OBSTETRICS

Does the human placenta delivered at term have a microbiota? Results of cultivation, quantitative real-time PCR, 16S rRNA gene sequencing, and metagenomics

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BACKGROUND: The human placenta has been traditionally viewed as sterile, and microbial invasion of this organ has been associated with adverse pregnancy outcomes. Yet, recent studies that utilized sequencing techniques reported that the human placenta at term contains a unique microbiota. These conclusions are largely based on the results derived from the sequencing of placental samples. However, such an approach carries the risk of capturing background-contaminating DNA (from DNA extraction kits, polymerase chain reaction reagents, and laboratory environments) when low microbial biomass samples are studied.

OBJECTIVE: To determine whether the human placenta delivered at term in patients without labor who undergo cesarean delivery harbors a resident microbiota ("the assemblage of microorganisms present in a defined niche or environment").

STUDY DESIGN: This cross-sectional study included placentas from 29 women who had a cesarean delivery without labor at term. The study also included technical controls to account for potential background-contaminating DNA, inclusive in DNA extraction kits, polymerase chain reaction reagents, and laboratory environments. Bacterial profiles of placental tissues and background technical controls were characterized and compared with the use of bacterial culture, quantitative real-time polymerase chain reaction, 16S ribosomal RNA gene sequencing, and metagenomic surveys.

RESULTS: (1) Twenty-eight of 29 placental tissues had a negative culture for microorganisms. The microorganisms retrieved by culture from the remaining sample were likely contaminants because corresponding 16S ribosomal RNA genes were not detected in the same sample. (2) Quantitative real-time polymerase chain reaction did not indicate greater abundances of bacterial 16S ribosomal RNA genes in placental tissues than in technical controls. Therefore, there was no evidence of the presence of microorganisms above background contamination from reagents in the placentas. (3) 16S ribosomal RNA gene sequencing did not reveal consistent differences in the composition or structure of bacterial profiles between placental samples and background technical controls. (4) Most of the bacterial sequences obtained from metagenomic surveys of placental tissues were from cyanobacteria, aquatic bacteria, or plant pathogens, which are microbes unlikely to populate the human placenta. *Coprobacillus*, which constituted 30.5% of the bacterial sequences obtained through metagenomic sequencing of placental samples, was not identified in any of the 16S ribosomal RNA gene surveys of these samples. These observations cast doubt as to whether this organism is really present in the placenta of patients at term not in labor.

CONCLUSION: With the use of multiple modes of microbiologic inquiry, a resident microbiota could not be identified in human placentas delivered at term from women without labor. A consistently significant difference in the abundance and/or presence of a microbiota between placental tissue and background technical controls could not be found. All cultures of placental tissue, except 1, did not yield bacteria. Incorporating technical controls for potential sources of background-contaminating DNA for studies of low microbial biomass samples, such as the placenta, is necessary to derive reliable conclusions.

Key words: bacteria, bacterial culture, contamination, low microbial biomass sample, microbiome, microorganism, pregnancy, tissue

Culture-independent sequencing technologies provide insight into the diversity of microbial communities that inhabit the human body[1–3] as well as other ecosystems such as soil[4,5] and oceans.[6–8] Studies derived from the Human Microbiome Project indicate that different human body sites are populated by site-specific microbiota ("the assemblage of microorganisms present in a defined niche or environment"[9]).[1,2] For example, the microbiota of the vagina[10–14] is different from that of the gut[15,16] and oral cavity.[17,18] The microbial burden of each of these body sites is large,[19–21] and samples derived from these niches are considered to have a high microbial biomass.[21,22] Results obtained with sequencing technologies of these samples are largely consistent, qualitatively, with those derived from cultivation techniques (ie, although molecular surveys of these sites typically capture far more microbial diversity than culture-based surveys, many of the prominent microbes in the molecular surveys have also been recovered through culture from these same sites).[23–27] In contrast, samples derived

EDITORS' CHOICE

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AJOG at a Glance

Why was this study conducted?

To examine whether there was evidence to support the existence of a microbiota in placentas delivered at term without labor via cesarean section.

Key Findings

- Placentas did not have a microbial DNA abundance exceeding that of background technical controls.
- 16S ribosomal RNA gene sequencing did not reveal consistent differences in the composition or structure of bacterial profiles between samples of the placenta and technical controls.
- Cultures were negative in 28 of 29 placentas.
- Metagenomic analysis of placental tissues largely yielded bacterial sequences from cyanobacteria, aquatic bacteria, and plant pathogens, which are microbes ecologically unlikely to populate the human placenta.

What does this add to what is known?

The findings of this study do not support the existence of a placental microbiota in patients who delivered at term without labor.

from sites with a low microbial biomass can give results that are difficult to distinguish from DNA present in reagents used for extraction, amplification, and sequence library preparation for molecular microbiology studies.[22,28–31]

Several reports have demonstrated that commercially available kits used to characterize the microbiota contain microbial DNA similar to that found in soil or water samples[28,29] and that this can affect the results of studies of low microbial biomass samples based on 16S ribosomal RNA (rRNA) gene amplicon or metagenomic sequencing.[22,28,29,31–33] DNA contamination of reagents is unavoidable, given the ubiquity of microorganisms and the fact that many reagents are products of microbial processes and engineering.[30] Therefore, the claim that body sites with a low microbial biomass have bacteria, based on the analysis of 16S rRNA gene surveys and metagenomic studies, requires rigorous exclusion of reagent contamination to avoid experimental artifacts and incorrect conclusions.[22,30,31]

The challenge of studying low microbial biomass samples is important, particularly in the female reproductive tract, because several investigators have viewed the endometrial cavity,[34,35] amniotic cavity,[36–57] and placenta[32,58,59] as being typically sterile.[60–63] With the application of molecular microbiologic techniques, the sterility of these sites, apart from cases of infection, has been questioned,[64–82] and functional hypotheses for potential mutualistic relationships between a microbiota and its human host are being considered.[78,83–87]

With respect to the placenta, microorganisms can invade the amnion and chorion[88–96] and the villous tree.[97–116] This is often associated with complications of pregnancy, such as preterm labor,[117–142] preterm premature rupture of membranes,[143–146] cervical insufficiency,[147–154] clinical chorioamnionitis,[155–172] and congenital infections.[97–101,173–187] The concept that most placentas have a microbial community emerged after a pioneering study that utilized sequencing techniques to analyze a large number of placentas.[64] Shortly after this report, questions were raised about this claim,[188] yet other investigators who used high-throughput sequencing strategies also reported the presence of a microbiota in the placenta.[65–75] The interpretation of these data has become a subject of controversy,[22,32,63,189,190] given the recognition that reagents used in molecular microbiologic techniques have their own microbiome (termed the *kitome*).[22,28–30,32,191] Recently, investigators have called for the application of rigorous and systematic methods to address DNA contamination in low microbial biomass samples.[22,30,31]

The objective of this study was to determine whether a microbiota exists in term placentas, delivered by cesarean section without labor, using multiple complementary modes of microbiologic inquiry: cultivation, quantitative real-time polymerase chain reaction (qPCR), 16S rRNA gene sequencing, and metagenomics.

Materials and Methods

Study design

This was a cross-sectional study in which the placenta was sampled from women not in labor at term (February–June 2016). The inclusion criteria were (1) cesarean delivery without labor at term (≥38 weeks), (2) singleton gestation, and (3) no antibiotic administration in the month before delivery, as determined by history and review of medical records. Each subject, however, did receive intraoperative prophylaxis before cesarean delivery (cefazolin or, if allergic, gentamicin and clindamycin), given the evidence that antimicrobial administration reduces perioperative complications.[192–194] Exclusion criteria consisted of multiple gestation, preterm delivery, fetal anomalies, and evidence of clinical infection.

The presence of bacteria in the placenta was determined using (1) cultivation, (2) 16S rRNA gene qPCR, (3) 16S rRNA gene sequencing, and (4) metagenomic sequencing. Placental histopathologic examinations were conducted according to protocols established by the Perinatology Research Branch.[59,93] The collection of samples and their use for research was approved by the Human Investigation Committee of Wayne State University and the Institutional Review Board of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development; all subjects provided written informed consent for participation.

Sample collection

After cesarean delivery, the placenta was placed in a sterile collection container with a sealed cover (Medline Standard C-Section Pack-LF, Mundelein, IL) within the sterile operating field. The placenta

was taken directly to a biologic safety cabinet located within 1 of 2 nearby rooms in Hutzel Women's Hospital, wherein study personnel (A.D.W., K.R.T.), wearing sterile surgical gowns, full hoods, and powder-free examination gloves (Kimberly-Clark, Roswell, GA) and using individually packaged, sterile, disposable scalpels (Surgical Design, Lorton, VA), forceps (TWD Scientific, Pleasant Prairie, WI), and surgical scissors (Sklar Instruments, West Chester, PA), collected a 1.5-cm^2 core sample from the placenta (ie, amnion and chorionic plate through to basal plate). The tissue sample was taken halfway between the umbilical cord insertion point and the edge of the placental disk, along the line that represented the longest distance from the cord insertion point to the edge of the disk. The tissue sample was transferred to a sterile polystyrene Petri dish (FB0875712; Fisher Scientific, Waltham, MA) and divided into 3 approximately equal aliquots, with each aliquot traversing the amnion, chorionic plate, villous tree, and basal plate. One aliquot was placed in a sterile 5.0-mL conical tube (Denville Scientific, Holliston, MA) on ice and stored at −80°C within 1 hour of initial placental collection. The 2 remaining aliquots were placed into Anaerobic Transport Medium Surgery Packs (Anaerobe Systems, Morgan Hill, CA) and 0.85% sterile saline solution tubes (Thermo Scientific, Waltham, MA) for anaerobic and aerobic cultures, respectively.

Bacterial culture of placental tissues

Placental tissue aliquots within anaerobic and aerobic transport containers were delivered to the Detroit Medical Center University Laboratories Microbiology Core, wherein they were processed the same day. To assess viability of a placental microbiota, placental tissues were homogenized and inoculated on growth media (trypticase soy agar with 5% sheep blood, chocolate agar, MacConkey's agar) under aerobic and anaerobic conditions and used in an assay for genital mycoplasmas. Detailed information on the cultivation protocols and taxonomic characterization of resultant bacterial cultivars is available in Supplemental Methods (Section 1).

DNA extraction from placental tissues

DNA extraction was performed to identify bacteria with molecular microbiologic techniques. During the process, study personnel wore sterile surgical gowns, gloves, and surgical masks (Soft Touch II; Kimberly-Clark, Roswell, GA) and used individually packaged, sterile, and disposable scalpels and forceps (DF 8988P-SPT; TWD Scientific, Pleasant Prairie, WI). For each placental tissue specimen, the amnion and chorionic plate (including a minimal amount of villous tissue) were separated from the placental villous tree, which remained attached to the basal plate. Genomic DNA was extracted from blocks of tissue that contained the amnion and chorionic plate and the villous tree and basal plate. The extraplacental chorioamniotic membranes were not sampled. DNA was extracted from the placental tissues (0.1−0.2 g) and background technical controls with the use of the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA), according to the manufacturer's protocol. The DNA extraction kit and the mass of placental tissue from which DNA was extracted were similar to those used in previous studies that addressed the issue of a placental microbiota.[32,64] Background technical controls included extractions performed on (1) DNA extraction kits without placental tissue, processed exactly as the placental samples (n=6), (2) extraction kits with bead tubes that had been exposed to a biologic safety cabinet for 20 minutes during placental biopsy collection or processing (n=16 samples from 3 biosafety cabinets), and (3) extraction kits with bead tubes that had been exposed for 20 minutes to an operating room or microbiology laboratory used in this study (n=21 samples from 3 operating rooms and 3 laboratories). These control samples therefore represented either 5 or 6 technical controls that reflected each potential source of background DNA contamination (ie, extraction kits, 3 biosafety cabinets, 3 laboratories, and operating rooms), with the 3 contiguous operating room environments being treated as a single potential contamination source. DNA concentrations of placental tissue and background technical control samples were 42.0±18.5 (standard deviation) ng/μL and ≤0.03 ng/μL, respectively. Purified DNA was stored at −20°C.

16S rRNA gene sequencing of DNA extracted from placental tissue and background technical control samples

The 16S rRNA gene is used widely as a phylogenetic marker to identify bacterial types present in clinical samples. A table of PCR 16S rRNA gene primers used in this study is available in Supplemental Methods (Section 2). We initially used the standard PCR and Illumina MiSeq (San Diego, CA) protocols described later; however, this approach did not produce sufficient quantities of amplified DNA to generate sequence libraries from placental tissue or technical controls and thus for 16S rRNA gene profile comparisons (Supplemental Methods Section 3; Supplemental Figure 1). Therefore, because of the very low microbial biomass in these human tissue samples, purified bacterial DNA was amplified with the use of a nested PCR approach.[195,196]

Nested PCR has been used recently to characterize low biomass microbiota in the lungs of mice,[197] sheep,[198] and chickens,[199] and in the middle ear fluid of children.[200,201] The first round in the nested PCR process included 20 cycles. Each reaction contained 0.4 μM (micromolar) each of the 16S rRNA gene broad-range primers 27f-CM (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-ACG GCT ACC TTG TTA CGA CTT -3′),[202,203] 12.5 μL of 2X GoTaq Green Master Mix (Promega, Madison, WI), and 3.0 μL purified DNA. Thermocycling was initiated by a 5-minute incubation at 95°C. Cycling parameters were 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 120 seconds. Products were then diluted 1:15 in nuclease-free water (Promega).

Amplification and sequencing of the V4 region of the 16S rRNA gene was

performed at the University of Michigan's Center for Microbial Systems (Ann Arbor, MI) with the use of the dual indexing sequencing strategy developed by Kozich et al.[204] Sequencing was performed on the Illumina MiSeq platform, with a MiSeq Reagent Kit V2 (500-cycle format; MS102-2003; Illumina), according to the manufacturer's instructions with modifications found in Kozich et al[204] and Caporaso et al.[205] AccuPrime High Fidelity Taq (12346094; Life Technologies) was used instead of AccuPrime Pfx SuperMix. Each PCR reaction (20 μL) contained 1.0 μM of each primer, 2.5 μL template DNA, 0.15 μL AccuPrime HiFi Polymerase, and DNase-free water to produce a final volume of 20 μL. PCR was performed under the following conditions: 95°C for 2 minutes, followed by 30 cycles at 95°C for 20 seconds, 55°C for 30 seconds, and 72°C for 5 minutes, with an additional elongation at 72°C for 10 minutes. Sequencing libraries were prepared according to Illumina's protocol for Preparing Libraries for Sequencing on the MiSeq (15039740 Rev. D) for 2 nM or 4 nM libraries. FASTQ files were generated for paired end reads. Sample-specific MiSeq run files have been deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive (BioProject ID PRJNA397876).

Processing of 16S rRNA gene sequence data

Mothur software (version 1.39.5) was used to assemble paired-read contiguous sequences, to trim, filter, and align sequences, to identify and remove chimeras, to assign sequences to bacterial taxonomies, and to cluster sequences into operational taxonomic units (OTUs) based on the percentage of nucleotide similarity (97% and 99%).[206] Detailed information on sequence processing is available in Supplemental Methods (Section 4).

Sequencing of DNA extracts of all samples and controls yielded 5,316,687 sequences. They clustered into 480 (209 singletons) and 35,503 (23,892 singletons) OTUs with the use of 97% and 99% sequence similarity cutoffs, respectively. The mean number of sequences for the placental tissue and technical control samples was 50,783 (range, 509−92,052) and 55,145 (2572−111,361), respectively. All raw count data for this study are available as supplemental material (Supplemental Data 1).

With the use of a 97% OTU nucleotide similarity cutoff, the Good's coverage values of all but 1 placental sample exceeded 99.7%; the exception was 98.8% (sample 25AC). Good's coverage values of all technical control samples exceeded 99.8%. For analyses of alpha diversity (microbial diversity within a sample), individual sample libraries were subsampled to the depth of the second least-represented sample (1997 sequences), and the least-represented sample (509 sequences for 25AC) was excluded. After subsampling for alpha diversity analyses, Good's coverage values of placental and technical control samples exceeded 99.4%.

qPCR of the 16S rRNA genes in DNA extracts of placental tissues and background technical controls

Bacterial DNA abundance within the samples was determined via qPCR amplification of the V1−V2 region of the 16S rRNA gene as described by Dickson et al,[207] with minor modifications. These included the use of a degenerative forward primer (27f-CM: 5′-AGA GTT TGA TCM TGG CTC AG-3′) and a degenerate probe containing locked nucleic acids (+) (BSR65/17:5′-56FAM-TAA +YA+C ATG +CA+A GT+C GA-BHQ1-3′). Amplifications were performed with an annealing temperature of 50°C to minimize amplification bias and to allow for a greater number of potential bacterial types, such as *Lactobacillus* and *Gardnerella* species.[203] Detailed information on the qPCR protocols are provided in the Supplemental Methods (Section 5).

