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Editor's Summary

Bacteria in Healthy Placentas

Contrary to the prevailing idea of a "sterile" intrauterine environment, Aagaard and coauthors demonstrated the consistent presence of a microbiome in placentas from healthy pregnancies. This microbiome was consistently different from those reported in other parts of the body, including the skin and urogenital tract. The placental microbiome was most similar to that of the oral cavity, but the clinical implications of this finding remain to be explored. In addition, the authors identified associations between the composition of the placental microbiome and a history of remote antenatal infection, as well as preterm birth, raising the possibility that the placental microbiome may play a role in these events.

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MICROBIOME

The Placenta Harbors a Unique Microbiome

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Humans and their microbiomes have coevolved as a physiologic community composed of distinct body site niches with metabolic and antigenic diversity. The placental microbiome has not been robustly interrogated, despite recent demonstrations of intracellular bacteria with diverse metabolic and immune regulatory functions. A population-based cohort of placental specimens collected under sterile conditions from 320 subjects with extensive clinical data was established for comparative 16*S* ribosomal DNA–based and whole-genome shotgun (WGS) metagenomic studies. Identified taxa and their gene carriage patterns were compared to other human body site niches, including the oral, skin, airway (nasal), vaginal, and gut microbiomes from nonpregnant controls. We characterized a unique placental microbiome niche, composed of nonpathogenic commensal microbiota from the Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria phyla. In aggregate, the placental microbiome profiles were most akin (Bray-Curtis dissimilarity <0.3) to the human oral microbiome. 16*S*-based operational taxonomic unit analyses revealed associations of the placental microbiome with a remote history of antenatal infection (permutational multivariate analysis of variance, $P = 0.006$), such as urinary tract infection in the first trimester, as well as with preterm birth <37 weeks ($P = 0.001$).

INTRODUCTION

Over the past 4 million or so years, hominids have coevolved with their microbiomes as physiologic communities composed of distinct body site niches. Recently published works by the Human Microbiome Project (HMP) Consortium (*1*–*4*) have provided the first reliable estimates of the breadth of structure, function, and diversity of the healthy ("reference") human microbiome across multiple body sites. Despite recent advances in cataloging the complexity of the adult human microbiome, there are limited population-based data as to how and when the human microbiome is established.

Characterizing the processes that govern the establishment of human microbial communities is essential for understanding human development and physiology. Studies have shown that neonates demonstrate complex microbial communities in the gut within the first week of life, with dynamic fluctuations in bacterial composition until a relatively mature equilibrium is reached around 1 to 3 years of age (*5*–*13*). What shapes these earliest microbial communities, and at what point the infant is first exposed to and colonized by its microbiome, remains unclear (*12*–*16*). In the first week of life, the full-term neonatal gut microbiome is largely colonized by the phyla Actinobacteria (including *Bifidobacterium*), Proteobacteria, Bacteroides, and, much less, Firmicutes (including the *Lactobacillus* spp., which dominate the vaginal flora) (*17*). In contrast, neonates who weigh <1200 g are dominated by both Firmicutes and Tenericutes phyla, with much less dominance of Actinobacteria (*18*). These collective observations raise the possibility that the infant may be first seeded in utero by a common shared low abundance source, such as the placenta, and this seeding may vary by length of gestation.

Establishment and maintenance of placental integrity and function are critical to fetal growth, development, and survival (*17*). Previous studies have largely used morphologic approaches and grossly documented the presence of bacteria in different regions of the placenta (*18*–*21*). A recent cross-sectional study of 195 patients (*18*) demonstrated Gram-positive and Gram-negative intracellular bacteria harbored in the basal plate (which comprises the tissue layer directly at and below the maternal-fetal interface). These were observed in nearly one-third of placental specimens, with a high prevalence among preterm deliveries <28 weeks of gestation but regardless of clinical or pathologic evidence of chorioamnionitis (*18*). Although current paradigms suggest that most intrauterine infections, which are associated with preterm birth, originate in the lower genital tract and ascend into an otherwise "sterile" intrauterine environment (*19*, *20*), most taxa detected in the placenta with DNA-based technology are not found in the urogenital tract, but rather represent commensal species common to the oral cavity (*21*–*24*). Some of these oral microbes, such as *Fusobacterium nucleatum* (a Gram-negative oral anaerobe), may facilitate hematogenous transmission during placentation as a result of their ability to bind vascular endothelium and alter permeability, thereby functioning as an "enabler" for other common commensals, such as *Escherichia coli* (*25*). These latter findings are of particular interest, given the long-standing strong association between periodontal disease [as well as upper and lower urinary tract infections (UTIs)] and increased risk of preterm birth (*26*–*29*).

Here, we have leveraged recent approaches and tools (*1*–*4*) for metagenomic analysis to characterize a rigorously collected set of placental specimens from 320 subjects in a case-cohort design. We present here an initial snapshot of the human placental microbiome and reveal which organisms are present, what they are capable of doing, and how the placental community is likely structured.

RESULTS

Baseline characteristics of the study subjects

Table S1 shows the baseline characteristics of the subjects in each case-cohort. By design, preterm birth cases significantly differed from their control cohort by virtue of gestational age (35 weeks versus 39.3 weeks; $P < 0.001$) and associated mean birth weight (2.59 kg versus 3.37 kg;

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P = 0 001), but not by mode of delivery, maternal body mass index (BMI), racial/ethnic classification, or maternal tobacco use. Similarly, by design, cases with a remote history of antenatal infection were significantly different from controls ($P < 0.001$) and included 68 sexually transmitted infections and 52 upper or lower UTIs. Because our population-derived case-cohort designations were based on either preterm birth or a remote history of antenatal infection, some preterm birth cases or their term cohort controls may have also experienced remote antenatal infections (and vice versa). There was no significant difference in the occurrence of antenatal UTI, pyelonephritis, or syphilis in the preterm birth cohort (table S1).

A unique microbiome niche in the placenta

We used the Meta Genome Rapid Annotation using Subsystem Technology (MG-RAST) to determine the taxonomic classification of the placental microbiome as characterized by whole-genome shotgun (WGS) (*30*). Figure S1 presents the relative abundance of commensal species with the highest occurrence in each placental sample. Although there is an observable interindividual variation in specific species, *E. coli* is the species with the highest abundance in most individuals. Several species of the oral microbiome were detected in the placenta, including *Prevotella tannerae* (gingival crevices) and nonpathogenic *Neisseria* species (mucosal surfaces) (*31*).

