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Placentas delivered by pre-pregnant obese women have reduced abundance and diversity in the microbiome

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Abstract

Maternal pre-pregnancy obesity may have an impact on both maternal and fetal health. We examined the microbiome recovered from placentas in a multi-ethnic maternal pre-pregnant obesity cohort, through an optimized microbiome protocol to enrich low bacterial biomass samples. We found that the microbiomes recovered from the placentas of obese pre-pregnant mothers are less abundant and less diverse when compared to those from mothers of normal pre-pregnancy weight. Microbiome richness also decreases from the maternal side to the fetal side, demonstrating heterogeneity by geolocation within the placenta. In summary, our study shows that the microbiomes recovered from the placentas are associated with pre-pregnancy obesity.

Importance: Maternal pre-pregnancy obesity may have an impact on both maternal and fetal health. The placenta is an important organ at the interface of the mother and fetus, and supplies nutrients to the fetus. We report that the microbiomes enriched from the placentas of obese pre-pregnant mothers are less abundant and less diverse when compared to those from mothers of normal pre-pregnancy weight. More over, the microbiomes also vary by geolocation within the placenta.

KEYWORDS

Microbiome, Obesity, Placenta, Pregnancy, 16S sequencing

Abbreviations: BMI, body mass index; DNA, deoxyribonucleic acid; HBV, hepatitis B virus; HIV, human immunodeficiency virus; NaOH, sodium hydroxide; OTU, operational taxonomic unit; PROM, preterm rupture of membranes; QC, quality control; QIIME, quantitative insights into microbial ecology; qPCR, quantitative polymerase chain reaction; rRNA, ribosomal ribonucleic acid; V4, fourth hypervariable region in 16S.

1 | INTRODUCTION

The human microbiome is the collection of microorganisms that reside on or in human organ systems. A delicate relationship exists between the microbiome and the human body. When the balance is maintained, symbiosis, or a beneficial relationship between humans and these microorganisms is achieved. Whereas in dysbiosis, the imbalance of the human microbiome has been associated with several diseases and abnormalities,[1] including pre-term birth. In particular, subjects who experienced pre-term labor had lesser *Lactobacillus* in their microbiome as compared to term gestation subjects.[2] In addition, wider bacterial diversity was noted in pre-term pregnancies as compared to controls, including those associated with the vaginal microbiome such as Lactobacillus species and those associated with the oral microbiome such as *Streptococcus thermophilus*.[2-4] Recent studies show that although placentas are unlikely to harbor microbes,[6,10,11] they could possibly contain pathobionts.[2-9]

We here conducted a study to determine if there is a relationship between maternal obesity status and microbiomes associated with placentas, from babies delivered by scheduled, non-labored cesarean sections. We divided the patients into cases and controls according to their pre-pregnancy weight: either pre-pregnant obese (BMI > 30) or normal-weighted (18.5 < BMI < 25). To account for the low bacterial biomass, we developed an optimized protocol to enrich the V4 region of bacterial 16S rRNA genes. Furthermore, we collected multiple placenta samples per patient, from the maternal, fetal and intermediate layers to examine the variations among them. The results show that microbiomes enriched in obese, and normal pre-pregnant weight women are different and could be biomarkers of maternal obesity.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Placenta samples were collected from pregnant mothers admitted for scheduled, non-labored, full-term cesarean section at ≥ 37 weeks gestation at Kapiolani Medical Center for Women and Children, Honolulu, Hawaii from November 2016 through September 2017. Such procedures minimized the introduction of other bacteria associated with vaginal births as well as bacterial contamination from air during births. The study was approved by the Western IRB board (WIRB Protocol 20151223). Women with preterm rupture of membranes (PROM), labor, multiple gestations, pre-gestational diabetes, hypertensive disorders, cigarette smokers, HIV, HBV, and chronic drug users were excluded from the study. Patients meeting inclusion criteria were identified from pre-admission medical records with pre-pregnancy BMI ≥ 30.0 (obese) or 18.5-25.0 (normal pre-pregnancy weights). Demographic and clinical characteristics were recorded, including maternal and paternal ages, maternal and paternal ethnicities, maternal pre-pregnancy BMI, pregnancy net weight gain, gestational age, parity, gravidity, and ethnicity. Placenta samples were obtained in areas equidistant from the cord insertion site and the placenta edge. Placenta samples were isolated (0.5 cm^3) from the maternal, fetal, and intermediate areas using sterile surgical. Additionally, samples were obtained by waving airswabs in the air in the operating room, in the pathology lab where the placenta biopsies were collected, and in the research laboratory where extraction was carried out. Unopened airswabs were also used as a control.

