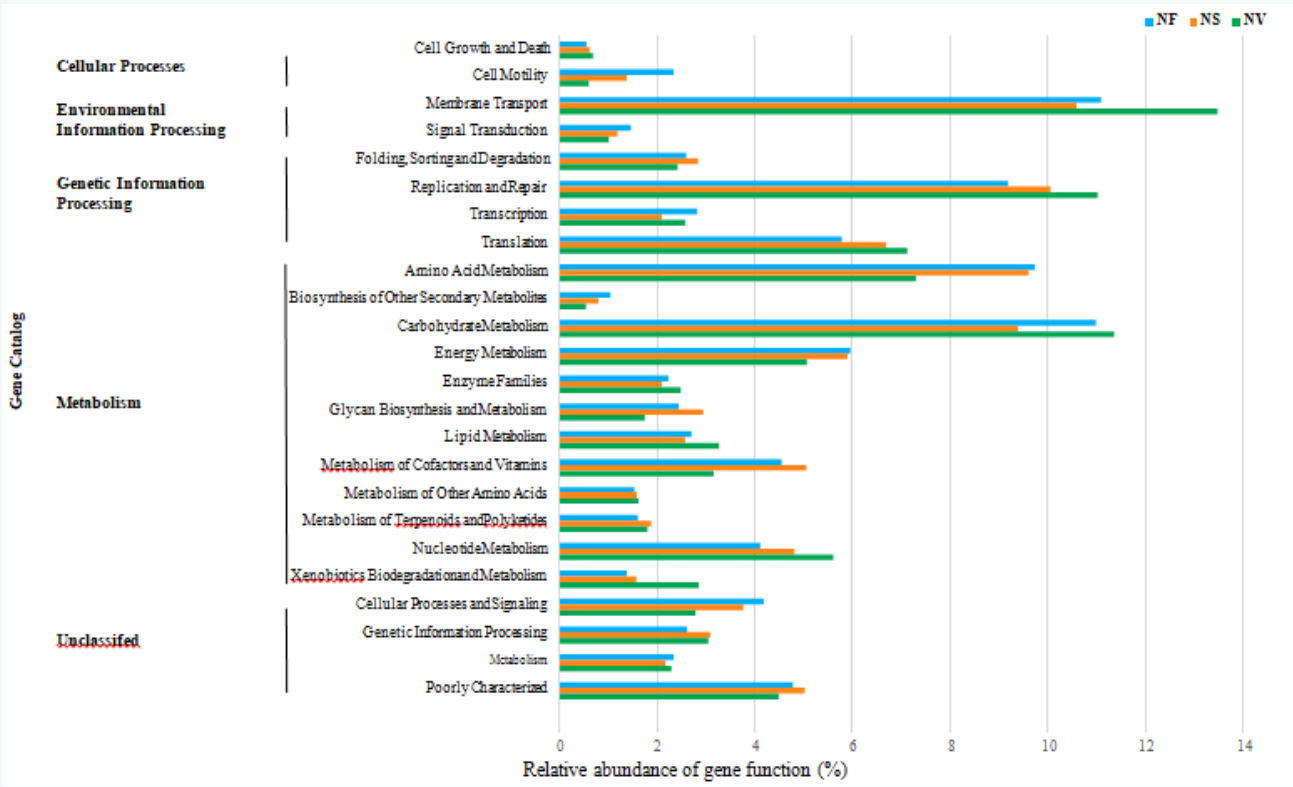

Figure 4 Predicted functions of the bacterial communities found on healthy women.

richness. The microbial reconstruction in late pregnancy resembles a disease-associated micro dysbiosis, such as obesity, inflammation, metabolism syndrome [30].

The phylum level microbiota in the healthy women gut was similar to the integrated reference catalog of the human gut microbiome [31]. Our study showed that in the healthy women gut (NF), the most abundant phylum is *Bacteroidetes*, which is followed by *Firmicute* 96% of bacteria in the healthy women. However, in the gut of PE women (PF), *Proteobacteria* is the most abundant bacteria, followed by *Firmicutes*, *Actinobacteria* and *Bacteroidetes*. *Firmicutes* and *Bacteroidetes* in total are less than 40% (Figure 1B). Bacteroides, *Faecalibacterium*, *Coprococcus* and *Roseburia*, are all dramatically decreased in PE women, while *Actinobacteria* (*Bifidobacterium*, *Eggerthella*) and *Proteobacteria* (*Escherichia*) significantly increased (Figure 2). The relative significant changes indicate that the microbiota changes with PE during pregnancy.

In gut microbiota, *Prevotella* was the most enriched genus in hypertension patients, while *Bacteroides* was the most enriched genus in healthy. *Oscillibacter, Faecalibacterium, Butyrivibrio* and *Roseburia*, which are indirectly linked to *Prevotella*, were all significantly decreased in in high blood pressure people. *Faecalibacterium* is also decreasing in colitis, obesity and asthma. F*aecalibacterium* and *Roseburia* are helpful to produce the anti-inflammatory butyric acid. It's suggested that the excessive growth of some pathogenic bacteria and the lack of synergistic beneficial bacteria may co-participate in the hypertensive disease process [15]. It is reported that *Coprococcus* can regulate the metabolic pathways of propionic acid and butyric acid, and then produces SCFAs. *Coprococcus* declined significantly, which may result in reduced production of propionic acid in the intestinal microenvironment, and eventually raise the risk of PE [32]. *Aerococcaceae* is one of the specific microbiota with significant changes in patients with schizophrenia (SCZ) in the gut microbiome. The mice receiving the fecal transplantation of this microorganism showed disorders related to amino acid and lipid metabolism, thus altering neurochemistry and neurologic function in ways that may be relevant to SCZ pathology [33]. Whether this pathway is related to hypertension or not, it is needed to further study.