To more robustly identify the possible origin or seed of the detected placental microbial community, we compared the microbiota detected in the placenta to those present in other body site niches as reported by the HMP. The phylum-level abundance has been previously used to compare taxonomic profiles from other body site samples with low microbial abundance (*32*), or samples from closely approximated body sites (*1*). We integrated the placental microbiome taxonomy profile identified by MG-RAST using WGS-generated taxonomic profiles for placental, oral, stool, skin, airway (nasal), and vaginal sites and calculated the Bray-Curtis distance at phylum-level abundance to reflect the similarity among body sites. Figure 1 demonstrates niche distinction by site, regardless of anatomic proximity. The placental microbiota show similarity to the oral phyla (derived from nonpregnant subjects) (*1*–*3*) notably among communities arising from the tongue, tonsils, and gingival plaques (Bray-Curtis index <0.3). By contrast, the potential anatomic contaminants during delivery of the placenta [stool and vagina, from both gravid (*4*) and nongravid (*1*–*3*) subjects] bore no evidence of similarity (Fig. 1). This held true for individual sample-level projections (fig. S2) and was replicated when alternately analyzed by ecologic indices specifically designed for WGS data [Jaccard (*33*) and Kulczynski (*34*, *35*)] (fig. S2). Results using SourceTracker (*36*) analysis similarly indicate that the primary related source to the placental microbiome tracked to known oral taxonomic classifications (mean oral source tracked classifications at 90%, SD 5%).

At the phylum level, each body site was characterized by one or a few signature phyla, such as Firmicutes in the vagina, Actinobacteria in the skin of the retroauricular crease and anterior nares, Proteobacteria and Firmicutes in all the oral sites, and Bacteroidetes in stool (Fig. 2A), whereas the placental microbiome is characterized by higher abundance of Proteobacteria and the unique presence of Tenericutes [which includes the known intrauterine genera of *Mycoplasma* and *Ureaplasma* (*37*)]. Detailed species-level comparison across body sites further characterized the unique properties of the placental microbiome phyla (Fig. 2B). In accordance with our data in fig. S1, *E. coli* is the most prevalent single species in the placenta. Although we did not characterize the infant microbiome in this study, *Escherichia* has been shown in high abundance in meconium from one recent study of neonates (*38*) and is a strong contributor to early-onset (within 48 hours of birth) sepsis among extremely low birth weight neonates (*39*).

Metabolic reconstruction of the placental microbiome

In contrast to the observable diversity in taxa, the relative abundance of high-level metabolic pathways reconstructed by HUMAnN (the HMP Unified Metabolic Analysis Network) (*1*–*3*) is much more constant across all body sites, except for the vaginal posterior fornix and placenta (Fig. 2C). In general, the metabolism of cofactors and vitamins is represented with greater relative abundance among placental functional gene profiles (Fig. 2C; corresponding with published observations in the infant gut) (*30*). When compared with proximal anatomic sites, such as the posterior fornix of the vagina or the stool, we observed

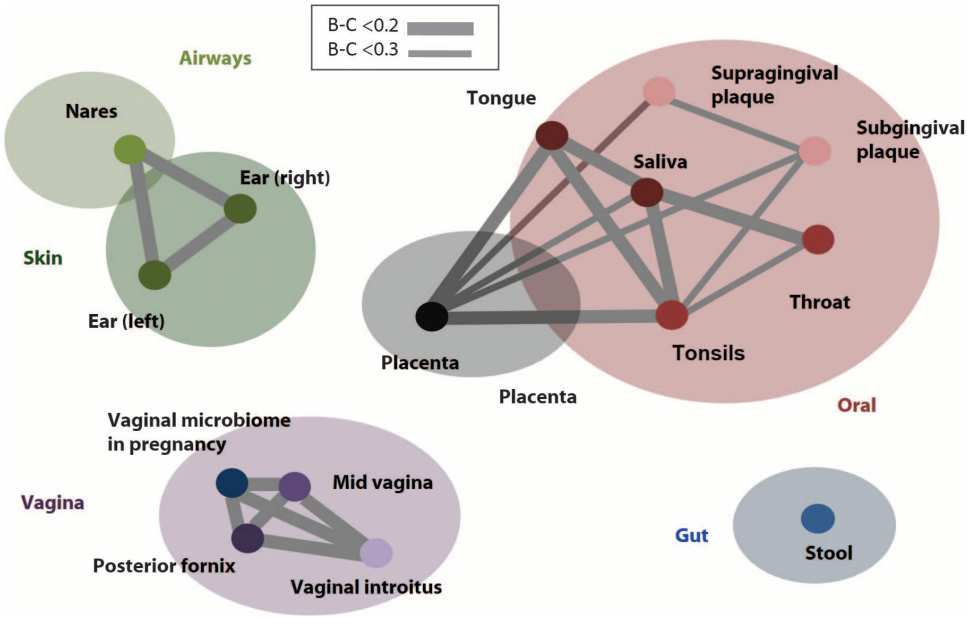

Fig. 1. The placental microbiome has a taxonomic profile that is similar to the oral microbiome. Bray-Curtis (B-C) dissimilarity was calculated using WGS-generated phylum-level abundance of bacteria from each body site, including placental data from this study; gut, vagina, posterior auricular skin, and nasal airways data from the HMP; and vaginal data from previously published gravidae (*1*–*4*). The thicker the connecting line, the greater the similarity of the taxonomic profile (Bray-Curtis <0.2). Strong phylum-level similarity was observed between the placenta and tongue, tonsils, saliva, and subgingival plaque taxonomic profiles. The colors of dots reflect the vicinity of the body sites.