2.2 | Extraction of genetic material

MOBIO PowerSoil DNA Kit (#12888-50) was used to extract DNA from placenta samples. Three hundred milligrams of placenta were homogenized, heated for 65°C and vortexed in a horizontal bead beater for 10 minutes. DNA was extracted from lysates by putting them through the MOBIO kit following the manufacturer's protocol. Extracted DNA was quantified and quality control checked using NanoDrop.

2.3 | Bacterial DNA enrichment

Given the very low bacterial mass, an enrichment step was performed to remove host DNA contamination and improve 16S specific amplification. (NEBNext Microbiome DNA Enrichment Kit, # E2612L). Samples were enriched in sets of 8 for the optimal enrichment of bacterial DNA. Samples were processed in sets of 8 for two main reasons: (1) Technical limitations of the enrichment kit prevented high throughput processing, such that we could not perform the enrichment on all samples simultaneously; (2) We performed the enrichment on sets of 8, so that every sample was receiving approximately equal exposure time at each stage of the enrichment treatment. We intermixed obese and normal samples in each of the six batches and included airswab samples into batch# 5 and 6. DNAs were incubated with NEBNext magnetic beads for 15 minutes. Beads containing human host DNA were precipitated using a magnet, leaving microbial DNA in the supernatant.

2.4 | qPCR amplification

qPCR was performed to determine 16S counts within extracted samples. Isolated microbial DNA was amplified using primers to the hypervariable V4 region of the 16S rRNA gene, similar to others.[7,12] Forward primer-TCGTCGGCAGCGTCAGATGTGTATAA GAGACAGG

TGCCAGCMGCCGCGGTAA. Reverse primer-GTCTCG TGGGCTCG GAGATGTGTATAAGAGACAGGGACTA CHVG GGTWTCTAAT. PCR was performed using KAPA HiFidelity Hot Start Polymerase; 95°C for 5 mins, 98°C for 20s, 55°C for 15s, 72°C for 1 minute for 25 cycles, 72°C for 5 minutes. After 25 cycles of amplification, V4 specific amplicons were observed by 2% agarose gels and Agilent Bioanalyzer traces. V4 amplicon was detected at the expected size of 290bp. Samples were pooled, size-selected, and denatured with NaOH, diluted to 8 PM in Illumina's HT1 buffer, spiked with 15% PhiX, and heat denatured at 96°C for 2 minutes immediately prior to loading. A MiSeq600 cycle v3 kit was used to sequence the samples, following the manufacturer's protocol.

2.5 | Bioinformatics analysis

The 16S rRNA gene reads were analyzed using a robust bioinformatics pipeline. Reads were stitched using PANDAseq[13] using 150 bp and 350 bp as the minimum and maximum lengths of the assembled reads, respectively. Operational taxonomic units (OTUs) were created by clustering the reads at 97% identity using UCLUST.[14] Representative sequences from each OTU were aligned using PyNAST,[15] and a phylogenetic tree was inferred using FastTree v. 2.1.3[16] after applying the standard lane mask for 16S rRNA gene sequences, Pairwise UniFrac distances were computed using QIIME.[17] Permutation tests of distance and principal coordinates analyses were performed using the MicrobiomeAnalyst, a web-based tool for comprehensive exploratory analysis of microbiome data.[18] Taxonomic assignments were generated by the UCLUST consensus method of QIIME 1.9, using the Greengenes 16S rRNA gene database v. 13_8.[19] We used the phyloseq R package to compute alpha and beta diversity.[20] We used SourceTracker (version 1.0.1) to estimate the percentage of OTUs in placental samples whose origin could be explained by their distribution in the airswabs.[21] We used Decontam[22] V1.4.0 to identify taxa microbiome that are more prevalent in airswabs than in placenta samples.