Metagenomic sequencing of extracted DNA from placental samples and background technical controls

By contrast with sequencing surveys that target a specific bacterial gene (eg, 16S rRNA gene), a metagenomic survey entails sequencing all of the genes in a clinical sample and assigning the protein-coding genes of bacterial origin to particular bacterial taxa. Nine placental and 11 technical control samples underwent metagenomic sequencing with the use of the Illumina HiSeq 4000, 150-base paired-end read protocol at the University of Michigan's DNA Sequencing Core (Ann Arbor, MI). The placental samples included amnion and chorionic plate as well as villous tree and basal plate samples from each of 4 subjects (subjects 14, 15, 22, and 30), and a villous tree and basal plate sample from 1 subject (subject 19). The technical control samples included 8 biologic safety cabinet and 3 blank extraction kit samples. Metagenomic sequence data were processed with MG-RAST.[208] Bacterial taxonomic assignments were made with the use of the GenBank database and the default MG-RAST parameters. Detailed information on metagenomic sequencing and sequence data processing protocols are available in Supplemental Methods (Sections 6 and 7). All raw genus-level count data are available as supplemental material (Supplemental Data 2).

Secondary DNA extractions and molecular analyses of placental tissues

After the primary 16S rRNA gene sequencing analyses did not yield evidence of a placental microbiota (see Results), secondary analyses were conducted to ensure that the primary sequencing results were not due to cross-contamination between DNA extracted from placental tissues and background technical controls during processing, or exclusively because of the use of a nested PCR approach for bacterial DNA amplification.

Secondary DNA extractions were performed on the collective villous tree and basal plate portion of each of the 29 placental samples. The extraction protocol was the same as that described earlier, except that at least 4 blank extraction kit controls were included in each of 4 rounds of extractions of the placental samples. Specifically, in the

first 3 rounds of extractions, we processed 8 placental and 4 technical control samples. In the fourth round, we processed 5 placental and 5 technical control samples. Additionally, we completed a fifth round of extractions composed entirely of 12 blank extraction kit controls, which were not exposed to the atmospheres of the biologic safety cabinets or the laboratories; they were processed exactly as the placental samples. DNA concentrations of placental tissue and blank extraction control samples were 56.0±24.3 ng/μL and ≤0.03 ng/μL, respectively. Purified DNA was stored at −20°C.

The secondary DNA extractions were used for 16S rRNA gene sequencing with the use of 3 amplification approaches: standard PCR, nested PCR, and touchdown PCR. For standard PCR, we aimed to generate the 16S rRNA gene profiles of DNA extracted from placental samples and background technical controls using 30, 35, and 40 amplification cycles. For nested PCR, we used a different primer pair for the first round of amplifications from that used in the primary analysis in this study and aimed to generate 16S profiles for these samples using 5, 10, and 20 cycles in the first round of amplification. The different primer set, 341F/1061R (Supplemental Methods, Section 2), was used for the first round of nested PCR in an attempt to eliminate potential underrepresentation[209] or selection against single bacterial species or groups of species[210] in placental samples. Specifically, in silico studies querying these selected primers against taxonomically diverse sequences in 3 popular 16S rRNA gene databases (ie, Greengenes,[211] Ribosomal Database Project,[212] and SILVA[213]) have shown these selected primers to be highly conserved.[209,214] Last, we aimed to generate 16S rRNA gene profiles for these samples using touchdown PCR.[215−217] Touchdown PCR can increase the sensitivity of PCR reactions in cases of very low microbial biomass and high background concentrations of host DNA.[215−217] Touchdown PCR was used recently to characterize the microbiota of the lung,[216−220] brain,[221] and blood[219] of mice and/or humans. The PCR cycle started with 2 minutes at 95°C, followed by (1) a touchdown PCR for 20 seconds at 95°C, 15 seconds at the annealing temperature (60°C in the first cycle and decreased by 0.3°C with each additional cycle), and 5 minutes at 72°C, and then (2) 20 cycles of a standard PCR with 20 seconds at 95°C, 15 seconds at 55°C, and 5 minutes at 72°C, with a final elongation step at 72°C for 10 minutes.

All template DNA was diluted 3-fold and transferred to the University of Michigan's Center for Microbial Systems for sequence library processing. Sequence library construction was done with the use of the dual indexing sequencing strategy developed by Kozich et al.[204] All reactions included 4 μL of template DNA. Based on visual inspections of amplified products that use gel electrophoresis, sequence library generation was unsuccessful with 30 and 35 cycles of standard PCR. Sequence library generation was also unsuccessful with 5 and 10 cycles in the initial amplification round for nested PCR. Therefore, for the secondary 16S rRNA gene analyses, we generated sequence libraries for placental samples and background technical controls using 40 rounds of standard PCR, nested PCR with 20 initial rounds of amplification, and touchdown PCR. Sample-specific MiSeq run files have been deposited into the NCBI Sequence Read Archive (BioProject ID PRJNA397876), and all raw count data for the secondary analyses are provided as supplemental material (Supplemental Data 3). Sequence data processing for the secondary analyses proceeded as described earlier; see Supplemental Methods (Section 4). The analyses presented herein are of sequence data clustered into OTUs based on a nucleotide similarity percentage of 97%. Results did not substantively differ with a 97% or 99% nucleotide similarity; therefore, only the results that used 97% similarity are presented for the secondary analyses. Raw data from sequence clustering based on a nucleotide similarity percentage of 99% are provided in Supplemental Data 3.

The abundances of 16S rRNA gene copies in each placental sample and blank extraction control in this secondary analysis were determined with qPCR, as described earlier, with minor alterations. Specifically, all samples were diluted 3-fold before analysis; each sample reaction was performed in triplicate, and, if a sample did not pass the threshold of quantification by 40 cycles, its cycle of quantification (Cq) value was assigned as 40.

Statistical analysis

16S rRNA gene profile alpha and beta diversity

Alpha diversity (ie, diversity within a single sample) was assessed with the use of Chao1 richness and Simpson heterogeneity indices.[222,223] Alpha diversity indices were calculated with Mothur software (version 1.39.5)[206] and statistically evaluated with Kruskal-Wallis tests and Mann-Whitney pairwise comparisons, if applicable, using Paleontological Statistics software (version 2.17c).[224−226]

Beta diversity (ie, diversity between 2 samples) was assessed with Jaccard and Bray-Curtis similarity indices to reflect 16S rRNA gene profile composition and structure, respectively. Bray-Curtis values were calculated with the use of percent relative abundance data for OTUs within samples. Beta diversity was visualized through principal coordinates analyses and heat maps and statistically evaluated with nonparametric multivariate analysis of variance (NPMANOVA),[225−227] with 9999 permutations. Principal coordinates analyses plots and NPMANOVA tests were conducted with Paleontological Statistics software (versions 2.17c and 3.14),[224] and heat maps were generated via Matrix2png.[228]

Linear discriminant analysis effect size (LEfSe)[229] was used to identify any OTUs that differed in relative abundance between the placental tissue and background technical control samples. Sourcetracker (version 1.0)[230] was used to estimate the percentage of OTUs in placental samples whose origin could be explained by their distribution in the background technical controls. For this analysis, we removed doubleton and singleton OTUs from the dataset.

16S rRNA gene qPCR

To assess differences in 16S rDNA abundance between the amnion and chorionic plate and the villous tree and basal plate samples among the 29 subjects, differences in the Cq were evaluated with paired *t*-tests. To assess variation in bacterial burden among individual sample types (ie, amnion and chorionic plate, villous tree and basal plate, operating rooms and laboratories, biosafety cabinets, and blank DNA extraction kits), analysis of variance tests, or Welch F tests in the case of unequal variances, were used for global assessment of variation in Cq, followed by Tukey's pairwise comparisons.[225,226] When data were not distributed normally, we used Kruskal-Wallis tests and Mann-Whitney pairwise comparisons. Statistical analyses were performed using Paleontological Statistics software (version 2.17c).[224]

TABLE 1
Descriptive and clinical characteristics of the 29 study participants

Variable	Median	Interquartile range
Age, y	29.0	25.5–33.0
Body mass index, kg/m^2[a]	32.8	24.7–36.1
Parity	2	1–2
Gestational age at delivery, wk	39.1	39.0–39.3
Birthweight, g	3450	3063–3905
Race, n (%)[b]		
African American	21 (80.8)	
Caucasian	5 (19.2)	
Clinical indications, n (%)		
Repeat elective cesarean delivery	23 (79.3)	
Large for gestational age fetus	3 (10.3)	
Breech presentation	2 (6.9)	
Myoclonus dystonia	1 (3.4)	

[a] Unreported for 7 subjects; [b] Race was self-reported by subjects; 3 subjects chose not to report.
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Results

Patient characteristics

Table 1 describes the demographic and clinical characteristics of the patients in this study. None of the placentas included in this study presented fetal or maternal inflammatory lesions, defined as stage 3 and/or grade 2 maternal and/or fetal inflammatory responses.[59,231]

Bacterial culture of placental tissues

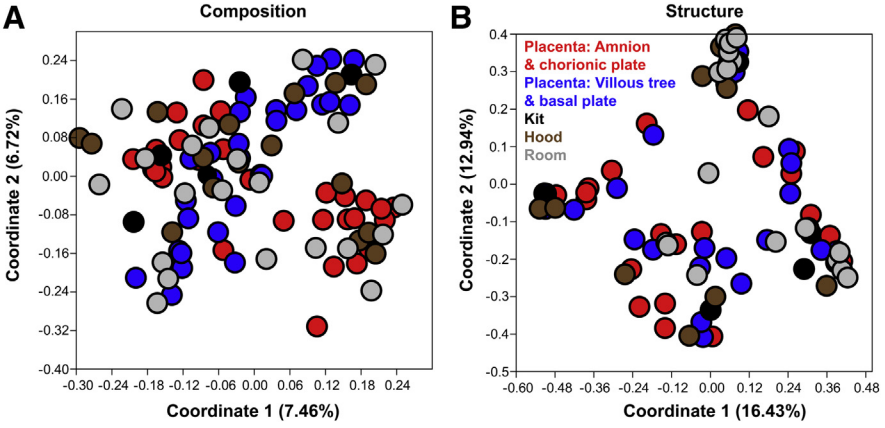
Twenty-eight of the 29 placental tissue samples did not yield any bacterial cultivars. One tissue sample (subject 25) yielded 3 colonies in the primary zone of the 5% sheep blood agar plate incubated aerobically: *Bacillus circulans*, *B pumilus*, and *Brevibacterium casei*. It did not yield colonies on other media under aerobic or anaerobic conditions or yield growth of genital mycoplasmas. Exact matches (ie, 100% nucleotide similarity) to the V4 region of the 16S rRNA genes of the 3 isolates recovered on the sheep blood agar plate were not found among any of the sequences from the primary (13,766 sequences; Good's coverage >99.9%) or the secondary (98,392 sequences; Good's coverage >99.9%) MiSeq 16S rRNA gene surveys of subject 25's placental tissues.

16S rRNA gene surveys of placental tissue and background technical control samples

Alpha diversity

There was no variation in OTU richness among the amnion and chorionic plate samples and the room, hood, and blank extraction kit controls (Chao1 index; Kruskal-Wallis test; H=4.114; *P*=.248), nor was there variation among the villous tree and basal plate samples and the various controls (H=3.871; *P*=.274).

FIGURE 1
Principal coordinates analyses illustrating similarity in 16S ribosomal RNA gene profiles among the amnion and chorionic plate, villous tree and basal plate, and technical control samples (i.e., blank DNA extraction kits ("Kit"), biological safety cabinets ("Hood"), and the operating rooms and laboratories ("Room") used in the study)



A, Plot of similarity in profile composition among placental and control samples based on the Jaccard index. **B,** Plot of similarity in profile structure among placental and control samples based on the Bray-Curtis index. Operational taxonomic units were generated with a 97% sequence similarity cutoff and the primary 16S ribosomal RNA gene nested polymerase chain reaction data set.
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There was also no variation in OTU heterogeneity between the placental and technical control samples (Simpson index; amnion and chorionic plate: H=3.384; *P*=.336; villous tree and basal plate: H=2.531; *P*=.470).

Beta diversity

There was no variation in the composition or structure of 16S rRNA gene profiles among the 3 biologic safety cabinets (NPMANOVA; Jaccard: F=0.846; *P*=.781; Bray-Curtis: F=0.880; *P*=.572), or among the different rooms used for sample processing (Jaccard: F=0.882, *P*=.833; Bray-Curtis: F=0.916; *P*=.602). Profile similarities among the amnion and chorionic plate samples, the villous tree and basal plate samples, and the 3 different types of technical controls (ie, blank extraction kits, biosafety cabinets, rooms) are illustrated in Figure 1. 16S rRNA gene profiles did not consistently vary among the amnion and chorionic plate samples, blank extraction kits, biologic safety cabinets, and processing rooms (Figure 1; Table 2). Similarly, 16S rRNA gene profiles did not vary among the villous tree and basal plate samples, blank extraction kits, biologic safety cabinets, and room controls (Figure 1; Table 2). Neither the 16S rRNA gene profiles of the amnion and chorionic plate samples nor those of the villous tree and basal plate samples differed specifically from those of the blank extraction kits (Table 2). These same patterns were found when an OTU nucleotide similarity cutoff of 99% was used (Supplemental Figure 2; Supplemental Table 1).

Sixteen of the 18 prominent OTUs (ie, those having an average relative abundance ≥1%) among the placental samples were classified confidently at the genus level (Figure 2). These OTUs were *Achromobacter*, *Delftia*, *Phyllobacterium*, *Clostridium*, *Propionibacterium*, *Stenotrophomonas*, *Acinetobacter*, *Blastomonas*, *Methylobacterium*, *Sphingomonas*, *Paracoccus*, *Ralstonia*, *Staphylococcus*, *Leucobacter*, and *Ureaplasma*. These 18 prominent OTUs accounted for 90.0% and 86.4% of total sequences obtained from the placental tissue samples and background technical controls,

TABLE 2
Nonparametric multivariate analysis of variance shows lack of variation in 16S ribosomal RNA gene profiles among the amnion and chorionic plate, villous tree and basal plate, and room, hood, and blank extraction kit technical control samples

	Composition		Structure	
Variable	F	*P* value	F	*P* value
Placenta: Amnion and chorionic plate				
Global	1.080	.261	1.128	.270
Rooms	1.367	.060	2.211	.028 (.077)
Hoods	1.310	.108	1.190	.275
Kits	1.018	.412	0.545	.873
Placenta: Villous tree and basal plate				
Global	1.051	.335	1.222	.189
Rooms	1.450	.037 (.223)	2.513	.007 (.043)
Hoods	1.149	.231	1.072	.351
Kits	0.944	.552	0.875	.529

Operational taxonomic units were generated with a 97% sequence similarity cutoff. 16S profile composition and structure were characterized with the use of Jaccard and Bray-Curtis indices, respectively. Results of overall global effect analyses are presented along with the results of pairwise comparisons that involve placental samples. Probability values for these permutation tests were not adjusted for multiple pairwise comparisons, because this can be overly conservative. However, for pairwise tests that were statistically significant, we present the Bonferroni corrected probability value in parentheses.

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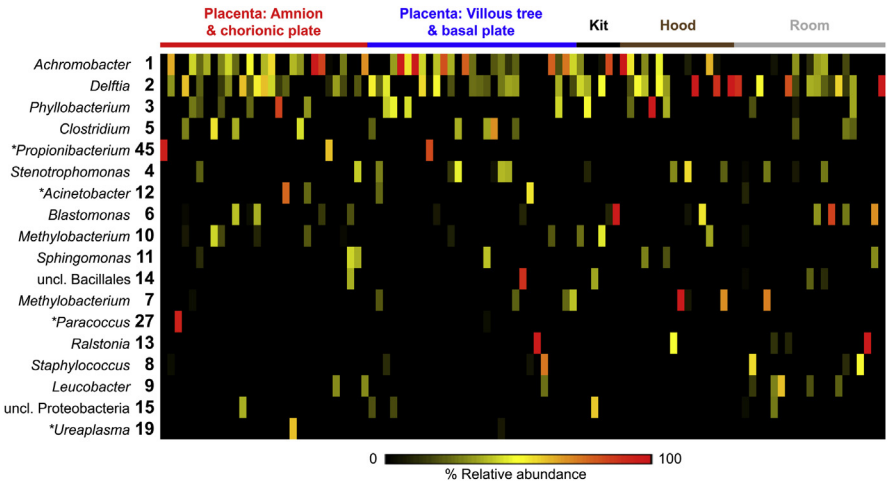
FIGURE 2
Heat map illustrating similarity in percent relative abundances of prominent operational taxonomic units among placental samples and technical controls



Prominent operational taxonomic units were defined as those having an average relative abundance ≥1% among the placental samples. Operational taxonomic units were generated with a 97% sequence similarity cutoff and the primary 16S ribosomal RNA gene nested polymerase chain reaction data set. *Asterisks* indicate operational taxonomic units prominent in placental samples but not in controls.