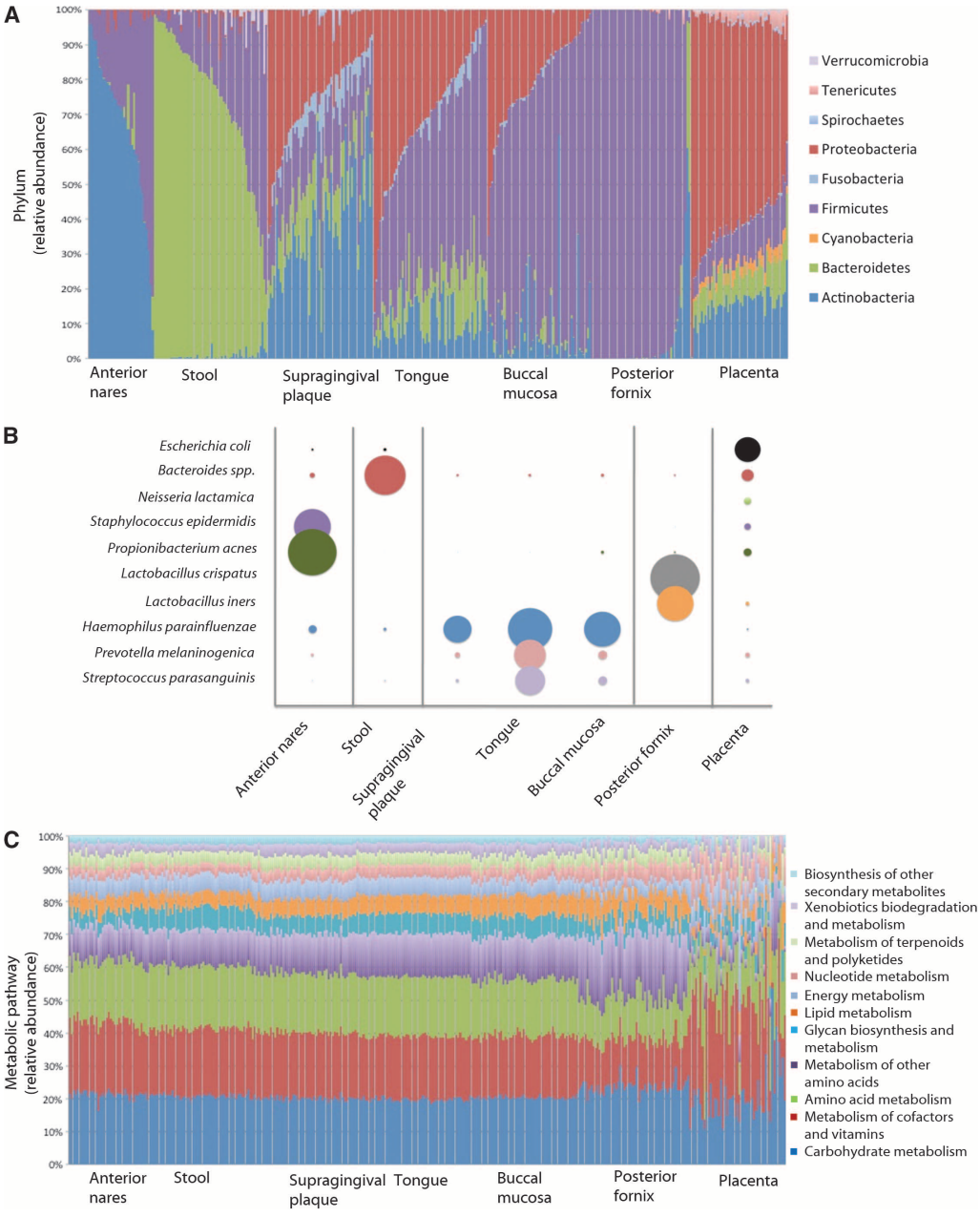

Fig. 2. Comparison of WGS-generated taxa and metabolic capacity among body sites reveals distinct features of the placental microbiome. (**A**) The phylum-level abundance (*y* axis) is represented by vertical bars indicating the dominant phylum for each of seven body sites, where each bar indicates an independent subject grouped by body site niche. The phylum-level abundance for specific taxa is relatively stable among all placental specimens when compared with that of the oral sites. (**B**) The bubble plot shows the prevalence and relative abundance of representative species at each body site, projected as a composite to scale. (**C**) The relative abundance of metabolic pathways generated by HUMAnN is similarly represented by subject (vertical bars), and demonstrates the functional profile variations of the placenta and vaginal posterior fornix as distinct from other body sites.

relatively lower abundance of carriage patterns of genes mapping to pathways involved in carbohydrate and amino acid metabolism (Fig. 2C). Further detailed comparisons of the variation in metabolic reconstructions associated with gestational age at delivery and a history of first- or second-trimester maternal infections are reported in Figs. 3 and 4 and described further below.

Variation in the placental microbiome associated with preterm birth

Consistent with the principles of a population-based case-cohort design, we examined beta diversity from our larger matched case-cohort placental specimens derived from healthy gravidae and women with complications of pregnancy (table S1, n = 320). Specifically, we interrogated beta diversity metrics in placental specimens from women with a well-documented but remote history of antepartum infections (primarily UTI in the first or second trimester), or having experienced a preterm birth at <37 weeks of gestation. The principal coordinate analysis (PCoA) was performed on all the operational taxonomic units (OTUs) generated by 16S ribosomal DNA (rDNA) sequencing that could be assigned at least to the phylum level and appeared in at least 5% of samples after chimera removal. As shown in Fig. 3A, we observed clustering in association with gestational age at delivery on jackknife-supported PCoA plots using weighted UniFrac distance. We demonstrate with statistical significance that the placental microbiome is associated with preterm birth <37 weeks [permutational multivariate analysis of variance (PERMANOVA), P = 0.001; permutational analysis of dispersion (PERMDISP), P = 0.855 (*40*)]. We also find that the placental microbiome is significantly associated with the interval week of delivery, when gestational age is alternately analyzed as a continuous variable (range of potential viability from 24 to 41 weeks; adonis, P = 0.001).

To identify placental taxa whose abundance is enriched or diminished among women who experienced a preterm birth, we applied Boruta feature selection on MG-RAST–generated taxonomy profiles derived from WGS data in a subset of placental specimens from 48 subjects (table S2). Several taxa, such as *Burkholderia*, demonstrated enrichment among gravidae delivered preterm. In contrast,