3 | RESULTS

3.1 | Demographic and clinical characteristics of the cohort

Our cohort consists of 44 women from three ethnic groups including Caucasians, Asians, and Native Hawaiians, who underwent scheduled full-term cesarean deliveries in Kapiolani Medical Center for Women and Children, Honolulu, Hawaii from November 2016 through September 2017. The patients were included based on the inclusion and exclusion criteria described earlier (Materials and Methods). In order to test if there is a microbiome difference associated with maternal pre-pregnancy obesity, the subjects were recruited into two groups: normal pre-pregnant weight (18.5 < BMI < 25) and pre-pregnant obese (BMI > 30) group. The patient demographical and clinical characteristics are summarized in Table 1. Maternal ages, gestational weight gain, and gestational age differences between the cases and controls are not statistically significant, excluding the possibility of significant confounding from these factors. Maternal pre-pregnant obesity, however, is associated with increasing parity and gravidity ($P < .05$). The variation in recruited cases vs controls in each ethnic background reflects the multi-ethnic population demographics in Hawaii.

TABLE 1 Clinical characteristics of the cohort

Variables		Obese (n = 26) Mean (SD)	Non-obese (n = 18)	*P* value
Maternal age, years		32.1(2.8)	31.4(0.7)	.72
Pre-pregnancy BMI, kg/m^2		34.1(5)	21.8(1.2)	.00000001
Gestational weight gain		31.4(7.7)	31.5(7.7)	.99
Maternal Ethnicity		Caucasian = 5 Asian = 5 HPI = 13	Caucasian = 7 Asian = 8 HPI = 3	.29
Parity	0	2	2	.01
	1	8	3	
	2	2	6	
	>3	0	8	

3.2 | Enrichment of the microbiome associated with placentas

Due to the low bacterial biomass, we developed a protocol to enrich the V4 region of bacterial 16S rRNA genes (see Materials and Methods). Before sequencing, we first performed qPCR to determine the 16S rRNA copy numbers within extracted samples. As shown in Figure 1A, placenta samples contain significantly more copies of 16S as compared to airswab or water negative controls (Log_{10} transformed values: Placenta: 12.2; Airswab: 2.1; Water: 1.1). The significant difference (P value < .05) of 16S transcript numbers between placentas and airswab/water suggests that the enrichment protocol was successful. Furthermore, V4 amplicons post-PCR on the agarose gel show the specific band of 290 bp—the expected size of V4 amplicons, providing additional support for the successful 16S amplification of microbiome associated with placenta samples (Figure 1D). Upon positive confirmation from qPCR,