In the vagina, normal flora is defined as *Lactobacillus* predominant [12,25,34]. There are differences in the vaginal microbial structure between normal pregnant and women of reproductive age, but they both are dominated by Lactobacillus spp. Meanwhile it is also showed that pregnancy is characterized by a higher degree of stability than observed in non-pregnant women, which might be good for the vaginal microbiological environment during pregnancy against ascending infection of the genital tract and reduced the risk factor for preterm birth [34]. Women who suffering from spontaneous preterm lacked a great number of Lactobacillus spp. of the vaginal microbiota and had higher relative abundance of *G. vaginalis, BVAB1* and *A. vaginae,* these bacteria are associated with bacterial vaginosis [35]. In our study, the predominant *Lactobacillus* in vagina is *L.*

iners and L. reuteri. It comprises 32.38%, 5.41% and 19.52%, 1.65% of the microbiome respectively in healthy and PE. Both of them are dominated by *Lactobacillus*. It is worth mentioning that *Collinsella* and *Peptostreptococcus* are increased in PE. *Collinsella* is correlation with insulin, C-peptide, and HOMA-IR in pregnancy. It is suitable for colonization in mucosal surfaces, metabolizes amino acids, and may directly interact with the host [36]. Whether *L. iners L. reuter, Collinsella* and *Peptostreptococcus* could contribute to Carbohydrate Metabolism and Replication and Repair to regulate hypertension is not clear. The potential role of in PE deserves further analysis. For the oral microbiota, *Streptococcus, Prevotella,* and *Corynebacteri* are all detected in both PS and NS. *Fusobacterium* and *Neisseria* are found in PS, while *Veillonella* and *Leptotrichia* are in NS. This suggests that *the* dominant genus in the second maxillary molars exudates of these women is similar with the previous report [37]. In previous reports, *Fusobacterium* and *Prevotella* in oral flora have been associated with preeclampsia [18]. *Peptostreptococcac* is normally found in the oral cavity, upper respiratory tract, intestinal tract and female genital tract. This study found that *Peptostreptococcac* in PS group was abnormally higher than that in NS group. It may be associated with periodontal disease and has not been reported to be related to preeclampsia. Its drawbacks in our study is that the PS1 sample cannot be detected, so only the biological information obtained from limited results could be analyzed. The oral microorganisms in preeclampsia are urged to continue research.

In the placentas, *Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria* and *Fusobacteria* are the most abundant phyla of both healthy and PE women (Figure 1B). We found that the placental microbiome profiles are most akin to the second maxillary molars exudates in the phyla [7]. Fusobacterium was enriched in both PS and PD, while *Prevotella* was reduced in NS and ND. The results are similar to some reports [7,18]. Furthermore, Fusobacterium and *Prevotella* were also detected in placental tissues, which have been associated with preeclampsia [18]. The research for whether placenta is sterile or not, which become a focus in recent years. The placenta, as low microbial biomass sample, is extremely prone to be contaminated, such as delivery, biopsy DNA extraction, DNA amplified reagents, sequencing, etc. [10]. In the genus level, Stenotrophomonas and Staphylococcus are detected in PD and ND. These two microbes are ubiquitous normal skin commensals, and could therefore originate from contamination during cesarean section. However, for pregnancy complications and infections, there are certain microorganisms and metabolites in the placenta. Therefore, we try our best to eliminate factitious contamination and use the human microenvironment to look for the relationship between diseases and microorganisms.

In our studies, the KO function can be predicted only in the healthy women group, however, no marker gene can be identified by PICRUSt in the PE group. PICRUSt is a computational method that can be used to predict the functional composition in a metagenome via marker gene set and reference genomes database. It provides some functional insights into the millions of existing samples where only 16S data is available [23]. Although the KO function is predictive, it indicated that disorder of human microbiota resulting in adverse perinatal outcomes through interference of Metabolic, immune and inflammatory functions [38-40]. The average abundance of KEGG modules differentially enriched in NF, NS, and NV microbiome in healthy pregnancy. The most three abundant KOs are related to Membrane Transport, Carbohydrate Metabolism, as well as Replication and Repair, which are involved in environmental information processing, metabolism and genetic information processing. It indicated that Carbohydrate Metabolism and Replication and Repair are very important given the elevated inflammation that can trigger preeclampsia.

This study has some limitations. There were only three samples from each size in pregnancies, it is extremely small to derive any significant information relating to the microbiome which is an extremely complex community affected by diet, age, antibiotics use, etc. However, our results are basically consistent with those reported in many studies, and the study still has certain reference value. Thus, it is necessary to collect a larger population with much more diversified body parts to sequence more regions of 16S rDNA, with even full length of 16S rDNA, which in turn generates a detailed microbiome database for Chinese pregnant women.

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Disclosure statement

KL conceived the study and directed project. KL and QF performed the data acquisition, sample collection and clinic support. KL performed data analysis, interpretation, and manuscript writing. All authors have approved the final manuscript as submitted and have no competing interests.

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