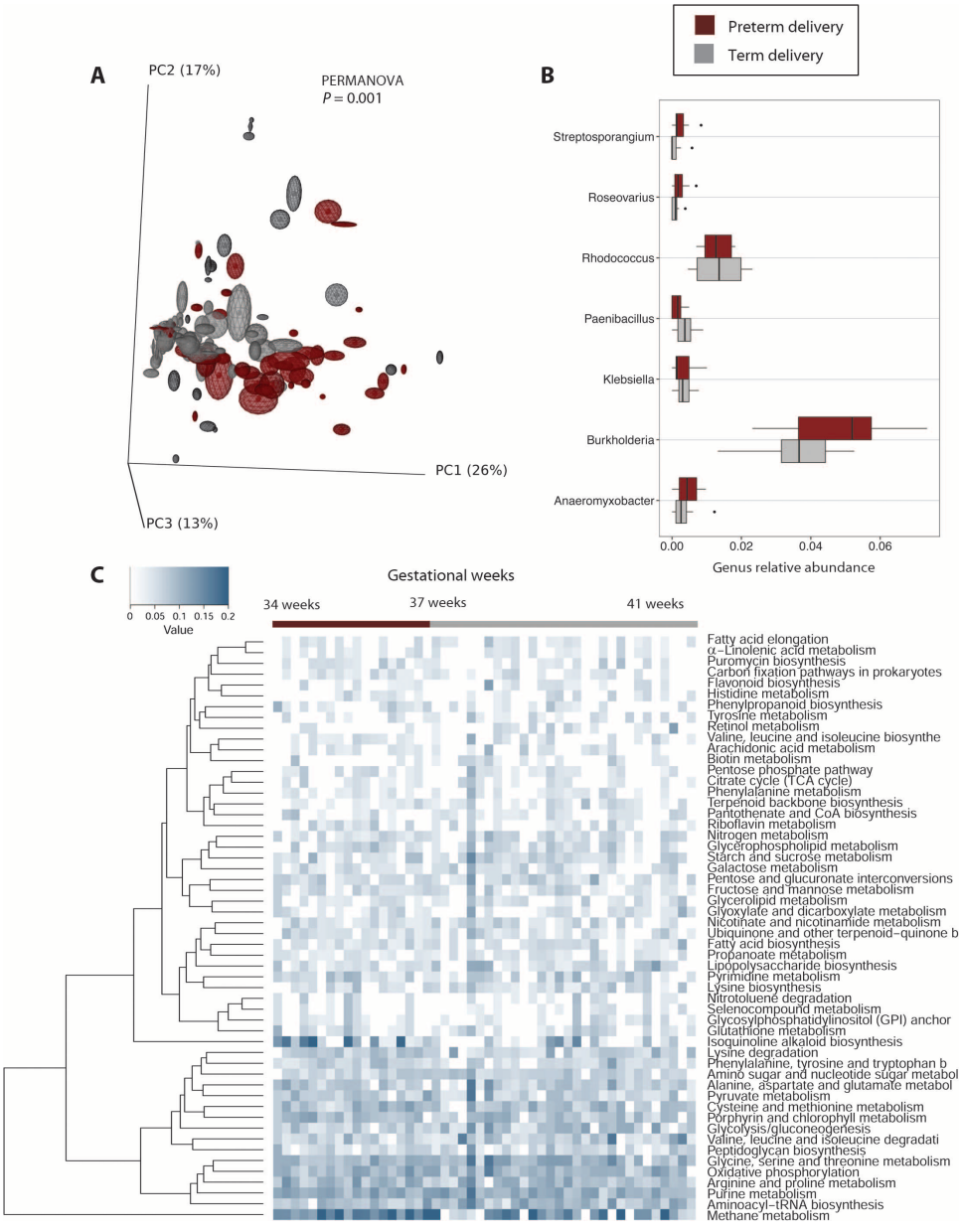

Fig. 3. The placental microbiome from pregnancies complicated by a preterm delivery demonstrates discrete taxonomic profiles and variations in metabolic pathways. (**A**) Jackknife-supported PCoA plot demonstrates the beta diversity of the placental microbiome community at the OTU level on weighted UniFrac distance from 16*S*-derived microbial sequences (PERMANOVA, $P = 0.001$). (**B**) Bacterial taxa derived from WGS sequence data identified as differentially abundant between placental specimens from preterm birth versus term gestations as analyzed by Boruta feature selection (box plots representing the first quartile, median, and third quartile). In (A) and (B), samples are labeled as preterm (red) and term delivery (gray). (**C**) Heat map demonstrating the MG-RAST–generated metabolic activity of the placental microbiome. Stronger intensity of blue indicates higher pathway activity. Pathways (rows) are hierarchically clustered on Manhattan distance. Samples (columns) were ordered on the basis of the gestational weeks.

Paenibacillus was enriched in term placental specimens (Fig. 3B). When we interrogated higher-level taxonomy by cladogram, the relative abundance of Actinomycetales and Alphaproteobacteria was increased in the preterm placenta (fig. S3). Moreover, when we projected the metabolic pathways as Manhattan distance on a heat map, we observed samples correlating with gestational age at delivery, with relative increased abundance of pathways involved in methane metabolism, isoquinoline alkaloid biosynthesis, and glycine/serine/threonine metabolism (Fig. 3C), and decreased biotin metabolism and glycosylphosphatidylinositol anchor pathways (Fig. 3C).

Variation in the placental microbiome associated with a remote history of antenatal infection

In Fig. 4A, we similarly analyzed placental microbial community clustering associated with a remote history of antepartum infection, with significant statistical support (PERMANOVA, $P = 0.006$; PERMDISP, $P = 0.07$). Group B *Streptococcus* (GBS) infection, infant gender, maternal BMI, and route of delivery (cesarean versus vaginal birth) failed to associate with clustering of the placental microbiome. The PCoA performed on the OTU table denoised by AbundantOTU (*4*) similarly indicated that the placental microbial community is distinctly shaped among gravidae who delivered preterm or who experience an antepartum infection in the first or early second trimester of pregnancy. To identify the placental microbiota community members in gravidae with a remote history of antepartum infection, we applied another metagenomic biomarker discovery approach called LEfSe (linear discriminate analysis coupled with effect size measurements) (*41*). We observed enrichment of *Streptococcus* and *Acinetobacter* in gravidae with a remote history of antepartum infection (Fig. 4, B and C). The same set of genera, including *Thioalkalivibrio* and *Pseudoalteromonas*, was identified by Boruta feature selection as well (Fig. 4D). Consistent with taxonomic observations, application of LEfSe to MG-RAST–generated gene carriage patterns from WGS data demonstrated an enrichment of genes involved in the lipopolysaccharide biosynthesis pathway in samples from individuals with remote history of antenatal infection (Fig. 4E).

DISCUSSION

The results of our study indicate that the placenta harbors a low-abundance but metabolically rich microbiome. The placental microbiome is largely