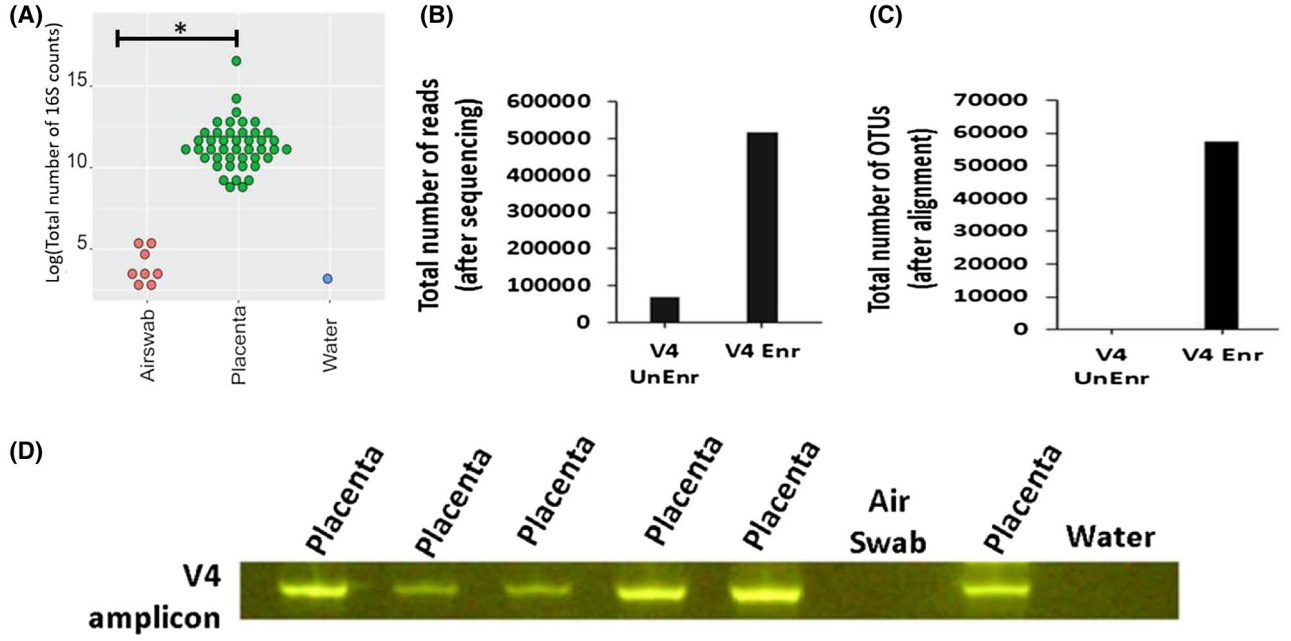

FIGURE 1 A, Log_{10}(total number of 16S counts) for placenta, airswab control, and water control samples. B, Total number of reads in enriched and unenriched samples while varying primer types. C, Total number of OTUs (after alignment to Greengenes database) in enriched and unenriched samples. D, Agarose gel run showing specific V4 amplicon (290bp) detected in placenta samples, which are below detection in airswab or water controls

we implemented a bioinformatics analysis workflow following 16S sequencing (Supplementary Figure S2). As shown in Figure 1B, unenriched V4 samples yield much lower total reads (median: 68,468) as compared to enriched V4 samples (median: 516,479), confirming the success of the experimental protocol. We aligned the 16S sequencing reads using the Greengenes database. The enriched samples using V4 primers detected 57,468 ± 2,859 operational taxonomic units (OTUs), compared to 233 ± 36 OTUs from un-enriched samples (Figure 1C), again highlighting the strength of the enrichment step following DNA extraction.

3.3 | Microbiomes associated with pre-pregnant obese women are less diverse than those of women of normal pre-pregnant weights

As many of the detected OTUs are unlikely to be of use when modeling the data, we removed OTUs that had low counts and variances. We removed 523 OTU features that had very few abundance levels (minimum counts) across samples and 3 OTUs that had low variance based on their interquartile ranges. As a result, a total of 26 taxa remained after the data filtering step (Figure 2A). Additionally, to check if the variation in data was driven by batch effects rather than by pre-pregnancy obesity status, we performed the principle component analysis of all samples (Figure 2B). We did not observe significant batch effects.

The heatmap of the microbiome associated with placentas of pre-pregnant obese mothers suggests the overall trend of less bacterial abundance and diversity, compared to that from the control group of normal pre-pregnant weights (Figure 2A). We then plot the Alpha diversity –Choa1 metric among samples (Figure 2C). Despite the variations, there appear to be higher diversities among the control samples compared to the cases. Moreover, the overall species richness, measured by alpha-diversity—Chao1 metric in the rarefaction curve, is less in pre-pregnant obese samples compared to control samples (*t* test, *P* value = 6.53E-05) across all read depths (Figure 2D). Next, we analyzed the taxonomic composition of the community through the direct quantitative comparison of relative abundance (Figure 2E). It is worth noting that control sample 66PI shows particularly high bacterial biomass compared to other control samples, possibly indicating an infection (Figure 2A). We thus excluded this sample from the following comparisons between cases and controls. The average relative abundance of *Lactobacillus* (Mann-Whitney *U* test, *P* value = .01) is significantly lower in obese samples compared to normal weight samples (Figure 2F), even though there are significant variations among individuals. Additionally, *Haemophilus* has a lower relative percentage in the obese group; however, the difference is not significant (*P* value = .24). Previously *Haemophilus* was observed to be less abundant in the salivary microbiome of obese subjects, compared to controls.[23]

Of note, as the UCLUST clustering method is known to call many OTUs, we used another popular method Dada2[26] which is more sensitive and specific to call OTUs than