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respectively. Fourteen of these 18 prominent placental OTUs were also prominent among the control samples (Figure 2). The 4 exceptions (OTUs classified as *Acinetobacter*, *Paracoccus*, *Propionibacterium*, and *Ureaplasma*) were OTUs that were either widely present among the technical control samples but at low relative abundances or abundant in only 1 to a few placental tissue samples. A full description of the distribution and relative abundances of these OTUs among placental samples and technical controls is provided in the Supplemental Results (Section 1).

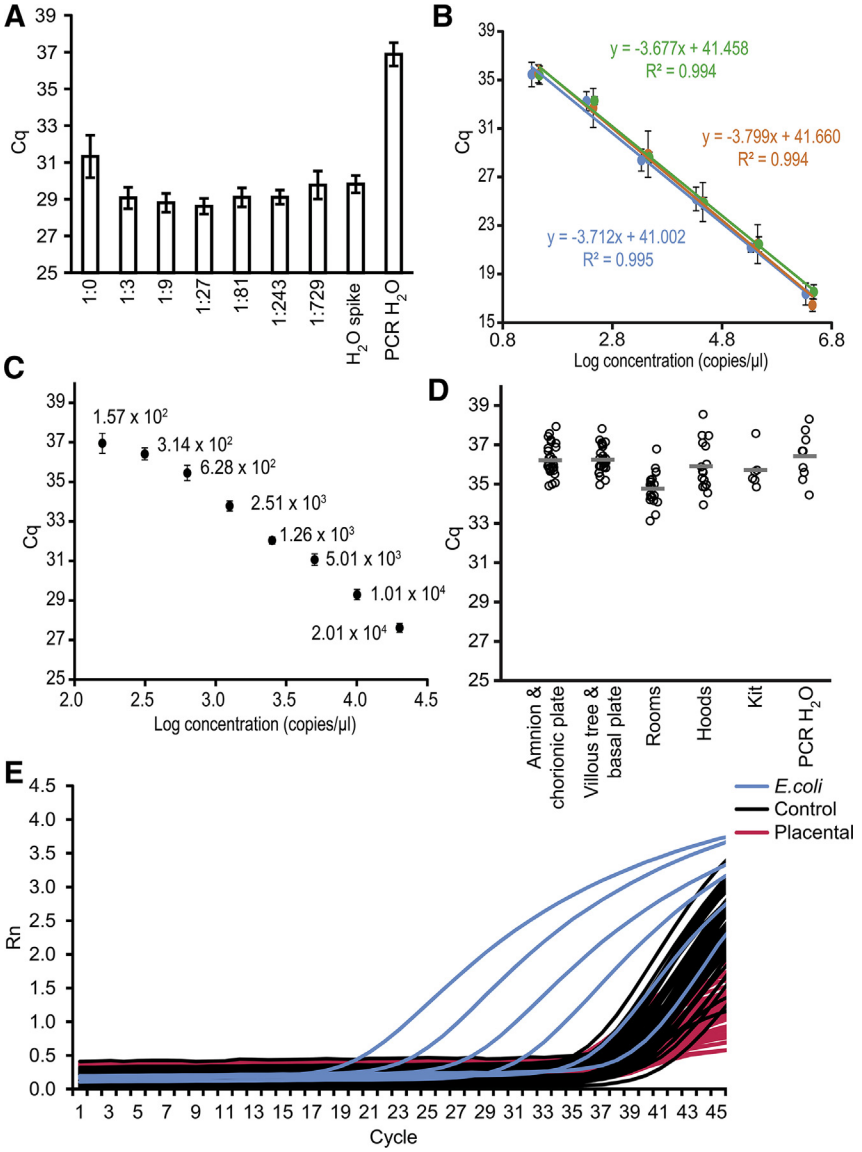
LEfSe indicated that 4 OTUs (classified as *Achromobacter*, *Blastococcus*, *Methylobacterium*, and *Caldalkalibacillus*) were more relatively abundant among the amnion and chorionic plate samples than the technical controls and that 3 OTUs (classified as *Achromobacter*, Burkholderiales, and *Herbaspirillum*) were more relatively abundant among the villous tree and basal plate samples than the controls (Supplemental Figure 3). The distribution and relative abundances of these OTUs among placental samples and technical controls is discussed in detail in Supplemental Results (Section 2).

SourceTracker analyses indicated that a median of 99.7% (interquartile range [IQR], 50.7%) and 99.9% (IQR, 8.2%) of OTUs in the amnion and chorionic plate and the villous tree and basal plate samples, respectively, could be attributed confidently to contaminating DNA in blank extraction kits, PCR reagents, and/or the rooms used for sample processing. Furthermore, when defining the core microbiota as those OTUs present in at least one-half of the samples of a particular sampling group,[69,72] every core OTU in the amnion and chorionic plate and the villous tree and basal plate samples was also a core OTU in the hood and blank extraction kit control samples (Supplemental Results, Section 3).

Real-time qPCR assays of 16S rRNA gene copy abundances in the placental tissues and background technical controls

Analysis of Cq values generated for broad-range standard curves and

FIGURE 3
Quantitative polymerase chain reaction analyses illustrating similarity in 16S ribosomal RNA gene abundance among the amnion and chorionic plate, villous tree and basal plate, and technical control samples



A, Comparison of quantification cycle values (mean±standard deviation) of serially diluted placental genomic DNA samples spiked with equal concentrations (5.7×10^3 copies per reaction) of genomic DNA from *Escherichia coli* ATCC 25922 illustrate that amplification inhibition is eliminated by diluting samples with nuclease-free water by a factor of ≥1:3. **B,** Standard curves for 3 10-fold dilution series (2.82×10^6 to 2.82×10^1 copies, 2.12×10^6 to 2.12×10^1 copies, and 2.97×10^6 to 2.97×10^1 copies) of *E coli* ATCC 25922 16S ribosomal DNA (mean quantification cycle values across all quantitative polymerase chain reaction runs). **C,** Standard curve for a 2-fold dilution series (mean quantification cycle values) of *E coli* ATCC 25922 DNA illustrate a limit of detection for the quantitative polymerase chain reaction assay between 1.57×10^2 and 3.14×10^2 16S ribosomal DNA copies per reaction (20 μL), as indicated by a standard deviation of replicate dilution samples >0.5 cycles. **D,** Comparison of mean 16S ribosomal DNA quantitative polymerase chain reaction quantification cycle values for placental and control samples. **E,** Amplification curves from placental samples, technical controls, and the serial dilution series of *E coli* DNA described in **B**.

Cq, quantification cycle; *PCR*, polymerase chain reaction; *Rn*, normalized fluorescence.

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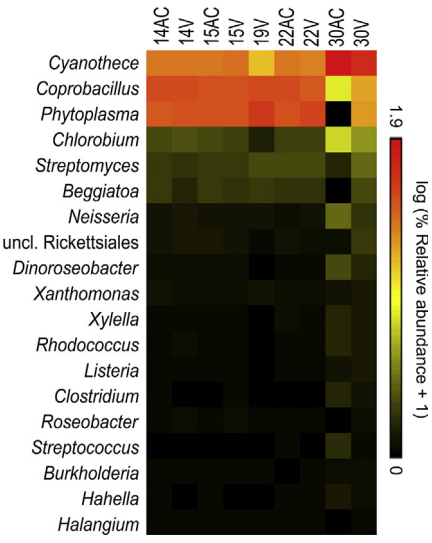
FIGURE 4
Heat map illustrating relative abundances of prominent bacterial genera among placental sample profiles as determined by metagenomic sequencing



Prominent genera are defined as those having an average relative abundance ≥0.1% among the placental sample profiles. *AC* indicates amnion and chorionic plate samples, and *V* indicates villous tree and basal plate samples, respectively.

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included across all qPCR runs indicated that the average amplification efficiency of the assay was 85.44%±1.91%. The regression curves were linear over a range of 10^1 to 10^6 gene copies, with slopes ranging from −3.88 to −3.62 and R^2 values ≥0.980 (Figure 3, B). Analysis of Cq values generated for the narrow-range standard curve that ranged from 2.01×10^4 to 1.57×10^2 revealed that standard deviation values reached 0.506 cycles for the most dilute replicate reactions (Figure 3, C), indicating that the limits of detection and quantification for the assay were between 1.57×10^2 and 3.14×10^2 copies (Figure 3, C).

qPCR revealed that 16S rDNA abundances within the majority of the placental and background technical control samples were beyond the detection and quantification limits of the qPCR assay (Figure 3, D and E). There were no differences in Cq between the amnion and chorionic plate and the villous tree and basal plate samples (paired *t*-test: n=29; *t*=−0.485; *P*=.631). For the background technical control samples, there was no variation in Cq values among the location-specific control samples from the rooms (analysis of variance: n=21; F=0.008; *P*=.999) or from the individual biological safety cabinets (n=16; F=0.063; *P*=.939). Therefore, these samples were combined within their respective groups for comparison to the amnion and chorionic plate and the villous tree and basal plate samples. Variation in Cq values was observed among the amnion and chorionic plate samples and the room, hood, kit, and water samples (Welch F test: n=81; F=7.683; *P*=.0005), and among the villous tree and basal plate samples and controls (F=9.572; *P*=.0001). In both cases, the variation was due to the room control samples having lower Cq values (ie, higher rDNA abundances) than the placental and water samples (Tukey's pairwise comparisons; amnion and chorionic plate vs rooms: Q=4.544; *P*=.016; villous tree and basal plate vs rooms: Q=5.108, *P*=.005; water vs rooms: Q=5.773; *P*=.001). Cq values did not differ between amnion and chorionic plate samples and blank extraction kits (*t*-test; *t*=−1.093; *P*=.282). They also did not differ between villous tree and basal plate samples and blank extraction kits (*t*=−1.535; *P*=.134). When a subset (n=32/43) of total control samples was diluted 1:9, there were no differences between the amnion and chorionic plate samples, villous tree and basal plate samples, and technical controls (*t*-tests: amnion and chorionic plate: *t*=−0.296; *P*=.768; villous tree and basal plate: *t*=0.048; *P*=.962). Differences were then also absent between the placental tissue samples and a subset (n=13/21) of room control samples (*t*-tests; amnion and chorionic plate: *t*=0.018; *P*=.985; villous tree and basal plate: *t*=0.354; *p*=.725).

Metagenomic surveys of placental tissues

At least 43,000,000 sequence reads were obtained from each of 9 placental tissue samples (61,027,678±9,572,214). On average, 0.05% of these sequences were classified as bacterial in origin. Good's coverage values (99.6%±0.004%) indicated that the bacterial profiles of these samples were characterized thoroughly from a taxonomic standpoint. The survey identified 267 bacterial genera, with 19 having an average relative abundance of ≥0.1% (Figure 4). Only 5 genera had an average relative abundance ≥1.0%: *Cyanothece*, *Coprobacillus*, "Candidatus phytoplasma," *Chlorobium*, and *Streptomyces*. *Escherichia* was present in each placental sample, with an average relative abundance of 0.05%. The functions of bacterial genes were characterized broadly as metabolism (amino acid, carbohydrate, vitamin, and energy metabolism), genetic (DNA translation, replication, repair, and degradation), and environmental (membrane transport and signal transduction) processing.

Given the necessary differences in metagenomic library preparation for the placental tissue and technical control samples, their broad bacterial profiles cannot be compared in a quantitative manner. However, it is reasonable to inquire if there are genera consistently identified in placental tissue samples that were not also widely present in the sequenced background technical controls. There were 36 genera present in all 9 sequenced placental tissue samples, and 89 genera present in at least one-half. Each of these genera was present in all 11 sequenced background technical controls. Of the 267 total genera, or approximate genus-level taxa, that were identified in placental tissue samples, only 1 was not found in every control sample: an unclassified Myxococcales, present in 1 placental sample with an abundance <0.01%.

Of the prominent genera identified in the primary 16S rRNA gene sequencing analysis (Figure 2), only *Clostridium* was present in placental metagenomic bacterial profiles at an average relative abundance ≥0.1% (Figure 4). *Achromobacter*, *Clostridium*, *Propionibacterium*, *Staphylococcus,* and *Stenotrophomonas* were present in the metagenomic profiles of at least one-half of the placental samples. However, each of these genera was also present

in the metagenomic profiles of all 11 sequenced background technical controls.

Secondary 16S rRNA gene sequencing and qPCR analyses

16S rRNA gene surveys with standard PCR

The median number of sequences obtained from the 29 placental samples was 89 (IQR, 15−3210), and no blank extraction kit controls yielded >100 quality sequences. Of the 29 placental samples, only 31% (9/29) yielded at least 1000 quality sequences and had Good's coverage values exceeding 99%. Their microbial profiles included 8 prominent OTUs (ie, average relative abundance ≥1%; Supplemental Figure 4). *Pelomonas* and *Sphingomonas* were most consistently abundant. These genera represented 2 of the 3 OTUs present in at least one-half of the 9 placental samples. The remaining core OTU (OTU001) was *Escherichia*, present in each of the 9 placental samples with a median relative abundance of 0.07% (IQR, 0.02−0.13%). Although the blank extraction kits had poor sequence yield, their bacterial profiles were dominated by *Escherichia* (median, 67%; IQR, 41−100%). Indeed, OTU001 was detected in 27 of 28 blank extraction kit controls that yielded sequence data.

Neither *Pelomonas* nor *Sphingomonas* was detected in the bacterial profiles of the 9 placental tissues characterized through metagenomic sequencing in the primary analyses described earlier.

16S rRNA gene surveys with the use of nested PCR

Fifty-seven of 58 placental and blank extraction kit control samples yielded ≥1000 sequences with a Good's coverage value that exceeded 99%. One blank extraction kit sample yielded 423 sequences and was excluded from analyses. The remaining placental samples and technical controls yielded 80,492±27,721 and 77,670±79,160 quality sequences, respectively. These sequences clustered into 207 OTUs. For alpha diversity analyses, each sample was subsampled to a depth of 4020 sequences. Alpha diversity did not differ between blank extraction controls processed alongside (n=16) or independent of (n=12) placental samples (Mann-Whitney; Chao1: U=67.5; *P*=.192; Simpson: U=90.0; *P*=.799). The richness (U=10.5; *P*<.0001) and heterogeneity (U=67.0; *P*=.0001) of blank extraction kit control samples, although very low, were greater than those of placental tissue samples (Supplemental Figure 5).

Extraction controls processed alongside placental samples did not have a different bacterial profile than those processed alone (NPMANOVA; Jaccard: F=0.863; *P*=.810; Bray Curtis: F=0.577; *P*=.940), which indicates that bacterial signals obtained from blank extraction kit samples were not simply due to DNA cross-contamination from placental tissue samples during processing. The bacterial profiles of placental tissue samples and blank extraction kit controls differed in both composition and structure (Supplemental Table 2; Supplemental Figure 6). However, OTU001, classified as *Escherichia*, accounted for 99.0% and 97.6% of the sequences obtained from placental samples and extraction controls, respectively. OTU009, an *Enterococcus*, was also found in all samples, with an average relative abundance of 0.11% and 0.33% among placental samples and blank extraction kit controls, respectively. OTU102, a *Clostridium*, was the only other OTU with an average relative abundance ≥0.1% among the placental tissue samples, and it was detected in only 3 of 29 of these samples. In addition to the 2 *Escherichia* and *Enterococcus* OTUs, OTU185, a *Shewanella*, was a third core OTU (ie, present in at least one-half of the samples) among placental tissue samples. LEfSe analyses indicated that OTU001, *Escherichia*, was the only OTU that was more relatively abundant among placental samples than technical controls. SourceTracker analysis indicated that a median of 100% (IQR, 99−100%] of the OTUs present in the 16S rRNA gene profiles of placental samples could be explained by their distribution among the profiles of technical controls.