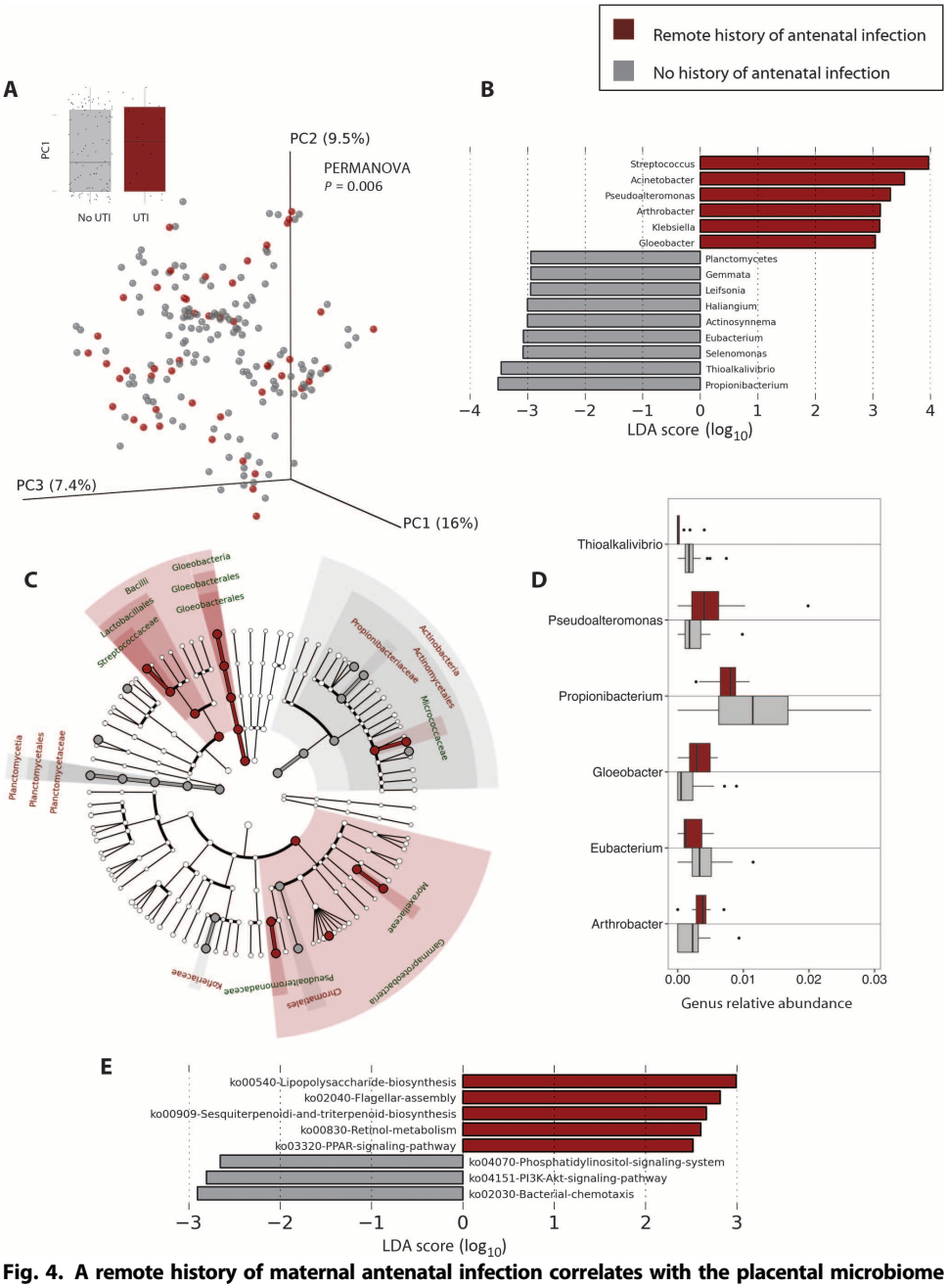

Fig. 4. A remote history of maternal antenatal infection correlates with the placental microbiome community. (**A**) PCoA plot shows the beta diversity of the placental microbiome community at OTU level with unweighted UniFrac distance derived from 16*S*-based sequencing data (PERMANOVA, $P = 0.006$). The first principal coordinates of the placental community structure from individuals with or without UTI are shown in the panel. (**B** to **D**) WGS-derived bacterial taxa were identified as differentially abundant between gravidae with and without a history of remote antenatal infection as analyzed (B) by LEfSe and (C) by LEfSe projected as a cladogram and (D) analyzed by Boruta feature selection. (**E**) The relative abundance of WGS-derived KEGG functional pathways was determined by MG-RAST and compared by LEfSe. As shown, lipopolysaccharide biosynthesis pathways are represented to a greater degree in the placental specimens from women with a remote history of antenatal infection. In all panels, red bars and dots are used to designate cases with a remote history of antenatal infection, and gray to designate noninfected controls.

composed of nonpathogenic commensal microbiota from the Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria phyla. In aggregate, the placental microbiome profiles were most akin (Bray-Curtis dissimilarity <0.3) to the nonpregnant human oral microbiome. 16*S*-based OTU analyses revealed correlation of the placental microbiome with a remote history of antenatal infection (PERMANOVA, $P = 0.006$), such as UTIs in the first trimester, as well as with preterm birth <37 weeks ($P = 0.001$).

There are some limitations to our study. One primary limitation is the comparison between our placental microbiome data and the oral, gut, nares, and posterior auricular microbiomes derived from nonpregnant subjects enrolled in the HMP (*1*–*3*). Our rationale for using this well-characterized reference cohort was largely practical: to sample multiple body sites in all 320 gravidae would have exceeded the resources available for this study and surpassed the total number of women enrolled in the HMP ($n = 150$) (*3*). However, given recent publications demonstrating an observed shift of the vaginal microbiome in pregnancy (*4*), we did include these available data sets in our analysis. As shown in Fig. 1, although there is measurable variance between the vaginal microbiome in pregnancy relative to the nonpregnant vaginal microbiome, the appreciable variance does not significantly alter Bray-Curtis dissimilarity measures between the vaginal and placental microbiota in aggregate.

A second limitation to our study is our inability to characterize the placental microbiome in early preterm gestation among women who ultimately deliver at term. This is a feasibility limitation because it would necessitate invasive sampling during ongoing gestation. Nevertheless, as shown in Fig. 3, there is an appreciable variation in the placental microbiome among all women who underwent a spontaneous preterm delivery at <37 weeks (PERMANOVA, $P < 0.001$; adonis, $P = 0.001$).

A third limitation to our study was the low number of subjects with periodontal disease ($n = 1$) and a relatively higher proportion of subjects with a remote history of UTI. We did not anticipate our finding a strong measure of similarity between the oral and placental microbiome, and thus did not specifically recruit subjects with periodontal disease. With respect to the definition of UTI, we used standard clinical definitions used in pregnancy, namely, clinical symptoms in combination with moderate colony-forming units of 10,000 to 100,000 (*4*, *18*–*20*). It remains a

formal possibility that potential exposure to antibiotics over the pre-pregnancy interval (data that we did not have access to), rather than documented infection and treatment (table S3) in the antenatal interval per se, could be driving our observations. In the absence of available data on all subjects outside of the index pregnancy, we cannot exclude this possibility. However, given the strong observed association demonstrated in Fig. 4 alongside the relative health of our subject cohort, we feel that such a misclassification of association is unlikely. Similarly, given the observational nature of this study, we cannot distinguish whether it was the remote antenatal infection or its clinically indicated antibiotic therapy that had an effect on the placental microbiome community.

A fourth potential limitation to our study was the overall low abundance (biomass) of microbiota in the placenta. On the basis of our recorded extracted specimen weights and resultant read counts, we estimate that 0.002 mg of bacterial DNA was extracted from every 1 g of placental tissue; this is accordant with recent morphometry estimates of intracellular bacterial burden in the normal human placenta (*20*). However, we were able to overcome the limitations of low biomass and the resultant risk of environmental contamination with our systematic application of three approaches: use of a large number of primary specimens in a population-based case-cohort design with strict adherence to sterile clean protocol, utilization of deep massively parallel sequencing with WGS metagenomics for delineation of community membership and metabolic pathways, and use of multiple biological and technical replicates.