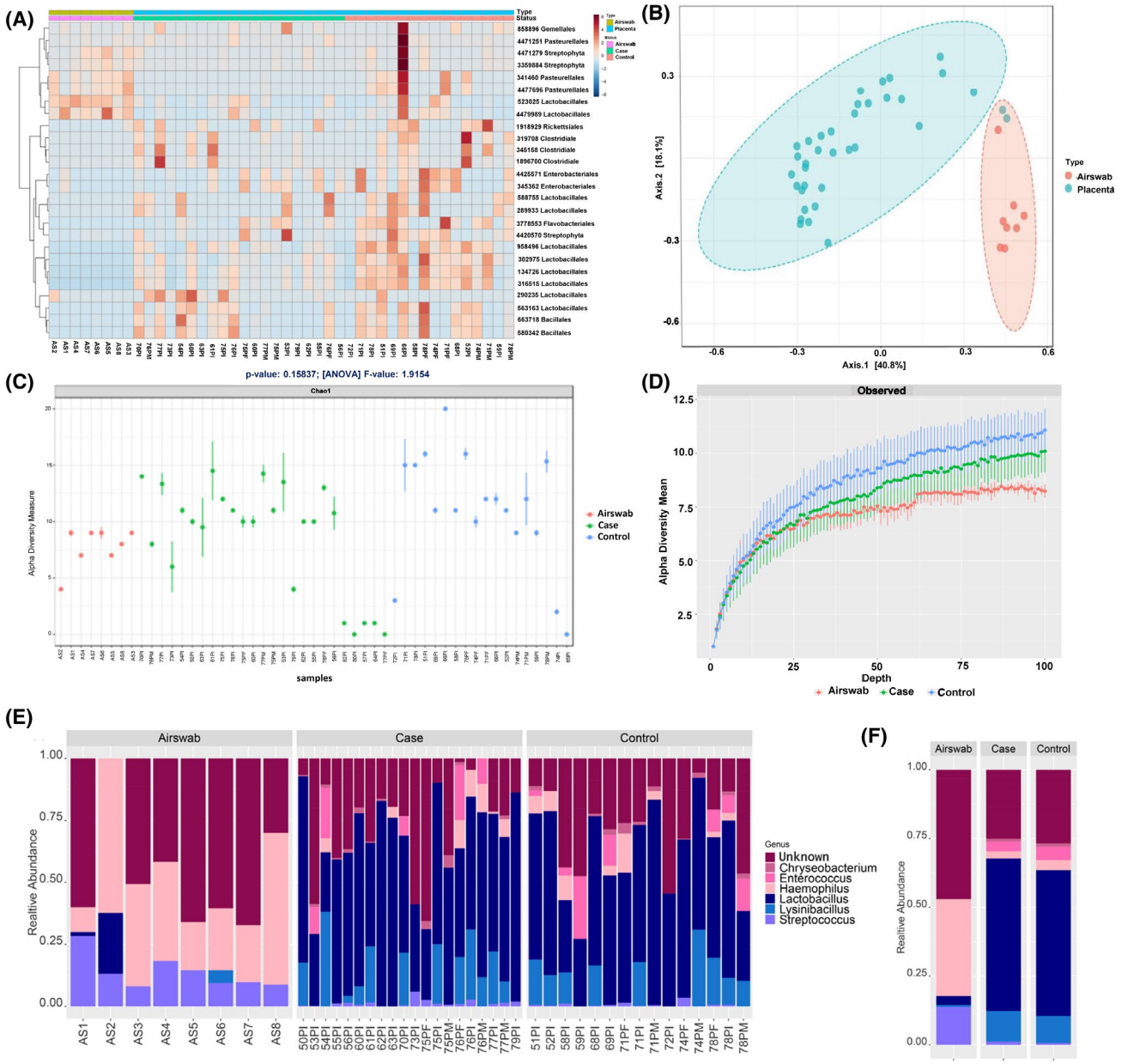

FIGURE 2 A, Heatmap showing the OTUs in placenta samples (pre-pregnant obese and normal pre-pregnant weighted groups) and airswabs. B, Principle coordinate (PCoA) plot of the placenta and airswab clusters, showing two distinct clusters. C, Alpha diversity –Choa1 metric among samples. D, The rarefaction curves of airswab (red), pre-pregnant obese (green), and normal pre-pregnant weight samples (blue), at different sequencing depths (x-axis) vs observed alpha diversity (y axis). E, The community structure of all placenta samples at genus level. F, Relative abundance of bacteria grouped by case, control and airswab

UCLUST, and obtained similar alpha and beta diversity results.