16S rRNA gene surveys with the use of touchdown PCR

Twenty-four of 29 placental tissue samples and 28 of 29 blank extraction kit controls yielded ≥1000 sequences with Good's coverage values exceeding 99%. The other samples were excluded from analyses. The remaining placental and extraction control samples yielded 14,602±12,641 and 38,817±35,710 quality sequences, respectively. These sequences clustered into 350 OTUs. For alpha diversity analyses, each sample was subsampled to a depth of 1060 sequences. Alpha diversity did not differ between controls processed alongside (n=17) or independent of (n=11) placental samples (Mann-Whitney; Chao1: U=81.5; *P*=.587; Simpson: U=78.0; *P*=.480). Alpha diversity also did not differ between placental samples and extraction controls (Chao1: U=168.5; *P*=.354; Simpson: U=190.0; *P*=.728).

The bacterial profiles of background extraction controls did not differ between controls processed alongside placental samples or alone (NPMANOVA; Jaccard: F=1.216; p=0.083; Bray Curtis: F=0.867, p=0.672). There was variation in the composition of bacterial profiles based on sample type and round of extraction (Supplemental Table 3; Supplemental Figure 7). Specifically, there was a modest observed difference in bacterial profile composition between placental sample and blank extraction controls in the first round of extractions (F=1.506; *P*=.040), but not in the second (F=1.032; *P*=.394), third (F=1.211; *P*=.122), or fourth (F=0.900; *P*=.734) round of extractions. In the first round, 5 of 6 and 4 of 6 of the placental samples contained OTU015 (*Ralstonia*) and OTU034 (an unclassified Enterobacteriaceae), respectively. These OTUs were not present in any of the 4 blank extraction controls processed in round 1. There was no difference in the structure of bacterial profiles between placental tissue samples and blank extraction controls (Supplemental Table 3; Supplemental Figure 7).

There were 21 prominent OTUs (ie, average relative abundance ≥1%) among placental samples (Supplemental

Figure 8). Eight of these OTUs were also prominent among blank extraction control samples. None of the 13 OTUs prominent among placental samples, but not prominent among technical control samples, was present in >21% (5/24) of the placental samples. LEfSe indicated that 3 OTUs were more relatively abundant among placental samples than blank extraction controls (Supplemental Figure 9). These OTUs were 15 (*Ralstonia*), 17 (*Chthoniobacter*), and 41 (*Anaerococcus*). OTUs 15 and 17 were among the prominent OTUs for blank extraction control samples. OTU041 was not prominent among either placental or technical control samples. It was present in 5 of 24 placental samples, with an average relative abundance of 1.79%. OTU041 was not present in any of the 17 blank extraction control samples processed alongside placental samples. However, it did account for 6.4% of the sequences from 1 blank extraction control processed independently of placental samples.

There were 5 core OTUs (ie, present in at least one-half of samples) among placental samples. Three of the 5 were also core OTUs among blank extraction controls (OTUs 1, 2, and 3). The exceptions were OTU015 (*Ralstonia*) and OTU017 (*Chthoniobacter*), which were nonetheless prominent among technical controls. Neither *Ralstonia* nor *Chthoniobacter* was detected in the bacterial profiles of the 9 placental tissues characterized through metagenomic sequencing in the primary analyses described earlier.

SourceTracker analyses indicated that a median of 24% (IQR, 0−76%) of OTUs in the placental samples could be attributed to background DNA contamination in the extraction kits and/or PCR reagents. The large degree of observed variation was due to whether the bacterial profiles of placental samples were dominated by 1 of the 4 most prominent OTUs among the placental samples (OTUs 3, 8, 15, and 2; Supplemental Figure 8). Among the 12 of 24 placental samples that derived at least 25% of their sequences from 1 of these 4 OTUs, 75% (IQR, 55−95%) of their OTUs could be attributed to background DNA contamination. The profiles of the 12 remaining placental samples were each dominated by a different OTU (Supplemental Figure 8). These OTUs were only sporadically present among the technical controls, so their distribution among the placental samples could not be attributed to background DNA contamination based on SourceTracker analyses (median, 0; IQR, 0).

qPCR

The secondary qPCR analysis did not indicate the presence of bacteria in placental samples. Although an increase in overall reaction efficiency was observed (96.7%) for the secondary qPCR analysis compared to the primary analysis, the sensitivity of the assay remained approximately 150 copies. As in the primary qPCR analysis, the vast majority of the placental and background technical control samples were beyond the detection limits of the assay. Mean Cq values for both placental sample and background technical controls were >37 cycles (Supplemental Figure 10). There was no difference in Cq values between blank extraction kit controls processed alongside (n=17) or independent of (n=12) placental samples (*t*-test: t=1.579; P=.126). Therefore, bacterial signals in blank extraction kit samples were not simply due to DNA cross-contamination from placental tissue samples during processing.

Comment

Principal findings of the study

Our principal findings were that (1) cultivation of the placental tissues did not yield viable bacteria in 28 of 29 cases; in the case in which it did, the microorganisms were not detected by 16S rRNA gene sequencing; (2) qPCR did not indicate a greater abundance of bacterial 16S rRNA genes in placental tissues than in technical controls (laboratory environments and reagents); (3) 16S rRNA gene sequencing did not reveal consistent differences in the composition or structure of bacterial profiles between placental samples and technical controls, and (4) metagenomic surveys of placental tissues largely yielded bacterial sequences from cyanobacteria, aquatic bacteria, and plant pathogens, which are microbes ecologically unlikely to populate the human placenta. The identification of *Coprobacillus*, *Streptomyces*, and other potentially clinically relevant genera in the metagenomic data, although intriguing, was not consistent with their absence or extreme rarity in the multiple 16S rRNA gene surveys of these samples. Overall, we did not find consistent evidence that the human placenta harbors a unique microbiota because microbial signals derived from placental tissues were similar to those observed in technical controls.

The claim that "the placenta harbors a unique microbiome"

In 2014, a key publication reported the results of a study of 320 placentas that used 16S rRNA gene sequencing and of a subset of these (n=48) that also underwent metagenomic sequencing.[64] The authors characterized "a *unique placental microbiome niche* composed of nonpathogenic commensal microbiota from the Firmicutes, Tenericutes, Proteobacteria, Bacterioidetes, and Fusobacteria phyla."[64] Placental microbiota profiles were more similar to those of the human oral cavity than those of the vagina, gut, and skin (Figure 1 in Aagaard et al[64]). *Escherichia coli* was most abundant in the placenta, followed by *Bacteroides* spp, *P acnes*, *Neisseria lactamica*, and *S epidermidis* (Figure 2 in Aagaard et al[64]). However, cultures were not used in this study; therefore, there is no information about the viability of the microbes from which sequences were detected. qPCR was also not part of the study; nonetheless, the authors emphasized that the placenta was a low microbial biomass site.[64]

This publication stimulated research into the existence of a placental microbiota. Twelve additional studies (Table 3) have interrogated placental samples at term with the use of sequence-based techniques to determine, at least in part, whether there is a placental microbiota.[32,65−75] Eleven of these studies have concluded that there is evidence of a placental microbiota at term based on 16S rRNA gene sequencing and/or metagenomics.[65−75] Thus, the

TABLE 3
Description of previous 16S ribosomal RNA gene or metagenomic studies of the human placental microbiota at term

Study	Author	Year	Central research questions	Mode of delivery (sample size)	Type of sample	Molecular microbiology methods	Was culture used?	Were DNA contamination controls included?
1	Aagaard et al[64]	2014	Is there a placental microbiota? Does it vary with antenatal infection and preterm birth?	Term cesarean (n=53); term vaginal (n=178); preterm cesarean (n=20); preterm vaginal (n=69)	Villous tree; collected <1 hour after delivery	16S ribosomal RNA gene sequencing; metagenomic sequencing (subset of 48 subjects)	No	One blank extraction kit processed per 11 placental samples; these blanks did not generate noticeable bands of amplified DNA and thus were not sequenced routinely; reagents from a limited number of blanks were sequenced, and their bacterial profiles reflected airway or nonhuman sources (data not provided)
				Conclusions: There is a placental microbiota at term and preterm, regardless of mode of delivery; the placental microbiota shares similarity with the microbiota of the oral cavity; the placental microbiota differs between women who deliver preterm and at term; the placental microbiota differs between women with and without a remote history of antenatal infection.				
2	Doyle et al[65]	2014	Does the placental microbiota differ between preterm and term deliveries?	Term cesarean without labor (n=4); term vaginal (n=6); preterm vaginal (n=14)	Amnion and chorion; time between delivery and processing not provided	16S ribosomal RNA gene sequencing	No	No
				Conclusions: There is a placental microbiota at term and preterm, regardless of mode of delivery; nevertheless, the microbial profiles of placental tissues differ between cesarean and vaginal deliveries; the placental microbiota differs between term and preterm deliveries.				
3	Antony et al[66]	2015	Does the placental microbiota vary with maternal obesity or excess gestational weight gain? If so, does its profile differ between preterm and term intervals?	Cesarean (n=54); vaginal (n=183); 62/237 subjects (26.2%) delivered preterm	Villous tree; processed immediately on delivery	16S ribosomal RNA gene sequencing; metagenomic sequencing (subset of 37 subjects)	No	Subgroup analysis of study 1
				Conclusions: There is a placental microbiota at term and preterm; among women who deliver preterm, differences in the placental microbiota exist between women with and without excess gestational weight gain.				
4	Zheng et al[67]	2015	Is the placental microbiota at term associated with neonatal birthweight?	Term vaginal normal birthweight (n=12); term vaginal low birthweight (n=12)	Villous tree; processed immediately on delivery	16S ribosomal RNA gene sequencing	No	No
				Conclusions: There is a placental microbiota at term; the placental microbiota differs between low birthweight and normal birthweight neonates.				

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TABLE 3
Description of previous 16S ribosomal RNA gene or metagenomic studies of the human placental microbiota at term *(continued)*

Study	Author	Year	Central research questions	Mode of delivery (sample size)	Type of sample	Molecular microbiology methods	Was culture used?	Were DNA contamination controls included?
5	Bassols et al[68]	2016	Is the placental microbiota at term associated with gestational diabetes mellitus?	Term vaginal without gestational diabetes mellitus (n=11); term vaginal with gestational diabetes mellitus (n=11)	Villous tree; processed immediately on delivery	16S ribosomal RNA gene sequencing	No	No
				Conclusions: There is a placental microbiota at term; the placental microbiota differs between women with and without gestational diabetes mellitus.				
6	Collado et al[69]	2016	Is the fetal gut colonized in utero by microbes from the amniotic cavity, placenta, and/or maternal gut?	Term cesarean without labor (n=15)	Placental tissue (unspecified); processed immediately on delivery	Denaturing gradient gel electrophoresis; 16S ribosomal RNA gene sequencing	Anaerobic culture was used; *Propionibacterium* and *Staphylococcus* cultured from the placenta	No
				Conclusions: There is a placental microbiota at term; the microbiota of the meconium shares similarities with the microbiota of the placenta and amniotic fluid.				
7	Lauder et al[32]	2016	Is there a placental microbiota at term?	Term cesarean (n=1); term vaginal (n=5)	Fetal side biopsy of the placenta; maternal side biopsy of the placenta (basal plate); processed immediately on delivery	Quantitative real-time polymerase chain reaction; 16S ribosomal RNA gene sequencing	No	Laboratory air swabs (n=11); sterile swabs (n=8); blank extraction kits (n=8); controls incorporated into statistical analyses
				Conclusions: Microbial signatures in placental tissues could not be distinguished from those of technical controls.				
8	Prince et al[70]	2016	Does the microbiota of the placental membranes vary in association with preterm birth and chorioamnionitis?	Term cesarean without chorioamnionitis (n=4); term vaginal without chorioamnionitis (n=11); term cesarean with chorioamnionitis (n=3); term vaginal with chorioamnionitis (n=9); preterm cesarean without chorioamnionitis (n=2); preterm vaginal without chorioamnionitis (n=11); preterm cesarean with chorioamnionitis (n=5); preterm vaginal with chorioamnionitis (n=26)	Swabs of the chorion and/or villous membranes adjacent to the fetal side of the placenta; processed on delivery	Metagenomic sequencing; targeted polymerase chain reaction of *Ureaplasma* and *Mycoplasma* serovars	Culture of *Ureaplasma* and *Mycoplasma* used; *Ureaplasma* cultured from the chorion of 8 subjects with chorioamnionitis and from 2 subjects who delivered preterm without chorioamnionitis; *Ureaplasma* not cultured from the chorion of subjects who delivered at term without chorioamnionitis	Only samples with high sequence yield and without concern for contamination included in analysis (specific methods not provided)
				Conclusions: There is a placental microbiota at term and preterm, regardless of mode of delivery; variation in the placental microbiota is associated with preterm birth and the presence and severity of chorioamnionitis.				

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(continued)

TABLE 3
Description of previous 16S ribosomal RNA gene or metagenomic studies of the human placental microbiota at term *(continued)*

Study	Author	Year	Central research questions	Mode of delivery (sample size)	Type of sample	Molecular microbiology methods	Was culture used?	Were DNA contamination controls included?
9	Doyle et al[71]	2017	Is there variation in the placental microbiota associated with gestational age, neonatal size, or chorioamnionitis? What is the origin of the placental microbiota (ie, oral cavity or vagina)?	1097 subjects; unreported percentages of subjects delivered via cesarean and/or preterm or with chorioamnionitis	Amnion and chorion; a sample of placental tissue at full thickness; some samples processed immediately on delivery; others processed 1–24 hours later, after being kept at room temperature	16S ribosomal RNA gene sequencing; quantitative real-time polymerase chain reaction	No	Reagents from 1 blank extraction kit processed and sequenced for every 10 extractions; operational taxonomic units detected in these negative controls were removed from the data set; only placental samples that had a positive quantitative real-time polymerase chain reaction value (equivalent to 40 colony-forming units/μL) were sequenced; 68.1% of amnion-chorion and 46.8% of placental samples had a positive quantitative real-time polymerase chain reaction value; a delay in sample processing increased the likelihood of a positive quantitative real-time polymerase chain reaction value
				Conclusions: There is a placental microbiota at term; the placental microbiota has more overlap with the microbiota of the vagina than with the oral cavity; variation in the placental microbiota is associated with severe chorioamnionitis and delivery of a smaller neonate.				
10	Gomez-Arango et al[72]	2017	What is the origin of the placental microbiota in overweight and obese pregnant women (ie, oral cavity or gut)?	Term cesarean (n=17); term vaginal (n=20); 13 overweight and 24 obese	Fetal side biopsy of the placenta; processed within 1 hour of delivery	16S ribosomal RNA gene sequencing	No	The reagents from 1 blank extraction kit and 1 polymerase chain reaction amplification control were pooled and sequenced for each kit type (each sample type [placenta, oral swab, stool] was processed with a different kit type); operational taxonomic units detected in these negative controls removed from the data set
				Conclusions: There is a placental microbiota at term, regardless of mode of delivery; the placental microbiota was more similar to that of the maternal oral than the intestinal environment, yet it was distinct from each.				

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TABLE 3
Description of previous 16S ribosomal RNA gene or metagenomic studies of the human placental microbiota at term *(continued)*

Study	Author	Year	Central research questions	Mode of delivery (sample size)	Type of sample	Molecular microbiology methods	Was culture used?	Were DNA contamination controls included?
11	Parnell et al[73]	2017	Is there a placental microbiota at term? Does it vary between the fetal membranes, placental villi, and basal plate?	Term cesarean (n=34); term vaginal (n=23)	Amnion and chorion; villous tree; basal plate; processed within 12 hours of delivery	16S ribosomal RNA gene sequencing; quantitative real-time polymerase chain reaction	No	Blank extraction kits (n=8); water controls (n=5);operational taxonomic units detected in these negative controls removed from the data set; only placental samples that had a positive quantitative real-time polymerase chain reaction value (>34 16S ribosomal RNA gene copies/μL) were included in sequence data analyses
				Conclusions: There is a placental microbiota at term, regardless of mode of delivery; the placental microbiota differs between the amnion −chorion and the basal plate; there may not be a resident microbiota in the villous tree.				
12	Zheng et al[74]	2017	Does the placental microbiota differ between cases of fetal macrosomia and control subjects?	Term cesarean without fetal macrosomia (n=10); term cesarean with fetal macrosomia (n=10)	Villous tree; processed immediately on delivery	16S ribosomal RNA gene sequencing	No	The amplification of 16S rDNA from blank extraction kits did not generate noticeable bands of amplified DNA; reagents from these kit controls were not sequenced
				Conclusions: There is a placental microbiota at term; the placental microbiota differs between cases of fetal macrosomia and normal birthweight control subjects.				
13	Leon et al[75]	2018	Does the placental microbiota differ between preterm and term deliveries?	Term cesarean (n=81); term vaginal (n=84); preterm cesarean (n=55); preterm vaginal (n=36)	Placental parenchyma (n=356 samples); villous tissue (n=44 samples); processed on delivery	16S ribosomal RNA gene sequencing	No	A blank extraction kit was processed for each round of extractions (reagents from 19 kits that yielded ≥500 sequences were analyzed); operational taxonomic units with ≥2 reads in ≥2 kit controls were removed (excluding *Lactobacillus*, *Veillonella*, and *Mycoplasma*)
				Conclusions: There was large overlap between the bacterial profiles of placental samples and technical controls; however, there are microbial signals in term and preterm placentas; mode of delivery impacted the microbial profiles of placental tissues; although there was not a unique preterm placental microbiota, some bacteria (ie, *Ureaplasma* and *Mycoplasma*) were enriched in preterm placentas.				