Other investigators likewise challenged to conduct metagenomic analysis of low-density and low-abundance microbial communities have used similar rigor to limit bias reduction when working at the boundary (DNA <1 pg/μl) of accurate microbiota detection. In our study, we followed the methodology of the HMP protocol but increased the amount of DNA in our 16*S* amplifications to a standardized 30 ng/μl (5 μl in each reaction) and compared all sequences to non-template controls. We limited our use of 16*S* data to generated PCoA plots of beta diversity and rigorously tested for significance. All remaining determinations of community membership by taxa and metabolic pathways, as well as comparative metagenomics, were conducted on WGS data to eliminate the potential for amplification artifacts and contamination from environmental sources. As with other investigators working in previously regarded sterile tissues such as the subepidermis of the skin (*42*), we have observed commensal bacterial communities that are shaped by host characteristics but are distinct from nearby and closely positioned microbial communities. Given the dissimilarity to the vagina and stool (regardless of mode of delivery) and significant similarity to specific sites in the oral microbiome (Bray-Curtis dissimilarity <0.3), we feel it is unlikely that our findings represent contamination at the time of delivery. Similarly, given the strong concordance of our findings by WGS and 16*S* (correlation coefficient of 0.56 at phylum-level abundance), we feel it is unlikely that this represents approach or analytic artifacts.

Our data are supported by other recent non–metagenomics-based observations. Stout *et al.* (*18*) demonstrated gross morphologic evidence (far below our culture-independent, metagenomic detection threshold) of Gram-negative intracellular bacteria harbored in the basal plate in placental specimens without clinical or histologic evidence of chorioamnionitis. Consistent with these and other investigators' observations, *Escherichia* has been shown in high abundance in meconium from one recent study of newborns (*38*), and is a strong contributor to early-onset sepsis among extremely low birth weight neonates (*37*). Detection of commensal *Escherichia* in the newborn meconium would be most consistent with intrauterine seeding, and our current study suggests that the placenta is a likely source.

We are intrigued by the potential biological relevance of our observed similarities between the oral and placental microbiomes. Other investigators working in murine models have also reported significant similarity between the microbial communities of these two spatially distinct niches, and postulated that this may indicate that the placental microbiome is established by hematogenous spread (*21*–*24*). Several lines of additional evidence support this view. First, in a case of clinical and histologic chorioamnionitis without cultivatable microorganisms, a single strain of *Bergeyella* was found to be 100% identical at the 16*S*-23*S* rRNA sequence level between the patient's periodontal subgingival plaque and her amniotic fluid (*22*), both of which were 99.7% identical to a previously deposited oral *Bergeyella* sequence. No species of *Bergeyella* was detected from any of this subject's multiple vaginal samples, and *Bergeyella* is not known to be commensal to any body site but the mouth (*22*). Second, tail vein injection of the oral anaerobe *F. nucleatum* in mice induces preterm and term delivery and stillbirth and localizes to the placenta, where in high doses it causes localized infection (*43*). *F. nucleatum* failed to colonize other organs, such as the spleen or liver, which suggests that the bacteria found in the placenta did not merely represent a low-grade bacteremia. Of interest, these same investigators have demonstrated that the *F. nucleatum* adhesin FadA binds cadherin on the vascular endothelium, increasing permeability and allowing for other microbial species such as *E. coli* to cross endothelial barriers (*25*). We have reported herein the relative abundance of *E. coli* species in the placental microbiome, as well as multiple *Fusobacterium* spp. Together, the available evidence suggests that the placental microbiome is likely established by hematogenous spread of oral microbiota, which would be anticipated to take up residence during early vascularization and placentation.

These observations are of interest to scientists and physicians alike. Although this study is not able to address the relationship between periodontal disease and the placental microbiome, we, as a community of obstetricians, have appreciated for decades the association between periodontal disease and preterm birth (*19*). A hallmark of periodontal disease is gingivitis (inflammation of the gums leading to bleeding) progressing to periodontitis (advanced pockets where the gum has pulled away from the tooth). It is tempting to therefore postulate that both the burden and the community profile of a hematogenously acquired low-abundance placental microbiome would rapidly vary and potentially result in overt inflammation and polymicrobial colonization in the setting of periodontal disease (*44*–*47*). In such a scenario, treatment of the periodontal disease with scaling and planing only once during pregnancy would not improve the rate of preterm birth since the pathophysiology would be long established (*44*–*46*). This is consistent with lack of proven efficacy of therapy for established periodontal disease in multiple randomized trials (*27*–*29*). However, primary prevention in the early reproductive years and potentially early treatment before placental architecture establishment may be efficacious (*44*, *45*), and future studies might benefit from metagenomics-based multiple modality therapies for the prevention and targeted treatment of periodontal disease for the prevention of preterm birth.

We similarly find it of interest that the placental microbiome is not structured by vaginal GBS colonization, maternal obesity, or mode of delivery. Rather, a remote history of antenatal infection was correlated

with community membership, which was also associated with preterm birth of <37 weeks (table S1). Other investigators have established that pathogenic microbes (predominantly pathogenic strains of *E. coli*) can establish occult intracellular reservoirs within the urinary tract epithelium by binding to uroplakins, potentially evading immune recognition by the host as well as culture-based detection (*48*, *49*). Upon inflammation or polymicrobial presence resulting in alteration in the endothelial barrier, this nonpathogenic status is altered as clinically symptomatic infection ensues. Similarly, it has been suggested that the nonpregnant endometrial epithelium may harbor occult microbes in women with asymptomatic bacterial vaginosis (*50*). It is interesting to speculate that a common inflammatory response (by host predisposition or clinical infection) may lead to altered niche habitats resulting in our observed structuring of the placental community in association with a remote history of antenatal infection. This would further concur with our observations demonstrating an association with preterm birth because host genomic variance in inflammatory and progesterone receptor pathways is strongly associated with recurrent preterm birth (*51*).

Our study was designed and executed using rigorous quality standards and state-of-the-science metagenomics, and demonstrates that the placenta harbors a unique low-abundance microbiome. In the coming years, human microbiomes of varying composition and abundance will be defined at many additional body sites and during different periods in the human life span. A metagenomic survey alone can describe the microbial communities present, but clinical context and advanced inference analyses will be required to understand the factors that affect community composition.

MATERIALS AND METHODS

Study design

The intent of this case-cohort study was to use metagenomics to characterize the placental microbiome and then compare the placental microbiome in healthy term pregnancies to pregnancies affected by a remote history of antepartum infection and those affected by preterm birth. All subjects were enrolled under Baylor College of Medicine Institutional Review Board (IRB) H-26364, and this work was undertaken with IRB approval H-26589. As part of the consent process, we discussed with participants the potential risks of participation, including the physical risks associated with specimen collection, and the possibility that protected health information or deidentified project data stored in a public repository could be accidentally released. The protocol and consent form described precautions taken to reduce these risks. Additional protections for participants included coding genomic and metagenomic specimens and sequence data, using controlled-access repositories for extracted nucleic acids and deidentified medical and human genome sequence data, and making efforts to remove any human sequence traces from the microbial data deposited in open access databases. If a participant withdrew consent after providing specimens, remaining specimens and extracted nucleic acids were to be destroyed; however, any metagenomic sequence data that were already published in open access databases could not be retracted.