3.4 | Variability of the microbiome from the maternal to fetal side of the placenta

The placenta samples were collected from three different regions of the placenta: maternal side, intermediate layer, and fetal side. We next investigated the microbiome abundance and compositions among these three regions. The overall richness (measured by alpha-diversity) is lower in the fetal side, compared to the maternal (P value = .01), and intermediate layer (P value = .03), as shown in the rarefaction curves (Figure 3A). There is no significant difference between maternal and intermediate layers. All three placenta regions share most genus types, as expected (Figure 3B). Among them, *Lactobacillus*, the dominant taxa in all three layers, shows a trend of decreasing relative percentages from the maternal to fetal side (Figure 3B,C).

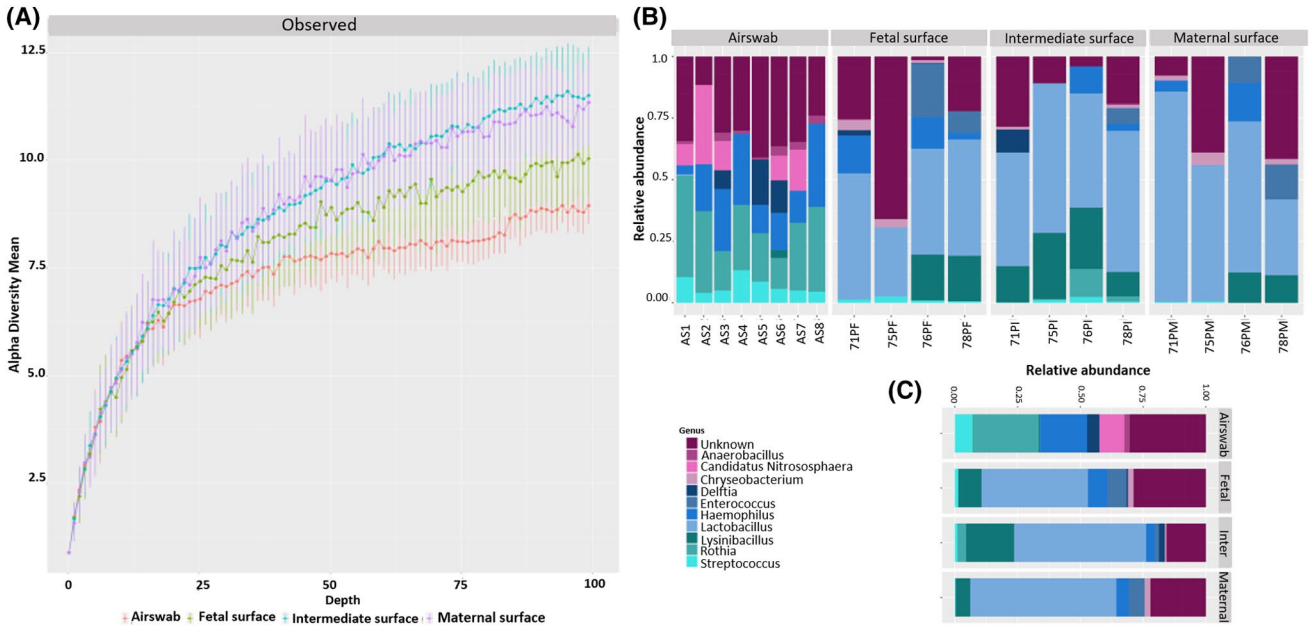

FIGURE 3 A, The rarefaction curves of airswab (red), fetal side (green), intermediate layer (blue), and maternal side (purple), at different sequencing depths (x axis) vs the alpha diversity (y axis). B, Community structure of placenta samples at genus level obtained from three different placenta layers (maternal surface, intermediate layer, and fetal surface). C, Summarized relative abundance of placenta microbiome at different placenta surfaces

4 | DISCUSSION

In this study, we sought to characterize the variations of the microbiome associated with the placentas of obese and non-obese women going through scheduled full-term cesarean deliveries. We found that pre-pregnant obese mothers have reduced bacterial diversity overall in placentas. Moreover, evidence also shows overall lower diversity of bacteria associated with the fetal side, compared to the maternal side. The microbiomes enriched from placenta samples are distinguishable from those in contamination controls. Our results demonstrate that reduced microbiome abundance and diversity in placentas, although in extremely low abundance, are identifiable features associated with maternal pre-pregnancy obesity.