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existence of a placental microbiota has become a majority view in perinatal microbiology at this time.

The limitations of molecular microbiologic techniques in low microbial biomass sites

Questions have emerged about the interpretation of microbiology studies based solely on sequencing techniques.[22,28–30] The detection of a nucleotide sequence from a bacterium or virus is not the same as the identification of a microorganism. These sequences can represent microbial breakdown products in the body (eg, DNA from dead microbes)[188] or background DNA contamination (eg, present in DNA extraction kits, PCR reagents, and laboratory environments).[22,30,31] Therefore, the demonstration of a microbiota requires (1) microbial signals beyond contamination, (2) reproducibility across methods (sequencing, qPCR, culture, and microscopic detection of the microorganisms in tissues, for example, through fluorescence in situ hybridization), and (3) ecologic plausibility.[22]

The microbial signals derived from the placenta are not distinguishable from those of technical controls

Lauder et al[32] sampled the placental tissues, vagina, and oral cavity of 6 women who delivered at term. For each woman, control samples included swabs waved in the air within the laboratory, sterile swabs, and blank extraction kits.[32] With the use of both qPCR and 16S rRNA gene sequencing, the bacterial profiles of placental tissues were not distinguishable from those of control subjects.[32] By contrast, the profiles of vaginal and oral samples differed from controls.[32] More recently, using 16S rRNA gene sequencing, de Goffau et al[22] showed that the microbial signals derived from placental tissues were largely due to the DNA extraction kits used. Additionally, in a recent sequence-based survey of targeted eukaryotic microbes in placental tissues, Lager et al[232] determined that sequenced DNA was due to technical artifacts and background DNA contamination, rather than a true signal of a placental microbiota. These studies highlight the need for addressing DNA contamination in sequence-based surveys and for using complementary techniques, such as cultivation.[22]

The findings of the study herein in the context of other reports

In this study, placental samples from 29 women who had a cesarean delivery at term without labor were examined for the presence of a placental microbiota. We included 72 background technical controls and used multiple complementary modes of inquiry: bacterial culture, 16S rRNA gene qPCR, 16S rRNA gene sequencing, and metagenomic surveys. Our results are consistent with those of Lauder et al,[32] de Goffau et al,[22] and Lager et al[232] in that we did not find evidence of a placental microbiota. The results are discussed later in detail.

Bacterial culture

The results of culture were negative. Only 1 of the placental cultures (3.4%) was positive, and the detected bacteria were *Bacillus circulans, Bacillus pumilus*, and *Brevibacterium casei*. *Bacillus* and *Brevibacterium* species are widespread bacteria that can be human commensals and opportunistic pathogens.[233–240] However, the 16S rRNA genes of the 3 cultured bacteria were not detected in the placental sample with the use of molecular techniques, which suggests that, in this study, these bacteria were laboratory contaminants. The congruence between the primers used in the primary nested PCR analysis (27F, 1492R) and the sequences of the bacterial cultivars is unknown because of the methods used to amplify the V4 region of their 16S rRNA genes. Nonetheless, the 16S rRNA genes of these bacteria had exact matches to the primers used in the secondary nested PCR analysis (341F/1061R; 515F/806R) and the primers used in the secondary standard and touchdown PCR analyses (515F/806R). Therefore, if *B circulans*, *B pumilus*, and *B casei* were present in the placental tissue sample, we should have detected their 16S rRNA gene sequences. In addition, this placenta, like others in this study, did not present severe/moderate acute inflammatory responses during the histopathologic examination.

qPCR

Consistent with Lauder et al,[32] qPCR analyses in this study indicated that placental tissue samples did not have a greater abundance of 16S rRNA gene copies than technical controls. Indeed, the abundances of 16S rRNA gene copies in both placental samples and control subjects were below the limit of detection in the qPCR assay.

16S rRNA gene sequencing

16S rRNA gene sequencing revealed similarity in the microbial profiles among placental tissues, blank extraction kits, biologic safety cabinets, and laboratory controls. In the primary 16S rRNA gene nested PCR analysis and the secondary 16S touchdown PCR analysis, the structures of the microbial profiles of placental tissues and technical controls did not differ. In the secondary 16S rRNA gene nested PCR analysis, the microbial profiles of placental tissues and controls were significantly different. However, 99% and 97.6% of the sequences obtained from placental tissues and controls, respectively, belonged to *Escherichia*. *Escherichia* was also widely present, although not highly abundant, in the primary 16S nested PCR analysis, the secondary 16S touchdown PCR analysis, and the secondary 16S standard PCR analysis in both the placental tissues and technical controls. *Escherichia*, especially *E coli*, has been identified previously as a principal member of the placental microbiota with the use of molecular surveys.[64,66,70] In a recent study, microbes were cultured from the fetal side of 20.7% (379/1832) of placentas obtained from cesarean deliveries at term without clinical chorioamnionitis; 13.5% of the placental samples (247/1832) yielded *E coli* cultures.[241] A valuable addition to that study would have been species-specific qPCR and/or 16S rRNA gene or metagenomic sequencing of the cultured placental samples to demonstrate that the absolute and relative abundances of *E coli* were indeed greater in samples that yielded *E coli* cultures than in those that did not.[241] This would provide verification of the culture results. In the current study, molecular signals of *Escherichia* were as widely distributed and relatively abundant among technical controls as among

TABLE 4
Genera indicated by linear discriminant analysis effect size as being more relatively abundant in placental tissues than technical controls

Data set	Genus	Ecologic and clinical description and the reported occurrence of the genus in previous sequence-based studies of the human placenta at term	Has the genus been documented as a DNA contaminant in previous sequence-based studies?
Primary 16S ribosomal RNA gene nested polymerase chain reaction: amnion and chorionic plate	*Achromobacter*	Generally aquatic and soil bacteria, but they can be infectious agents in immunocompromised hosts,[267,268] especially patients with cystic fibrosis[269]; *Achromobacter* was identified in 1 placental microbiota study at low abundance (0.05%)[74] and in 3 others at low, yet unreported, abundances.[66,68,72]	Yes[28]
	Blastococcus	Typically associated with rocks or marine environments[270,271]; an isolate was obtained from human stool[272]; *Blastococcus* was identified in 1 placental microbiota study at low abundance (<0.01%).[69]	Yes[29]
	Methylobacterium	Generally aquatic and soil bacteria, but they can be infectious agents in immunocompromised hosts[273]; *Methylobacterium* was identified in a previous placental microbiota study at low abundance (<0.01%).[69]	Yes[28,32,72]
	Caldalkalibacillus	Thermoalkaliphilic environmental bacteria[274,275]; *Caldalkalibacillus* has not been identified in previous studies of a placental microbiota.	No
Primary 16S ribosomal RNA gene nested polymerase chain reaction: villous tree and basal plate	*Achromobacter*	Generally aquatic and soil bacteria, but they can be infectious agents in immunocompromised hosts,[267,268] especially cystic fibrosis patients[269]; *Achromobacter* was identified in 1 placental microbiota study at low abundance (0.05%)[74] and in 3 others at low, yet unreported, abundances.[66,68,72]	Yes[28]
	Herbaspirillum	Typically found in soils, but can be opportunistic pathogens of immunocompromised hosts[276,277]; *Herbaspirillum* was identified in a previous placental microbiota study at a low, yet unreported, abundance.[68]	Yes[28]
Secondary 16S ribosomal RNA gene nested polymerase chain reaction: villous tree and basal plate	*Escherichia*	Common human commensals and opportunistic pathogens, including of the urogenital and reproductive tracts[278–280]; *Escherichia* was reported as a principal member of the placental microbiota in 3 studies,[64,66,70] at low abundances in 2 others (<0.05%),[67,69] and at a low, yet unreported, abundance in another.[68]	Yes[28,75]
Secondary 16S ribosomal RNA gene touchdown polymerase chain reaction: villous tree and basal plate	*Ralstonia*	Common environmental and aquatic bacteria that can be agents of nosocomial infections[281,282]; *Ralstonia* was widespread among placental samples in 2 previous studies,[70,73] at low abundance in a third study (<0.01%),[69] and at a low, yet unreported, abundance in a fourth study.[72]	Yes[22,28,57,75]
	Chthoniobacter	This genus has a single species, a soil bacterium[283,284]; *Chthoniobacter* was identified in a low, yet unreported, abundance in 1 previous study of a placental microbiota.[70]	No
	Anaerococcus	Human commensals that can be opportunistic pathogens, including in the urogenital tract[285–287]; *Anaerococcus* was identified in low abundance (0.02%) in 1 previous study[69] and present at low, yet unreported, abundances in 2 others.[68,75]	Yes[29]

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placental tissues, and *Escherichia* was not cultured from any of the placental tissues.

In addition to the community level analyses, LEfSe was used to identify OTUs more relatively abundant in placental tissues than in technical controls. Other than *Escherichia*, the identified genera have been detected in low relative abundance in only a few previous sequence-based studies of placental microbiota. Most of these bacteria are also considered common DNA contaminants in sequence-based studies (Table 4).

Metagenomic sequencing

The metagenomics data obtained in the current study were also consistent with DNA contamination that had a marked influence on the microbial profiles of placental tissues. Specifically, 63.4% of the bacterial sequences recovered from placental tissues came from *Cyanothece*, "Candidatus Phytoplasma," and *Chlorobium*. In a recent commentary that emphasized the effect of DNA contamination in microbiome studies, it was recommended that data from sequence-based investigations of low microbial biomass environments be interpreted through the lens of microbial ecology.[22] One example provided by the authors is to consider that sequence data indicating that photosynthetic bacteria inhabit internal organs in the human body ought to be questioned because residency in these organs precludes photosynthesis. *Cyanothece* is a photosynthetic cyanobacterium,[242] and *Chlorobium* is a photosynthetic green sulfur bacterium.[243] Furthermore, members of "Candidatus Phytoplasma" are obligate plant pathogens restricted to the phloem of plants and phloem-feeding insect vectors. Among the remaining 16 prominent ($\geq$0.1%) bacterial genera identified through metagenomic sequencing of placental tissues, there were aquatic bacteria (*Beggiatoa*, *Roseobacter*, *Hahella*, *Halangium*), additional plant pathogens (*Xanthomonas*, *Xylella*), and an algal symbiont (*Dinoroseobacter*). Therefore, it is unlikely that the human placenta is a suitable niche for these microorganisms.

Some metagenomic data warrant discussion. *Coprobacillus* represented 30.5% of the bacterial sequences identified in placental samples. Although *Coprobacillus* has been detected in 2 sequence-based studies of term and preterm placentas at low abundance,[66,75] this microorganism was not detected in any of our 16S rRNA surveys. Although the primers used to target the 16S rRNA gene in the first round of amplification in the primary and secondary nested PCRs (27F/1492R; 341F/1061R) were not an exact match for *C cateniformis* (Japan Collection of Microorganisms (JCM) 10604), the only member of the genus *Coprobacillus*,[244] the primers used for the secondary standard and touchdown PCRs (515F/806R) were a perfect match for this bacterium. Therefore, if *Coprobacillus* was present in placental tissues, and if its 16S rRNA gene sequence was similar to that of the lone characterized representative of this genus (ie, *C cateniformis*), we should have identified it in the standard 16S rRNA gene PCR and touchdown PCR analyses.

Streptomyces represented, on average, 1% of bacterial sequences obtained from placental tissues through metagenomic sequencing. Although *Streptomyces* was previously identified in placentas at term with the use of sequencing techniques,[64,66,69] in the current study, only 2 16S rRNA gene sequences in all the 16S surveys of placental tissues were assigned to *Streptomyces*. However, given that our 16S rRNA gene V4 primers (515F/806R) were perfect matches for 98.6% (580/588) of the type strains of *Streptomyces* included in the Ribosomal Database Project,[212] we should have detected these microorganisms more frequently in the standard 16S rRNA gene PCR and touchdown PCR analyses. Other bacterial genera (>0.1% average relative abundance) identified through metagenomics in this study and previously detected in sequence-based studies of placental tissues at term were *Neisseria*,[64,66] *Rhodococcus*,[64,67] *Clostridium*,[71] *Streptococcus*,[70,72] and *Burkholderia*.[66] Nevertheless, in the current study, sequences for these microorganisms were detected in all placental samples and in all background technical control samples. It is noteworthy that these sequences have been reported previously as DNA contaminants in sequence-based studies.[28,29,31] There is not sufficient evidence to conclude that bacterial signals identified through metagenomic sequencing represent evidence of a placental microbiota or bacterial ecosystem in this organ.

Similar to the placenta, there is a lack of evidence for an amniotic fluid microbiota in normal pregnancy at term without labor

Some studies claim that amniotic fluid is not sterile and has a microbiota similar to that of the placenta.[69,245] However, recent studies have shown that there is not an amniotic fluid microbiota in normal term pregnancies. For example, in a prospective investigation of 344 asymptomatic women between 15 and 22 weeks of gestation, amniotic fluid samples were negative for the presence of genital mycoplasmas, bacteria, or fungi with the use of species-specific and broad-range PCR techniques.[54] Furthermore, in a recent study of 10 women who underwent elective cesarean deliveries without labor, the bacterial loads (assessed through digital droplet PCR of the 16S rRNA gene) of amniotic fluid samples were comparable to those of background technical controls.[57] Also, these amniotic fluid samples did not yield bacterial isolates. Conversely, amniotic fluid samples from 14 women with previous rupture of membranes had bacterial loads 10 times higher than those of technical controls, and these samples yielded bacterial isolates 50% of the time.[57] In addition to these recent clinical investigations, a logical argument against the existence of either a placental or an amniotic fluid microbiota is the generation of germ-free mammals through sterile cesarean delivery and germ-free technology (ie, incubators, water, food).[63,246] This has also been extended to a human infant affected by severe combined immunodeficiency syndrome.[246–249]

The lack of a microbiota in the placenta or amniotic fluid does not exclude fetal exposure to microbial products

The absence of a resident microbiota in the placenta or amniotic fluid does not rule out exposure of the fetus to microbial metabolites. Using germ-free

pregnant mice transiently gestational-colonized with *E coli* HA107 (a genetically engineered bacterium[250]), Gomez de Agüero et al[251] showed that microbial metabolites are transferred from the mother to the fetus through the placenta. Yet, no live microorganisms were found in the placenta or the offspring.[251] Fetal exposure to microbial metabolites from the mother was mediated, in part, by antibodies, given that antibody-deficient dams ($J_H^{-/-}$ mice) had a reduced concentration of such microbial products.[251] These microbial metabolites shaped the innate immune system of the offspring as evidenced by an increased number of intestinal group 3 innate lymphoid cells (ILC3s) and macrophages in neonates born to transiently gestational-colonized dams.[251] Of interest, fetal ILC3s are present in the amniotic cavity where they seem to participate in the host defense mechanisms against microbial invasion.[252,253] In addition, Gomez de Agüero et al showed that neonates born to transiently gestational-colonized mothers had an enhanced ability to clear bacteria,[251] which suggests that microbial metabolites of the mother influence the innate immune fitness of the offspring.