For comparisons in this study, two case-cohort data sets with high-integrity samples were created. For the first (preterm birth case-cohort), the PeriBank database was queried to identify subjects spontaneously delivering at less than 37 weeks. Spontaneous preterm birth was defined by standard obstetrical definitions as adequately documented gestational age >24 and <37 weeks (by last menstrual period with <20-week gestation sonogram, or by <14-week sonogram with subsequent second-trimester sonogram), and no evidence of maternal or fetal comorbidity indicating induction of labor (such as preeclampsia, placenta previa, or hydramnios). For the second case-cohort data set (remote history of antenatal infection case-cohort), the database was independently queried for subjects with laboratory evidence of antepartum (first or early mid-trimester) clinically symptomatic infections including sexually transmitted infections such as gonorrhea, chlamydia, or syphilis; upper or lower UTIs with documented culture speciation and antibiotic sensitivity; and resistance profiling, periodontal disease, or systemic infections, such as pneumonia. The control groups for cohort comparison were thereafter created by a 1:1 match with previously identified case subjects (preterm birth or a remote history of antepartum infection). All case-cohort subject specimens were matched for gestational age, mode of delivery, race/ethnicity, BMI, age, and diabetic status but not for any of the case definitions (preterm birth or a remote history of antenatal infection). In this manner, we enabled preterm birth cases to potentially carry a diagnosis of a remote history of antenatal infection, and vice versa; this is consistent with the principles of a population-based case-cohort design.

Subjects with fatal fetal anomalies, fetal growth restriction, prelabor preeclampsia, placenta previa, and multifetal gestation were excluded because of the likelihood of indicated preterm birth. Subjects with intrapartum chorioamnionitis suspected clinically and treated with intrapartum antibiotics and confirmed by positive maternal or infant culture were excluded from this analysis.

Subjects

Subjects were recruited by trained PeriBank study personnel who approach eligible gravidae at time of admission to labor and delivery. After consent was obtained, expanded variables of clinical information (clinical metadata) were directly extracted from the electronic medical record and accompanying prenatal records alongside directed subject questioning. Data entry into PeriBank was completed by trained study nurses and research staff. Subsets of charts were routinely audited by a board-certified maternal-fetal medicine physician scientist (K.A.) to ensure quality of the data. For this study, extracted and analyzed clinical metadata included the presence and type of infection, gestational age(s) at the time of infection, the presence or absence of *Streptococcus agalactiae* (GBS) on rectovaginal swab, or bacteriuria. Here, maternal group B streptococcal positivity on routine screening was not considered to constitute an antenatal infection (case) and was thus alternately classified as a clinical variable among both cases and control cohort subjects. The presence of noninfectious asymptomatic bacteriuria and bacterial vaginosis was noted, in addition to the subjects' age, race/ethnicity, BMI, diabetic status, and mode and gestational age at delivery.

The placental samples from 48 subjects were randomly selected from the overall case-cohort (n = 320) to undergo WGS sequencing, generating a nested case-cohort subset consistent with our overarching population-based design. About one-third of these samples were from the preterm birth case-cohort, one-third were from the remote antenatal infection case-cohort, and the remaining one-third were from the controls. Selected nested cases and controls were matched (as feasible) for gestational age (where appropriate), mode of delivery, race/ethnicity, BMI, age, and diabetic status, but not for the case definitions (preterm birth or a remote history of antenatal infection). Baseline characteristics of these 48 of the initial 320 study subjects who

underwent WGS analysis are shown in table S2. Notably, the nested 48 subjects did not significantly differ from their derived cohort counterparts by any measure other than their case definition (not for maternal age, mode of delivery, race/ethnicity, BMI, maternal comorbidities, or tobacco use; Student's t test on continuous variables and χ^2 analysis for categorical variables with lack of significance designated at $P > 0.05$).

Sample collection

All samples were collected by perinatal and placental pathology-trained personnel in our universal perinatal database and biospecimen repository (PeriBank) under strict uniform protocol. A working group of board-certified obstetrician-gynecologists, maternal-fetal medicine specialists, and pathologists with molecular, surgical, perinatal, and placental pathology and microbiology expertise developed and refined the collection protocol, consent forms, and the manual of procedures for PeriBank. This manual provides detailed, standardized instructions to ensure consistency and clinical laboratory quality in enrollment sampling and specimen processing, and ensure compliance with regulatory and data management requirements. Briefly, following standard obstetrical practice, the placenta is delivered and immediately passed off to trained personnel in a sterile clean container. In a separate room, four to six 1-cm × 1-cm × 1-cm cuboidal sections are circumferentially excised from separate areas of the placenta, each located 4 cm from the cord insertion site. The excision was performed by pathology-trained personnel donning facial masks and sterile gloves and using a sterile scalpel and instruments; these clean and sterile conditions are standardized technique in the banking of all PeriBank specimens. The placental surfaces (namely, the maternal decidua and fetal chorion-amnion) were thereafter excised and discarded to avoid potential contamination from maternal, fetal, or environmental surface exposure during delivery or at the time of specimen transport. Over the interval of this study, PeriBank enrolled 85 to 93% of all gravidae delivering at the Baylor College of Medicine–affiliated hospital, Ben Taub General Hospital (Harris Health System).

Specimen processing and microbiome analyses

For this study, all primary specimens (placental samples) were collected within 1 hour of delivery and under clean and sterile conditions as detailed above. After collection, placental specimens were immediately placed on dry ice in sterile closed vials, transported to the laboratory, and then stored at −80°C until DNA extraction. In a strictly controlled, decontaminated, and sterile environment, genomic DNA was isolated in triplicate using the standard protocol from the MO-BIO PowerSoil DNA Isolation Kit (MO-BIO Laboratories) as previously described (*1*–*4*). About 100 to 150 mg of frozen placental tissue was taken from each sterile sample vial under ongoing strict decontamination conditions (personnel using masks and gloves, and decontaminated clean sterile equipment in a sterile ventilated hood, Labconco Class II Type B2), homogenized and heated, and then processed using PowerSoil DNA Isolation Kits (MO-BIO) according to protocols benchmarked as part of the National Institutes of Health (NIH) HMP (*1*–*4*). All extracted DNA samples were stored in kit solution C6 at −20°C. DNA was quantified by NanoDrop and PicoGreen assays. We obtained sufficient quantities of DNA from all specimens, repeating the extraction when necessary until sufficient quantity was achieved. Time between delivery, specimen collection, storage, and extraction was examined as a possible confounder, and no bias was observed. One nontemplate blank extraction was performed for every 11 samples, and a subset was used in both WGS- and 16*S*-based metagenomic sequencing (on the Roche 454-Ti or Illumina platforms) to control for potential environmental or source reagent contamination; no 16*S* sequences were generated from nontemplate controls (fig. S4).