The key characteristic of our study which makes it different from other studies is the enrichment process of the bacterial DNA. When we started analysing the 16S sequencing data from unenriched samples, very little OTUs were detected. We then used the NEBNext Microbiome DNA enrichment kit, a kit shown to be effective in enriching microbial DNA,[24] allowing for a 100-fold enrichment of bacterial DNAs and successful detection of a large number of OTUs (Figure 1B,C). Our results demonstrate the usefulness of enrichment tools when testing samples that contain extremely low microbial biomass, such as the placenta.[24-25,27]

Over all, the placenta microbiome associated with obese pre-pregnant women shows lower diversity compared to non-obese women, consistent with previous findings that also associate obesity with lower microbiome diversity. In the gut microbiome, it was reported that lower alpha diversity was associated with higher BMI[32] and that low fecal microbial diversity was associated with higher body adiposity content.[33] The oral microbiome was also less abundant in obese subjects, compared to normal-weight controls.[23] Of note, other groups have also reported associations between the placental or pregnancy microbiome and other comorbidities such as gestational weight gain,[2] gestational diabetes,[30] diet,[31] as well as earlier gestational age.[29] Excess gestational weight gain was described as a contributor to adverse pregnancy outcomes through adverse placental changes.[2] Gestational diabetes and diet were reported to affect the maternal gut microbiome composition and normal metabolic functions and these changes which occurred during pregnancy persisted after birth and had lasting effects on offspring.[30,31] In our study, the potential effects from gestational weight gain and pregnancy complications are minimized owing to the optimized experimental design and patient inclusion and exclusion criteria. As shown in Table 1, gestational weight gain between the cases and controls is not statistically significant. The women recruited in this study were all healthy without clinical complications such as gestational diabetes. Their diet could be different; however, these women were fasted before surgery, which minimized the confounding factors in the microbiome due to immediate dietary effects.

Another interesting finding is that *Lactobacillus,* a type of gram-positive bacteria, was significantly lower in placentas

delivered from pre-pregnant obese women, as compared to normal weighted pre-pregnant controls. *Lactobacillus,* typically referred to as "good bacteria," contributes to symbiosis in the digestive system where they convert sugar to lactic acid. It was postulated that *Lactobacillus* could transfer from the maternal gut to the placenta, possibly through hematogenous, enteric or enterohepatic transfer.[28] While the exact mechanisms have not been fully elucidated, other groups have similarly shown an endometrial microbiome which was also enriched in *Lactobacillus* and further the reduction in *Lactobacillus* was associated with increased occurrence of endometriosis.[34]

Our results show that the placenta microbiome differs from the maternal to fetal side, despite the small sample size. Several earlier studies have also revealed the geo-variations of the placental microbiome.[35-37] It was reported that the basal plate which is in closest contact to the maternal uterine wall was dominated by Proteobacteria species, while the fetal membranes which were farthest away from the uterine wall were dominated by Firmicutes species.[35,36] Villous tissue which is in between the basal plate and fetal membranes did not have a dominant species and contained a mixture of bacteria, confirming our observation of a microbiome gradient in the placenta. Furthermore, 16S in situ hybridization experiments confirmed the presence of bacteria primarily in villous parenchyma placental tissue and to a less extent in the chorion and maternal intervillous spaces.[37] Since we observed more *Lactobacillus* in the maternal side compared to the fetal side, this may suggest that the origins of the placenta microbiome may be from the mother, possibly through circulation from the maternal gut.

5 | CONCLUSION

Using an enrichment protocol optimized for low bacterial biomass samples, we show that microbiome associated with placentas of pre-pregnant obese weighted mothers is less abundant and less diverse compared to the mothers of normal pre-pregnant weights.

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CONFLICT OF INTEREST

The authors disclose no conflict of interest exists.

AUTHOR CONTRIBUTIONS

L.X. Garmire envisioned the project, obtained funding, designed and supervised the project and data analysis. R.J. Schlueter, I.Y. Chern collected the samples. P.A. Benny, F.M. Al-Akwaa, T.K. Wolfgruber, and C. Dirkx carried out the experiments and analyzed the data. All authors have read, edited, revised, and approved the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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