Strengths of the current study

First, in our attempt to determine whether there is a placental microbiota during normal pregnancy, we limited our investigation to women who delivered at term without labor. Thereby, we avoided the introduction of bacteria into the amniotic cavity during labor at term.[157,254] Second, we used samples collected after cesarean delivery to prevent microbial colonization or 16S rRNA gene contamination of placental tissues during vaginal delivery.[75] Third, we excluded placentas from preterm gestation, given that molecular surveys have identified a potential placental microbiota linked to preterm delivery.[64,65,70,75] Fourth, we used multiple modes of inquiry: bacterial culture, 16S rRNA gene qPCR, 16S rRNA gene sequencing, and metagenomic surveys. We further bolstered our initial 16S rRNA gene sequencing analyses with secondary analyses using alternative amplicon-library generation techniques. Each approach used in this study has its own strengths and weaknesses; however, these approaches are complementary, and their ultimate agreement herein provided a more robust conclusion than any of them could have provided in isolation. Fifth, we included thorough controls for potential background DNA contamination that included conducting numerous extractions without any biologic template, extractions after exposure to circulating air within our biologic safety cabinets, and extractions after exposure to our broader operating rooms and laboratory environments. Importantly, we incorporated the sequence data from these background technical controls into graphic and statistical analyses.

Limitations of the current study

First, all subjects necessarily received intraoperative antibiotic prophylaxis, typically cefazolin, at cesarean delivery, so we cannot rule out a subsequent inhibitory influence on cultivation results.[192,194,255–262] Second, for part of the study, we used nested PCR, an approach that can facilitate the amplification of the very low concentrations of bacterial DNA present in relation to the high background concentrations of host DNA.[196,197] Given that nested PCR entails 2 separate rounds of amplification, it can increase the likelihood of amplification bias and thereby promote similarity among characterized bacterial profiles.[196,263] However, this would require that characterized samples contain the same preferentially amplified gene variants. In our primary 16S rRNA gene analysis, our sample coverage was thorough, and we amplified and characterized 16S rRNA gene variants from numerous genera previously identified in molecular surveys of placental tissues (ie, *Acinetobacter*, *Actinomyces*, *Bacillus*, *Bacteroides*, *Burkholderia*, *Clostridium*, *Corynebacterium*, *Enterococcus*, *Escherichia*, *Fusobacterium*, *Lactobacillus*, *Lactococcus*, *Mycobacterium*, *Neisseria*, *Prevotella*, *Propionibacterium*, *Pseudomonas*, *Rhodococcus*, *Staphylococcus*, *Streptococcus*, and *Ureaplasma*).[64,66,67,69–73] However, no gene variants from these genera were more widely distributed among placental tissues than among background technical controls. Also, we followed up this primary analysis with secondary analyses, in which we used a second, highly conserved primer pair for the first round of amplifications in nested PCR and additionally used standard PCR and touchdown PCR approaches. We further complemented these approaches with metagenomic surveys of placental tissues, which minimize amplification bias.[264] Third, we focused exclusively on bacteria; eukaryotic pathogens and viruses were not targeted. Fourth, our study did not use morphologic techniques, such as fluorescence in situ hybridization,[265] to visualize bacterial cells in these placental tissues. However, using fluorescence in situ hybridization, we did not detect bacteria in the placental tissues of a different set of women who had elective cesarean deliveries at term without labor (Alexander Swidsinski, Universitätsmedizin Berlin, written communication). Fifth, a valuable negative control here would have been extraction of alternative presumed sterile human tissues, thereby controlling for any potential influence of competition between host and microbial DNA during extraction and amplification processes. Nevertheless, the specific kit we used to perform extractions and the masses of placental tissues on which extractions were conducted were consistent with previous studies that investigated the existence of a placental microbiota.[32,64] A positive control would have been extraction of alternative human tissues with a confirmed very low microbial biomass, such as the lung.[216,217] Such negative and positive control tissues would require the use of animal models. An alternative approach would be to include placental tissue samples spiked with known numbers of bacterial cells to ascertain the specific limits of microbial detection in the study.[22,266]

Criteria to establish the presence of a resident microbiota in low biomass sites such as the placenta

A fundamental question that emerges from the debate about the existence of a

unique placental microbiota is: what are the requirements to demonstrate the presence of such a microbial ecosystem? The existence of a microbiota would be supported by the following evidence: (1) identification of microbial DNA sequences in tissues or fluids through multiple modes of inquiry, such as 16S rRNA gene sequencing and metagenomics, and the profiles of these microbial DNA sequences are distinct from those detected in technical controls (eg, DNA extraction kits, PCR reagents, laboratory environments), (2) confirmation of microbial burden through qPCR, (3) demonstration of the viability of the microorganisms, either through culture or the transcription of specific microbial genes, (4) visualization of the microorganisms in tissues or fluids with the use of microscopic techniques, such as fluorescence in situ hybridization with eubacterial or, ideally, species-specific probes, and (5) residency of the microorganisms in the tissues or fluids is ecologically plausible in that they are likely to survive in the niche in which they have been found.

Conclusions

Through multiple modes of microbiologic inquiry, we did not find consistent and reproducible evidence of the existence of a placental microbiota at term. We have not shown definitively that microbes do not inhabit the placentas of term pregnancies; it is difficult to prove the null hypothesis, and there are limits of detection inherent in the contemporary survey techniques we used. However, using multiple investigative approaches, incorporating technical controls, and focusing on placental tissues that were obtained through cesarean delivery at term without labor, we detected no consistent evidence for bacterial communities in the placental tissues beyond the signals also present in the technical controls. This study bolsters the arguments for the necessity of substantively incorporating technical control samples into studies of very low microbial biomass,[22,30] such as those targeting a placental microbiota,[22,32,75] and for starting with the null hypothesis that microbial signals in these biologic samples are background DNA contamination.[22,30,32,63] The optimization of cultivation techniques, in concert with molecular survey approaches, will be important in the evaluation of the existence of a microbiota in low microbial biomass body sites.[22,63,189] ■

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Glossary

Human microbiota

Microbiota—The assemblage of microbes present in a defined environment.

Microbiome—Microbiome has 2 common definitions. It can refer to the genomic content of a microbiota or, more comprehensively, to a habitat, its microbiota, and the genomic content of this microbiota.

Microbial alpha diversity

Alpha diversity—Diversity within a single microbial community. In this study, we characterized alpha diversity through 2 indices, Chao1 and Simpson. The Chao1 index estimates the richness of microbial communities; the Simpson Index estimates their heterogeneity.

Richness—Number of different microbial types (eg, species) in a mixed microbial community.

Chao1 index—An estimate of microbial community richness. It is calculated as $S_{obs}+(F_1^2/2F_2)$, wherein S_{obs} is the number of species in the sample, F_1 is the number of species that are singletons, and F_2 is the number of species that are doubletons. A singleton is any species occurring only once in a sample; a doubleton is any species occurring twice in a sample.

Heterogeneity—A measure of microbial community alpha diversity that takes into account both the richness and evenness (ie, relative abundances) of community members.

Simpson index—An estimate of microbial community heterogeneity. It is calculated as $\sum (n_i [n_i-1]/N[N-1])$, wherein n_i is the number of individuals in the i^{th} species, and N is the total number individuals sampled.

Microbial beta diversity

Beta diversity—Diversity between 2, or among multiple, microbial communities. In this study, we characterized beta diversity through 2 indices, Jaccard and Bray-Curtis. The Jaccard index describes the composition of microbial communities. Specifically, it describes the extent to which 2 communities share the same species. The Bray-Curtis index describes the structure of microbial communities. It describes not only the extent to which 2 communities share the same species, but also the extent to which the species they do share are present in the same relative abundances in the 2 communities.

Jaccard index—A measure of similarity in composition (ie, shared species membership) between 2 microbial communities. It is calculated as a/(a+b+c), wherein "a" is the number of shared species between the 2 communities; "b" is the number of species unique to the first community, and "c" is the number of species unique to the second community.

Bray-Curtis index—A measure of similarity in structure (ie, shared species membership and relative abundances of shared species) between 2 microbial communities. It is calculated as $2W/(N_1+N_2)$, wherein W is the sum of the lower values of the 2 abundances for species shared between the 2 communities; N_1 is the number of individuals sampled in the first community, and N_2 is the number of individuals sampled in the second community.

Characterizing microbial diversity through 16S ribosomal RNA (16S rRNA) gene sequencing

16S rRNA gene—A housekeeping and phylogenetic marker gene present in all bacteria. It is critical in protein manufacturing and therefore is highly conserved. Nevertheless, it has regions of hypervariability. The conserved regions of the gene evolve slowly and therefore can serve as targets for polymerase chain reaction (PCR) primers, although the hypervariable regions afford researchers information on the evolutionary relationships among bacterial types. 16S rRNA gene surveys are used very commonly to characterize the bacterial types (eg, genera) within mixed bacterial communities in clinical and environmental samples.

16S rRNA gene survey—Characterization of mixed bacterial communities in samples based on patterns in the presence and/or relative abundance of variants of the 16S rRNA gene, a phylogenetic marker gene present in bacteria.

Mothur—A software program providing quality filtering, alignment, clustering, and taxonomic classification of DNA sequence reads, such as variants of the 16S rRNA gene. Clustering entails grouping sequence reads into operational taxonomic units (OTUs) based on their percent nucleotide similarity.

OTU—A group of DNA sequence reads, for example of the 16S rRNA gene, that share a certain percent nucleotide similarity (eg, 97%). OTUs are generally viewed as bacterial types or variants. OTUs commonly are used in microbiome studies because many 16S rRNA gene variants amplified and sequenced from mixed microbial communities cannot be confidently assigned to taxa with a high degree of resolution (eg, genus or species identity).

Good's coverage—An estimate of the extent to which a microbial community has been sufficiently sampled. With respect to next-generation sequencing surveys, for each community, Good's coverage is calculated as (1−[#singleton OTUs/#total sequences for sample])×100%. It reveals the percentage of sequence reads in a sample that were not in singleton OTUs; a higher percentage indicates higher sample coverage.

Singleton—An OTU represented by only 1 sequence read. A read is a single sequenced amplicon of the targeted gene (eg, 16S rRNA gene).

Doubleton—An OTU represented by only 2 sequence reads.

Nested PCR—A modified PCR approach aimed at reducing nonspecific amplification and increasing recovery of target amplicons, for example amplicons of the 16S rRNA gene. In the first round of PCR, a large gene fragment is targeted for amplification. In the second round of PCR, a smaller gene fragment within this larger fragment is targeted for amplification. The second round of PCR selects against nonspecific amplicons and promotes targeted gene products.

Touchdown PCR—A modified PCR approach aimed at reducing the initial amplification of nonspecific sequences during early steps of amplification by using a relatively high primer annealing temperature in relation to the melting point of the primers. As cycling proceeds, the annealing temperature is decreased incrementally, which allows for increased amplification efficiency. The increased initial specificity of the reaction at higher annealing temperatures permits amplification of sequences with the greatest primer specificity to outcompete amplification of nonspecific sequences as cycling proceeds at lower annealing temperatures.

Characterizing microbial diversity through metagenomic sequencing

Metagenomics survey—Characterization of bacterial communities in samples based on patterns in the presence and/or relative abundance of all genes of bacterial origin. In contrast to surveys based on phylogenetic marker genes, like the 16S rRNA gene, all genomic DNA in samples is sequenced. Those sequences determined to be of bacterial origin are classified taxonomically, often times even at the species level, and the metabolic and functional potential of sampled mixed bacterial communities can be characterized.

Meta Genome Rapid Annotation using Subsystem Technology (MG-RAST)—An analysis server for quality filtering, taxonomically classifying, and functionally annotating and comparing metagenomic datasets.

Quantifying microbial abundance through quantitative real-time PCR

Quantitative real-time PCR—A molecular technique that monitors the amplification of a targeted gene (eg, 16S rRNA gene) in real-time, across multiple cycles of PCR. In this study, it was used to compare the relative abundances of 16S rRNA gene copies in placental and background technical control samples.

Cycle of quantification (Cq)—In quantitative real-time PCR, the cycle number at which a sample's amplification curve exceeds a predefined minimum threshold based on background fluorescence levels. It is the point at which the signal from the sample has exceeded a baseline level for the assay. The more abundant the targeted gene is within a sample, the lower the sample's Cq value will be.

Degenerate primer—A PCR primer sequence in which ≥1 nucleotide positions has several possible bases. It enables capturing variation in nucleotide combinations for a target gene (eg, 16S rRNA gene) within mixed microbial communities.

Supplemental Methods

Section 1: bacterial cultivation and characterization of resultant isolates

Placental tissues were homogenized with the use of a Covidien Precision Disposable Tissue Grinder (3500SA; Minneapolis, MN) and plated via an inoculating loop on 3 growth media (trypticase soy agar with 5% sheep blood, chocolate agar, MacConkey's agar) at 35°C under anaerobic (5% CO_2, 10% H_2, 85% N_2) and aerobic (8% CO_2) conditions for at least 4 days. A genital mycoplasma cultivation assay (Mycofast US, Logan, UT) was also conducted for each placental sample.[1] Characterization of the taxonomic identities of resultant isolates was done with Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF)[2] within the Detroit Medical Center Microbiology Laboratory. Submission of cesarean-delivered placental tissues from 1 subject who delivered at term, spiked with an innocuous *Escherichia coli* strain (ATCC 25922), to whose presence the Microbiology Laboratory personnel were blind, yielded growth on anaerobic and aerobic growth media.

Genomic DNA was extracted from resultant bacterial isolates with an UltraClean Microbial DNA Isolation kit (MoBio Laboratories, 12224, Carlsbad, CA) according to the manufacturer's protocol. The 16S rRNA gene was amplified from purified DNA with the 27F/1492R primer set and bidirectionally sequenced with the 515F/806R (V4 hypervariable region) primer set. Forward and reverse reads were trimmed with the use of DNA Baser software (http://www.dnabaser.com/) with default settings. Contiguous sequences were assembled using CAP, a contig (ie, overlapping sequence reads) assembly program in BioEdit software (v7.2.5; http://www.mbio.ncsu.edu/BioEdit/bioedit.html), with default settings. To screen for the presence of each bacterial isolate in its respective placental tissue sample, the 16S rRNA gene sequence of each isolate was trimmed to exclude the 515F/806R primer regions, and identical sequences for each bacterial isolate were identified by searching for similarity through BLAST[3] against a database of that placenta sample's 16S rRNA gene-V4 sequences. These sequences were generated before the preclustering step in the mothur protocol described later.

Section 2: polymerase chain reaction (PCR) primers used in assays for the amplification of 16S rRNA genes in placental tissue and background technical control samples

Primers for nested PCR round 1a and 1b refer to the study's primary and secondary analyses, respectively. A "+" precedes the position of a locked nucleic acid base.

Section 3: results of sequencing libraries for placental tissues and background technical controls with the use of standard PCR protocols, as described in the general Methods section

Sequencing of sample libraries generated with the use of only 30 cycles of standard PCR and 5 μL of template DNA yielded 13,064 sequences. With a 97% sequence similarity cutoff for operational taxonomic units (OTUs), there were 199 OTUs, with 29 of these being singletons. With the removal of OTUs present in background technical control samples,[4] there remained 33 OTUs in the placental samples. These 33 OTUs constituted 187 sequences and included 13 singletons and 3 doubletons. Thirty-three (56.9%) placental samples had no data remaining, and only 5 samples (8.6%) had $\geq$10 sequences remaining. Thirty of the 33 remaining OTUs were found in only a single placental sample. The other 3 OTUs were found in 2 placental samples each. These OTUs were *Herbaspirillum*, *Cupriavidus*, and *Brevibacillus*. All 3 genera are soil and environmental bacteria that have been implicated as contaminants in 16S rRNA gene sequencing surveys.[5,6]

No placental samples (0/58) and only 4 of 43 control samples, each of which was a room sample, yielded a library with at least 500 quality-filtered sequences and a Good's coverage value of 97%. Only 10 placental (17.2%; each an amnion and chorionic plate sample) and 31 control (72.1%) samples yielded a library with at least 100 quality-filtered sequences with a Good's coverage value of at least 97%. Among these samples, there was no evident difference in the composition (Jaccard; nonparametric multivariate analysis of variance; F=0.896; *P*=.694) or structure (Bray-Curtis; =0.812; *P*=.651) of 16S rRNA gene profiles between placental and technical control samples (Supplemental Figure 1). OTU029, an *Escherichia*, was present in every placental and technical control sample. OTUs 2, 8, and 12 (*Delftia*, *Staphylococcus*, and *Acinetobacter*, respectively) were also found in at least one-half of the placental and background technical control samples. These 4 genera have been implicated as contaminants in next-generation sequencing surveys.[5,6] OTU001, an *Achromobacter*, was also present in at

Assay	Forward primer (5′−3′)	Reverse primer (5′−3′)	Probe (5′−3′)
V4 region PCR	515F: GTGCCAGCMGCCGCGGTAA	806R: GGACTACHVGGGTWTCTAAT	Not applicable
Nested PCR round 1a	27F: AGAGTTTGATCMTGGCTCAG	1492R: ACGGCTACCTTGTTACGACTT	Not applicable
Nested PCR round 1b	341F: CCTACGGGNGGCWGCAG	1061R: CRRCACGAGCTGACGAC	Not applicable
Quantitative real-time PCR	27F: AGAGTTTGATCMTGGCTCAG	357R: CTGCTGCCTYCCGTAG	BSR65/17: 56FAM-TAA+YA+CATG+CA+AGT+CGA-BHQ1

least one-half of the placental samples. *Achromobacter* species are widespread environmental bacteria and can also be opportunistic nosocomial pathogens.[7,8] It was present in 32.3% (10/31) of the background technical control samples.