HMP data retrieval

The 16*S* and WGS data for body sites other than placenta were obtained from http://hmpdacc.org. The 16*S* OTU tables were downloaded from http://hmpdacc.org/HMQCP/. The WGS taxonomy profile was downloaded from http://hmpdacc.org/HMSMCP/, and metabolic profile was downloaded from http://hmpdacc.org/HMMRC/. The detailed procedure for data processing is as previously described (*1*, *2*). Briefly, raw 16*S* sequences were demultiplexed using QIIME (*40*). Taxonomy was assigned using the RDP classifier v2.2 (retrained with GreenGenes) (*52*). The WGS-based taxonomy profile was generated by MetaPhlAn (*53*). For metabolic reconstruction, HUMAnN (*54*) was used to infer KEGG-based community functions from WGS reads.

WGS sequencing and analysis

Community genomic DNA isolated from 48 placental samples (randomly selected but matched to maternal baseline characteristics as described above) was sheared to ~700 base pairs (bp). Illumina adapters were ligated to the sheared fragments. DNA fragments were paired-end sequenced (150-bp reads) in multiplexed pools on the Illumina HiSeq 2500 platform in the Baylor College of Medicine Human Genome Sequencing Center (BCM-HGSC). Resultant data demonstrated an average of 26.5 million reads per sample, and up to 100 million reads per sample.

Consistent with other microbiome profiles derived from low-biomass tissue samples, there was a large amount of human sequence in our placental metagenomics data sets (>99%). By comparison, we have previously demonstrated that samples from the human vagina have >96% human contamination (*1*). Thus, the first step in processing WGS reads was to remove human contamination and thereby ensure quality and privacy of the data set. Human reads were identified and removed using BMTagger (Best Match Tagger) through a heuristic approach (*1*). The remaining reads were searched against a protein database of all the ORF from prokaryotic organisms in KEGG v54 (*55*) using BLASTX. BLASTX output was filtered on the basis of HMP standards (*1*, *2*) and used as input reads to metabolic pathway reconstruction. The metabolic pathway reconstruction was directly inferred on the basis of short reads using HUMAnN (*54*). For taxonomy identification using WGS reads, quality-filtered reads were uploaded to MG-RAST (*56*). Taxonomy assignment was based on best-hit classification using the M5NR database as an annotation source with a maximum *e*-value cutoff of 1, minimum percent identity cutoff of 50%, and minimum alignment length cutoff of 15. The relative abundance of each species was calculated on the basis of the number of hits for that species. MG-RAST was also used to generate low-level KEGG pathway abundance for comparison between case- and control-cohort samples. MG-RAST–generated taxonomy and pathway abundance were further normalized by number of reads mapped to each sample.

16*S* rDNA sequencing and analysis

From DNA (30 ng/μl), variable (V) region amplicons were sequenced as previously described in the HMP (*1*–*4*). The V1-V3 region of the 16*S* rRNA gene was amplified by polymerase chain reaction (PCR) using

bar-coded universal primers 27F (F, forward primer) and 534R (R, reverse primer), which contain linker sequences for 454 pyrosequencing (*1*–*4*). Samples containing no template and those containing known 16*S* rDNA sequences were used as positive and negative controls in these PCR steps (fig. S4). V-region amplicons were quantified by PicoGreen (Invitrogen) and pooled in equimolar amounts. Multiplex sequencing was performed at the BCM-HGSC on a 454-FLX Titanium sequencer (Roche). Because of our concern for bias in low-abundance samples, all specimens were thrice extracted and thrice run to enable triplicate extraction and sequencing, and sequences from all subjects were combined for analysis. Similarly, we do not report species-level data for our 454-derived 16*S* data because we do not feel we can do so reliably in these low biomass samples.

Concordant with ours and others' previous work, all 16*S* data were quality-filtered to attain high-quality reads (*1*, *4*). Sequences from each standard flowgram format (sff) file were preprocessed to remove sequences with length less than 200 nucleotides or longer than 1000 nucleotides and sequences with minimum average quality score less than 20; sequences without precisely matched 5′ primers were also excluded. Sequences were thereafter assigned to their sample of origin on the basis of barcode matches, and the 5′ primer and barcode were excised after filtering. RDP classifier v2.2 (retrained using the release 4 February 2011 of the GreenGenes taxonomy) (*52*) was used to assign taxonomy for each read at various levels. We used reads with confidence level greater than 50% at the lowest level of assignment for further analysis. The read counts at each taxonomy level (phylum, order, family, genus) were normalized to relative abundance.

For OTU analysis, quality- and RDP-filtered reads were analyzed with the QIIME (*40*) pipeline and AbundantOTU (*57*), and each read was assigned to OTUs. OTU picking was performed by QIIME, with error correction and OTU centroid choice at 97% similarity. Chimeric sequences were identified using ChimeraSlayer (*58*) and removed from subsequent analyses. AbundantOTU was used as a denoising approach to eliminate the artifact caused by sequencing error. OTUs were analyzed by RDP classifier v2.2 as well to find out the corresponding taxonomy. The number of reads assigned to each OTU was counted and normalized. The generated OTU table combined with the clinical metadata was used as input for follow-up analysis.

Beta diversity was calculated on the normalized OTU table using phylogenetic (such as UniFrac) and nonphylogenetic (such as Bray-Curtis) distance matrices with the PCoA, and PCoA with jackknife support pipeline provided by QIIME (*40*). PERMANOVA was used to confirm that the strength and statistical significance of sample grouping use the same distance metrics. Boruta feature selection was used to identify the most important taxa involved in discriminating the two groups of samples. LEfSe (*41*) was also used to detect significant features (taxonomy and pathway abundance), which differentiate groups and rank these features by effect size. The α value used for the factorial Kruskal-Wallis test was 0.05. The threshold used on the logarithmic linear discriminate analysis score for discriminative feature was 2.0 (*4*).

Statistics

Student's *t* test was used to determine the significance of continuous metadata for study subjects. χ^2 test was used to determine the significance of the categorical metadata of study subjects. The threshold of statistical significance was set at $P < 0.05$. PERMANOVA was used to test the significance of beta diversity. Because PERMANOVA is sensitive to differences in dispersion among the groups, PERMDISP was used as a companion to PERMANOVA to exclude the possibility that the significant difference is caused by the difference in dispersions.

SUPPLEMENTARY MATERIALS

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Fig. S1. The placental microbiome harbors a variety of nonpathogenic commensal species.

Fig. S2. PCoA plot demonstrates individual sample niche-specific beta diversity.

Fig. S3. LEfSe-generated cladogram indicates the enrichment of placental microbiome taxa in gravidae who experience a spontaneous preterm birth.

Fig. S4. Representative gel image of 16*S* amplicon from placental samples demonstrates the appropriate positive and negative controls.

Table S1. Baseline characteristics of the entire case-cohort of study subjects (n = 320).

Table S2. Baseline characteristics of the study subjects undergoing WGS (n = 48).

Table S3. Characterization and treatment of remote antenatal infections.

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