Section 4: processing of 16S rRNA gene sequence data with mothur software

Quality-filtered sequences (<300 base pair [bp], no ambiguous base calls, and homopolymer runs ≤8 bp) were aligned to the SILVA 16S rDNA reference database (release 102), and sequences falling outside the target alignment space were removed.[9,10] The average length of all bacterial sequences in the final dataset was 253 bp. A preclustering step (differences (diffs)=2) was performed to reduce the potential impact of sequencing errors, and chimeras were identified via UCHIME[11] and removed. The remaining sequences were classified taxonomically with the SILVA reference database with a k-nearest neighbor approach and a bootstrap cutoff of 80%, and sequences that were derived from an unknown domain, Eukaryota, Archaea, chloroplasts, or mitochondria were removed. OTUs were defined by clustering sequences at dissimilarity levels of 3% and 1% by cluster.split (splitmethod=classify; taxlevel=4; cutoff=0.20). Representative sequences for OTUs were classified with the use of the SILVA reference database.

Section 5: quantitative real-time PCR (qPCR) of 16S rRNA genes

All qPCR reactions were performed in duplicate (20 μL each), with each reaction containing 0.6 μM of 27f-CM primer, 0.6 μM of 357R primer, 0.25 μM of BSR65/17 probe, 10.0 μL of 2X TaqMan Environmental Master Mix 2.0 (Life Technologies, Carlsbad, CA), and 4.0 μL purified DNA. All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Thermocycling was initiated by a 10-minute incubation at 95°C. Reactions were run for 45 cycles (94°C for 30 seconds; 50°C for 30 seconds; 72°C for 30 seconds) to ensure amplification; fluorescent readings were taken at the end of each cycle on an ABI 7500 Real-Time PCR System (Applied Biosystems, Waltham, MA). Raw amplification data were normalized to the ROX passive reference dye and analyzed with Standard Curve 3.3.0-SR2-build15 (Thermo Fisher Cloud), with a threshold setting of 0.05 and the automatic baseline setting.

Quantification cycle values, defined as the average number of cycles required for normalized fluorescence (Rn) to exponentially increase, were calculated.

DNA derived from *Escherichia coli* ATCC 25922 that contained 7 16S rRNA gene copies per genome (GenBank accession: CP009072) was quantified with the use of a Qubit 3.0 fluorometer with a Qubit dsDNA HS Assay kit (Life Technologies, Carlsbad, CA) and used for the generation of standard curves. The standard curves were used to evaluate the performance of the qPCR assay by estimation of its efficiency based on the slope of regression lines and to assess the dynamic range and sensitivity of the assay based on standard deviation of replicate dilution samples according to Svec et al.[12] Additionally, concentrations of 16S rDNA gene copies in placental and control samples were estimated relative to standard curve concentrations. To assess the dynamic range and sensitivity of the qPCR assay, a narrow-range standard curve that contained 8 2-fold serial dilutions (5 replicates each) that ranged from 2.01×10^4 to 1.57×10^2 copies was included in a single run. To estimate qPCR efficiency and determine the concentration of 16S rDNA copies in placental and control samples, 1 of 3 independently diluted broad-range standard curves that contained 6 10-fold serial dilutions (2 replicates each) that ranged from 2.82×10^6 to 2.82×10^1 copies, 2.12×10^6 to 2.12×10^1 copies, or 2.97×10^6 to 2.97×10^1 copies was included in each run.

In preliminary observations in our laboratory, we noted inhibition of qPCR-amplification of bacterial DNA in placental samples that resulted from the carryover of unknown inhibitors and/or nonspecific amplification. To address this issue, we performed a qPCR assay on 4 3-fold dilution series of *E coli* DNA-spiked (5.7×10^3 16S rDNA copies) genomic placental DNA obtained from 2 amnion and chorionic plate samples and 2 villous tree and basal plate samples. Additionally, both spiked and unspiked nuclease-free water (Promega, Madison, WI) samples were included in the assay to allow for comparison of differences in amplification of placental and non-placental samples. This preliminary experiment indicated that modest inhibition was evident in the most concentrated placental samples (Figure 3, A). We therefore diluted placental DNA samples by a factor of 1:9 before qPCR analysis.

Section 6: metagenomic sequencing of placental and technical control samples

DNA was fragmented to 500 bp with standard Covaris sonication (Covaris, Woburn, WA). Fragmented DNA was then prepared as a standard Illumina library with the use of IntegenX (now WaferGen, Fremont, CA) reagents on the Apollo 324 instrument; fragments were end repaired, A-tailed, and adapter ligated. The samples were PCR amplified for 10 cycles and pooled. Library preparation for placental samples was effective, but not for control samples as was expected. Therefore, for control samples, a high sensitivity ThruPlex DNA-Seq kit (Rubicon Genomics, R400428; Ann Arbor, MI) and 17 amplification cycles were used for library preparation. Final libraries were checked for quality and quantity by TapeStation (Agilent, Santa Clara, CA) and qPCR using Kapa's library quantification kit for Illumina Sequencing platforms (KK4835, Kapa Biosystems, Wilmington, MA). The libraries were clustered on the cBot (Illumina) and sequenced on an Illumina HiSeq 4000, 150 paired-end run, according to manufacturer's protocols.

Section 7: metagenomic sequence data processing with the use of MG-RAST

The 20 metagenomic sequence libraries were submitted to the MG-RAST metagenomes analysis server at the Argonne National Laboratory.[13] Forward and reverse reads were combined into joined paired-end reads, as applicable, yet those

with nonoverlapping paired-ends were retained as well. Default pipeline parameter options were used: assembled (no), dereplication (yes), and screening (*Homo sapiens*, NCBI v36). Dynamic trimming was also set to default, except lowest quality base phred score was set to 10, and sequences were trimmed to contain at most 7 bases with a phred score of 10.[14] Reads >2 standard deviations from the mean read length were discarded, as were poor quality and artificial duplicate reads.[13] As the final processing step, sequences were screened for host DNA, in this case those that matched *Homo sapiens*, via NCBI v36. SortMeRNA[15] was used to identify rRNA genes with a 70% identity cut-off; CD-HIT[16] was used to cluster those with a 97% nucleotide similarity. The longest representative from these clusters was run through a BLAST-like alignment tool[17] similarity search against the M5rna database for rRNA identification.[13] Sequences of potential protein coding regions were identified via FragGeneScan,[18] and predicted protein coding sequences were clustered at 90% identity with CD-HIT and run through a BLAST-like alignment tool search against the M5NR protein database. Protein features were excluded if they overlapped with ribosomal RNA features. Identified rRNA and protein sequences were annotated and mapped back to the original sequences. Taxonomic assignments were made with the use of the GenBank database and the default MG-RAST parameters: maximum e-value cutoff of 5, minimum percent identity cutoff of 60%, minimum alignment length cutoff of 15, minimum abundance of 1, and representative-hit classification.[13] For gene function characterization, sequences were mapped to the KEGG Orthology database.[19]

Supplemental Results

Section 1: distribution of operational taxonomic units (OTUs) that were prominent (ie, average relative abundance ≥1%) among placental samples but not among background technical controls

Fourteen of the 18 prominent OTUs among placental samples also had an average relative abundance ≥1% among technical control samples (Figure 2). The exceptions were OTUs 12, 19, 27, and 45. OTU012 was an *Acinetobacter* found in 53.5% (23/43) of technical control samples with an average relative abundance of 0.26%. OTU019, an *Ureaplasma*, constituted 60.9% and 7.3% of the sequences from subject 20's amnion and chorionic plate and villous tree and basal plate samples, respectively. Its abundance in both suggests the possibility of asymptomatic presence of this bacterium. However, this subject was negative for *Ureaplasma* culture, and the cycle of quantification (Cq) values from quantitative real-time PCR for this subject were not uniquely low compared with other placental samples (11th and 41st lowest of 58 for amnion and chorionic plate and villous tree and basal plate samples, respectively). OTU019 was found in 11 of 56 of the remaining placental samples and in 5 of 43 controls, but at <0.006% abundance in each case. OTU027, a *Paracoccus*, was extremely abundant (94.9%) in 1 placental sample (3AC), was abundant in another (4.4% in 18V), and was present but rare (<0.008%) in 10 of 56 of the remaining placental samples and 5 of 43 of the technical controls. OTU045, a *Propionibacterium*, was very abundant in 3 placental samples (95.3% in 1AC, 84.2% in 10V, and 62.3% in 25AC). It was present in 11 of 55 of the remaining placental samples and 15 of 43 of the controls, but at <1% abundance in each case. Although not a prominent OTU, as defined by ≥1% average relative abundance among placental samples, *Escherichia* (OTU029) was found in 94.8% (55/58) and 86% (37/43) of placental samples and technical controls, respectively, but in all but 5 instances at a relative abundance of <1%.

Section 2: distribution of OTUs more abundant in placental samples than background technical controls, as determined by linear discriminant analysis effect size

OTU001, an *Achromobacter*, was more prevalent in both amnion and chorionic plate and villous tree and basal plate samples than in technical controls (Supplemental Figure 3). However, this OTU was present in every placental and technical control sample, often abundantly so. OTUs 10, 47, and 144 (*Methylobacterium*, *Blastococcus*, and *Caldalkalibacillus*, respectively) were more relatively abundant in amnion and chorionic plate samples than in technical controls. OTU010 was among the core OTUs (ie, present in at least one-half of samples) for control samples. OTU047 was present in 27.6% (8/29) of amnion and chorionic plate samples, but at a relative abundance of <1% except for a single sample (13.9% in 22AC). It was present in 9.3% (4/43) of technical controls. OTU144 was present in 24.1 (7/29) and 7.0% (3/43) of amnion and chorionic plate and technical control samples, respectively, however, always at a relative abundance <1%. In addition to OTU001, OTUs 285 and 23 (unclassified Burkholderiales and *Herbaspirillum*) were more relatively abundant in villous tree and basal plate samples than in technical controls (Supplemental Figure 3). OTU285 was present as a single sequence in 3 villous tree and basal plate samples but was not found in any control samples. OTU023 was present in 31.0% (9/29) and 9.3% (4/43) of villous tree and technical control samples, respectively. However, outside of 1 sample (56.3% of sequences in 17V), it was at a relative abundance <0.01%.

Section 3: distribution of core OTUs (ie, present in at least one-half of samples) among placental samples and background technical controls

Every core OTU in amnion and chorionic plate and villous tree and basal plate samples was also a core OTU in hood and blank extraction kit controls. Only 1 core OTU among placental samples was not a core OTU among room control samples, and this OTU (OTU010) was present in 47.6% (10/21) of room control samples. Notably, OTU029, an *Escherichia*, was a core OTU in every placental and technical control sample group. OTU015, an unclassified Proteobacteria, was a core OTU in amnion and chorionic plate samples but not in villous tree and basal plate samples. OTU007, a *Methylobacterium*, was a core

OTU in villous tree and basal plate samples but not in amnion and chorionic plate samples. Both of these OTUs were core OTUs in all 3 technical control sample groups.

Supplemental Data Legend

Supplemental data 1

Raw count data for operational taxonomic units, with the use of both 97% and 99% nucleotide similarity cutoffs, detected in placental tissues and background technical controls in the primary 16S rRNA gene analyses for this study.

Supplemental data 2

Raw count data for placental tissue and background technical control metagenomic sequences at the genus level.

Supplemental data 3

Raw count data for operational taxonomic units, with the use of both 97% and 99% nucleotide similarity cutoffs, detected in placental tissues and background technical controls in the secondary 16S rRNA gene analyses for this study.

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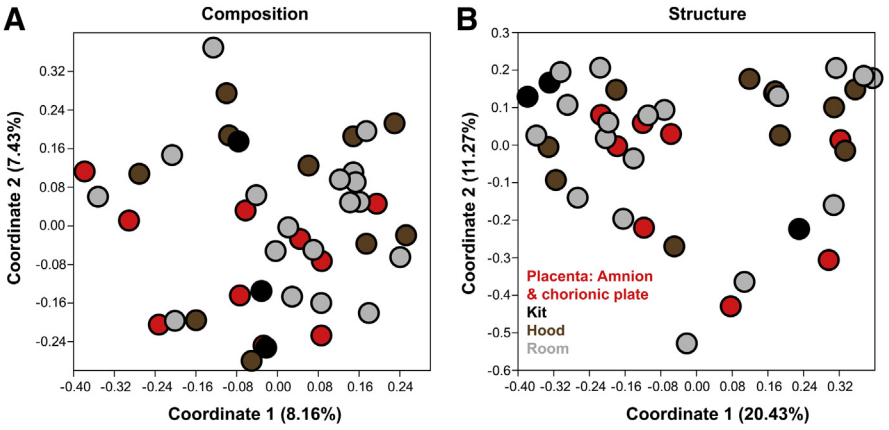
SUPPLEMENTAL FIGURE 1

Principal coordinates analyses illustrating similarity among placental and technical control samples that yield at least 100 sequences and have a Good's coverage value exceeding 97% via standard polymerase chain reaction approaches with 30 cycles



A, Plot of similarities in composition of 16S ribosomal RNA gene profiles among placental and control samples based on the Jaccard index (nonparametric multivariate analysis of variance; F=0.896; *P*=.694), **B,** Plot of similarities in structure of 16S ribosomal RNA gene profiles among placental and control samples based on the Bray-Curtis index (F=0.812; *P*=.651). Operational taxonomic units were generated with a 97% sequence similarity cutoff.

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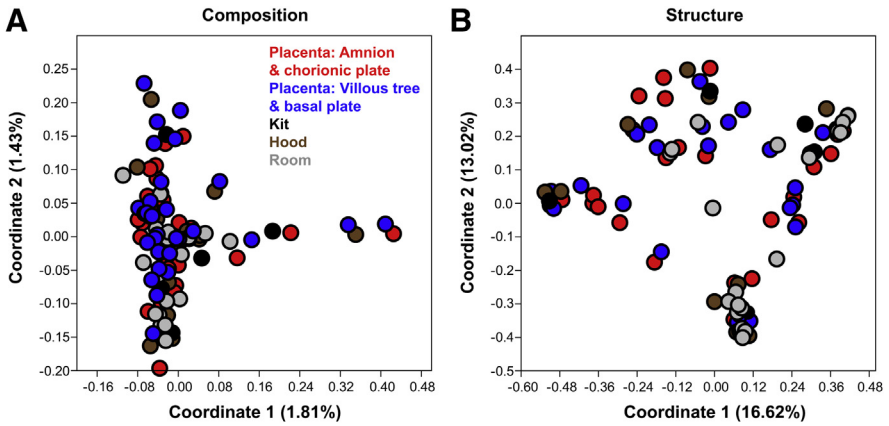
SUPPLEMENTAL FIGURE 2

Principal coordinates analyses illustrating similarity among amnion and chorionic plate, villous tree and basal plate, and technical control samples



A, Plot of similarities in composition of 16S ribosomal RNA gene profiles among placental and control samples based on the Jaccard index. **B,** Plot of similarities in structure of 16S ribosomal RNA gene profiles among placental and control samples based on the Bray-Curtis index. Operational taxonomic units were generated with a 99% sequence similarity cutoff and the primary 16S ribosomal RNA gene nested polymerase chain reaction data set.

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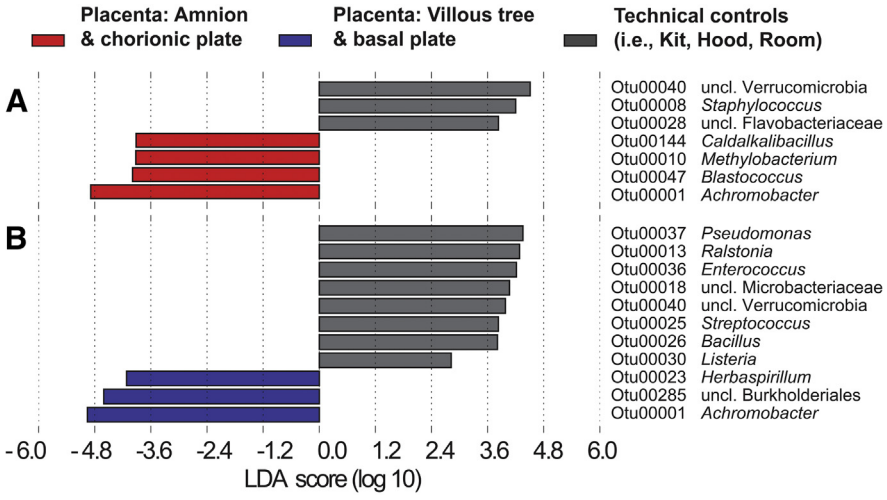
SUPPLEMENTAL FIGURE 3

Linear discriminant analysis effect size analyses that indicate differential relative abundance of operational taxonomic units between placental samples and technical controls



Operational taxonomic units were generated with a 97% sequence similarity cutoff and the primary 16S ribosomal RNA gene nested polymerase chain reaction data set. All plotted operational taxonomic units were distributed differentially between placental and technical control samples ($P<.05$). Panels show differential distribution of operational taxonomic units between **A,** amnion and chorionic plate samples and all controls and **B,** villous tree and basal plate samples and all controls.

LDA, linear discriminant analysis; *Otu*, operational taxonomic units; *uncl.*, unclassified.

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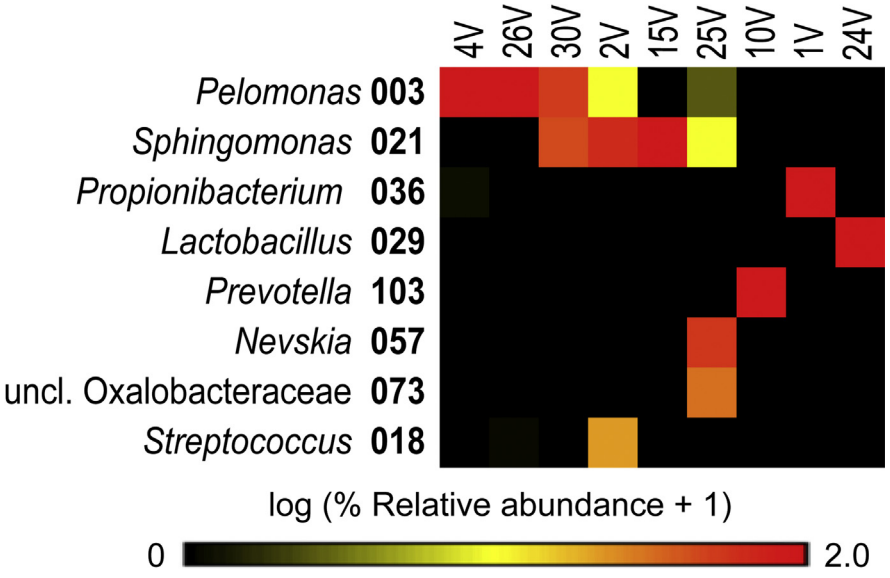
SUPPLEMENTAL FIGURE 4

Heat map illustrating relative abundances of prominent operational taxonomic units among 9 placental samples profiled with the use of 40 cycles of standard polymerase chain reaction



Prominent operational taxonomic units were defined as those having an average relative abundance ≥1.0% among the placental (*V* indicates villous tree and basal plate) samples.

uncl., unclassified.

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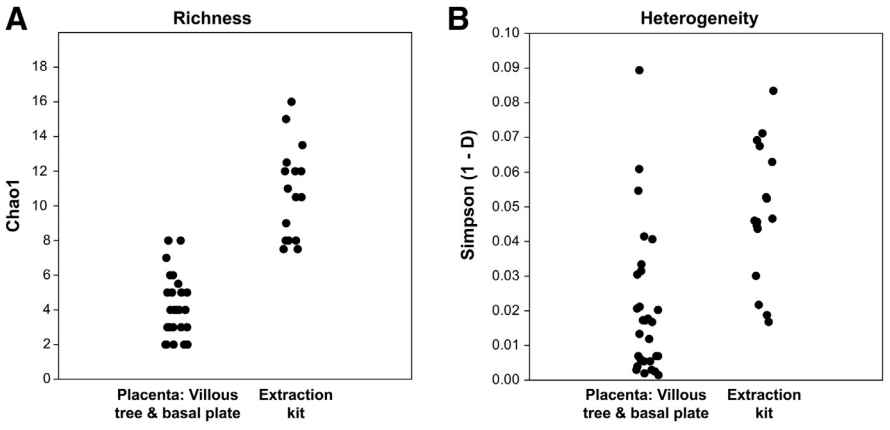
SUPPLEMENTAL FIGURE 5

Plot of richness and heterogeneity differences between placental samples and blank extraction kit controls profiled via the secondary nested polymerase chain reaction analysis



A, Richness based on the Chao1 index, **B,** Heterogeneity based on the Simpson (1-D) index.

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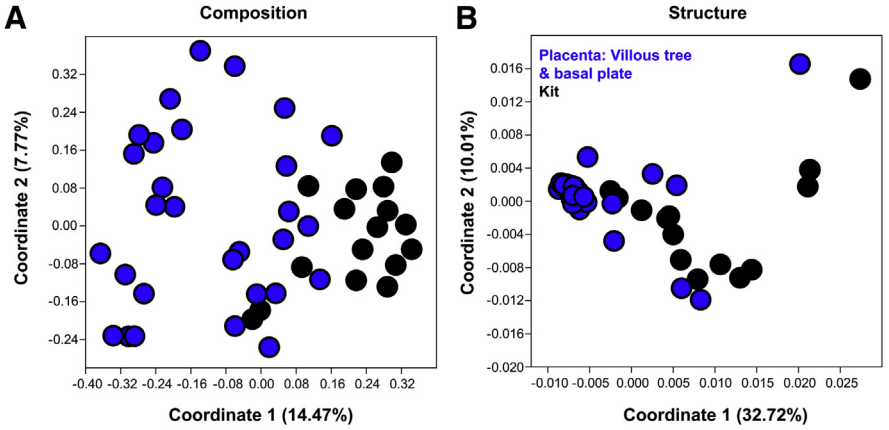
SUPPLEMENTAL FIGURE 6

Principal coordinates analyses illustrating differences in bacterial profile between placental samples and technical controls profiled via the secondary nested polymerase chain reaction analysis



A, Plot of similarities in composition of 16S ribosomal RNA gene profiles among placental and blank extraction kit control samples based on the Jaccard index. **B,** Plot of similarities in structure of 16S ribosomal RNA gene profiles among placental and control samples based on the Bray-Curtis index. Operational taxonomic units were generated with a 97% sequence similarity cutoff.

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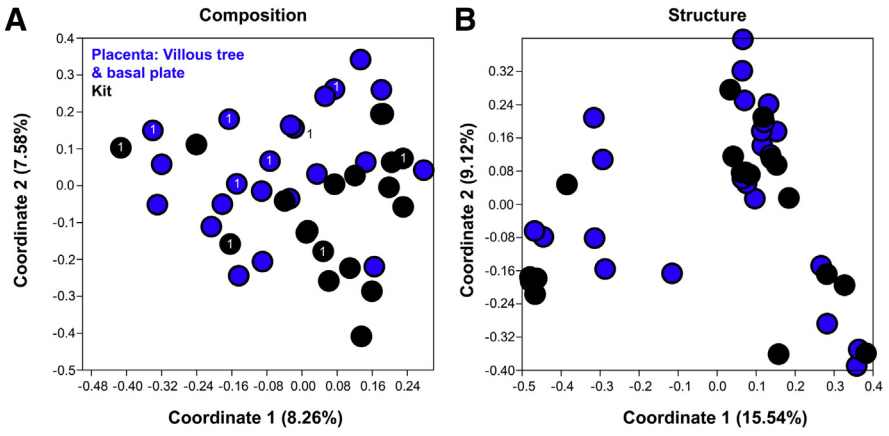
SUPPLEMENTAL FIGURE 7

Principal coordinates analyses illustrating overall similarity in bacterial profile between placental samples and technical controls profiled via the secondary touchdown polymerase chain reaction analysis



A, Plot of similarities in composition of 16S ribosomal RNA gene profiles among placental and blank extraction kit control samples based on the Jaccard index. Samples generated in the first of 4 rounds of extractions are indicated by the number 1. **B,** Plot of similarities in structure of 16S ribosomal RNA gene profiles among placental and control samples based on the Bray-Curtis index. Operational taxonomic units were generated with a 97% sequence similarity cutoff.

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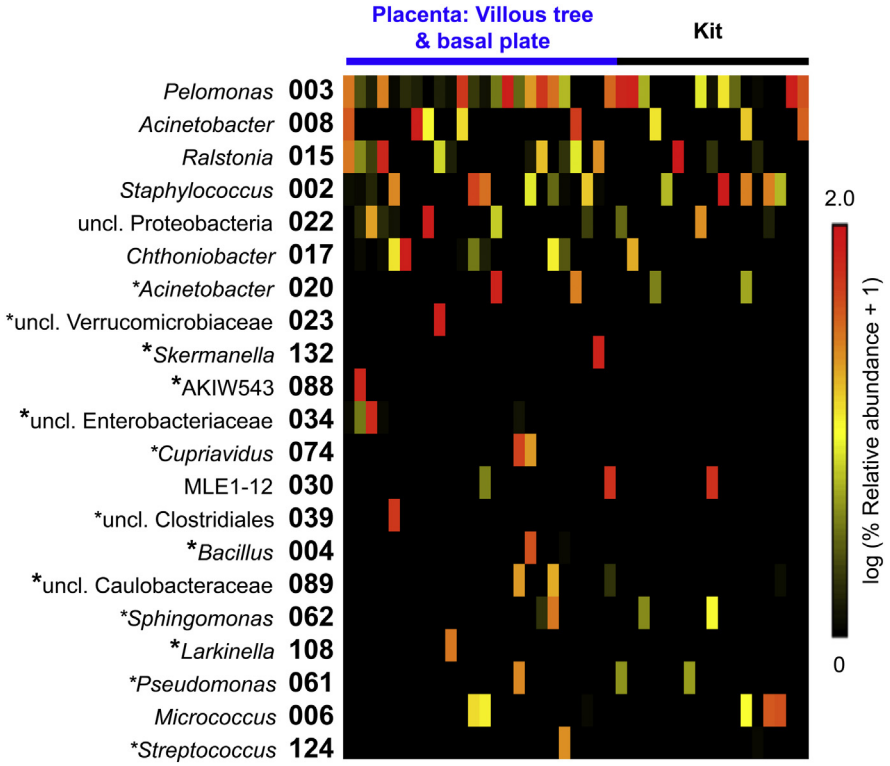
SUPPLEMENTAL FIGURE 8

Heat map illustrating relative abundances of prominent operational taxonomic units among placental samples and their corresponding abundances in blank extraction kit controls



Prominent operational taxonomic units were here defined as those having an average relative abundance ≥1.0% among the placental samples in this study's secondary 16S ribosomal RNA gene touchdown polymerase chain reaction data set. *Asterisks* indicate operational taxonomic units that were prominent in placental samples, but not in controls.

uncl., unclassified.

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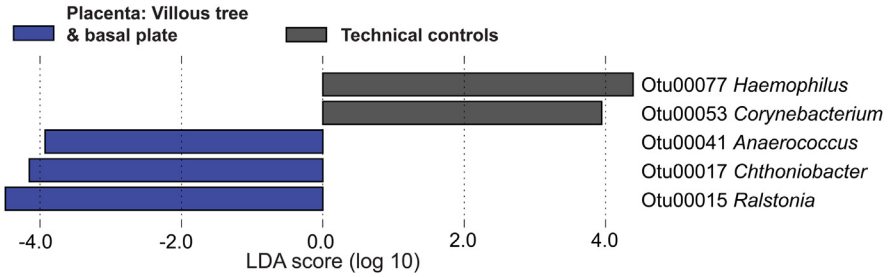
SUPPLEMENTAL FIGURE 9
Linear discriminant analysis effect size analyses indicating differential relative abundance of operational taxonomic units between placental samples and background technical controls



Operational taxonomic units were generated with a 97% sequence similarity cutoff and this study's secondary 16S ribosomal RNA gene touchdown polymerase chain reaction data set. All plotted operational taxonomic units were distributed differentially between placental and technical control samples ($P<.05$).

LDA, linear discriminant analysis; *Otu*, operational taxonomic unit.

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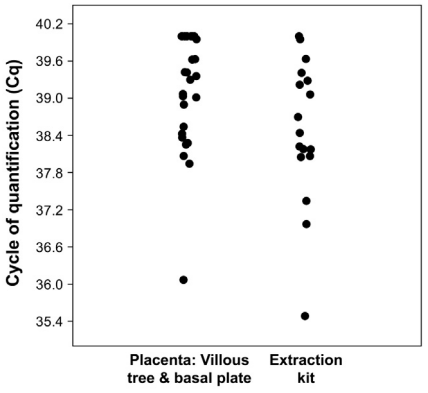
SUPPLEMENTAL FIGURE 10
Plot of 16S ribosomal DNA quantitative real-time polymerase chain reaction cycle of quantification values for placental samples and blank extraction controls from this study's secondary analysis



Comparison of 16S ribosomal DNA quantitative real-time polymerase chain reaction cycle of quantification values for placental and control samples.

Cq, cycle of quantification.

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SUPPLEMENTAL TABLE 1

Nonparametric multivariate analysis of variance shows lack of variation in 16S ribosomal RNA gene profiles among amnion and chorionic plate, villous tree and basal plate, and room, hood, and blank extraction kit control samples

	Composition		Structure	
Variable	F	*P* value	F	*P* value
Placenta: Amnion and chorionic plate				
Global	0.992	.790	1.064	.356
Rooms	1.012	.205	2.025	.020 (.118)
Hoods	0.997	.520	1.184	.280
Kits	0.982	.856	0.542	.885
Placenta: Villous tree and basal plate				
Global	1.003	.345	1.301	.114
Rooms	1.048	.004 (.026)	2.625	.003 (.019)
Hoods	1.003	.394	1.382	.173
Kits	0.995	.595	0.940	.472

Operational taxonomic units were generated with a 99% sequence similarity cutoff. 16S profile composition and structure were characterized with the use of Jaccard and Bray-Curtis indices, respectively. Results of overall global effect analyses are presented along with the results of pairwise comparisons that involve placental samples. Probability values for these permutation tests were not adjusted for multiple pairwise comparisons, because this can be overly conservative. However, for pairwise tests that were statistically significant, we do present the Bonferroni corrected probability value in parentheses.

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SUPPLEMENTAL TABLE 2

Two-way nonparametric multivariate analysis of variance shows difference in 16S ribosomal RNA gene profiles between villous tree and basal plate samples and blank extraction kit controls, independent of the round in which extractions occurred

	Composition		Structure	
Source	F	*P* value	F	*P* value
Sample type	3.561	.0001	6.141	.0001
Round	0.632	.917	1.065	.116
Interaction	−1.695	.069	−1.099	.118

The analyses were based on the secondary 16S ribosomal RNA gene nested polymerase chain reaction dataset and included 29 placental samples and 16 technical controls across 4 rounds of extractions. Operational taxonomic units were generated with a 97% sequence similarity cutoff. 16S profile composition and structure were characterized with the use of Jaccard and Bray-Curtis indices, respectively.

Theis et al. Lack of evidence for a microbiota in the human placenta at term. Am J Obstet Gynecol 2019.

SUPPLEMENTAL TABLE 3

Two-way nonparametric multivariate analysis of variance shows a difference, within 1 of 4 rounds of extractions, in the composition of 16S ribosomal RNA gene profiles between villous tree and basal plate samples and blank extraction kit controls

	Composition		Structure	
Source	F	*P* value	F	*P* value
Sample type	1.345	.010	0.721	.769
Round	1.186	.005	0.859	.590
Interaction	0.073	.068	0.158	.058

There were no differences in the structure of 16S profiles between placental samples and controls. The analyses were based on the secondary 16S ribosomal RNA gene touchdown polymerase chain reaction dataset and included 24 placental samples and 17 technical controls across 4 rounds of extractions. Operational taxonomic units were generated with a 97% sequence similarity cutoff. 16S profile composition and structure were characterized with the use of Jaccard and Bray-Curtis indices, respectively.

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