OBSTETRICS

No evidence for a placental microbiome in human pregnancies at term

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BACKGROUND: The placenta plays an important role in the modulation of pregnancy immunity; however, there is no consensus regarding the existence of a placental microbiome in healthy full-term pregnancies.
OBJECTIVE: This study aimed to investigate the existence and origin of a placental microbiome.
STUDY DESIGN: A cross-sectional study comparing samples (3 layers of placental tissue, amniotic fluid, vernix caseosa, and saliva, vaginal, and rectal samples) from 2 groups of full-term births: 50 women not in labor with elective cesarean deliveries and 26 with vaginal deliveries. The comparisons were performed using polymerase chain reaction amplification and DNA sequencing techniques and bacterial culture experiments.
RESULTS: There were no significant differences regarding background characteristics between women who delivered by elective cesarean and those who delivered vaginally. Quantitative measurements of bacterial content in all 3 placental layers (quantitative polymerase chain reaction of the 16S ribosomal RNA gene) did not show any significant difference among any of the sample types and the negative controls. Here, 16S ribosomal RNA gene sequencing of the maternal side of the placenta could not differentiate between bacteria in the placental tissue and contamination of the laboratory reagents with bacterial DNA. Probe-specific quantitative polymerase chain reaction for bacterial taxa suspected to be present in the placenta could not detect any statistically significant difference between the 2 groups. In bacterial cultures, substantially more bacteria were observed in the placenta layers from vaginal deliveries than those from cesarean deliveries. In addition, 16S ribosomal RNA gene sequencing of bacterial colonies revealed that most of the bacteria that grew on the plates were genera typically found in human skin; moreover, it revealed that placentas delivered vaginally contained a high prevalence of common vaginal bacteria. Bacterial growth inhibition experiments indicated that placental tissue may facilitate the inhibition of bacterial growth.
CONCLUSION: We found no evidence to support the existence of a placental microbiome in our study of 76 term pregnancies, which used polymerase chain reaction amplification and sequencing techniques and bacterial culture experiments. Incidental findings of bacterial species could be due to contamination or to low-grade bacterial presence in some locations; such bacteria do not represent a placental microbiome per se.

Key words: 16S RNA gene, amniotic fluid, bacterial culture, contamination, feces, in utero colonization, microbiome, placenta, pregnancy, quantitative polymerase chain reaction, saliva, vaginal fluid, vernix caseosa

Introduction

In the last century, it has been assumed that the intrauterine environment in healthy pregnancies is sterile. It was not until the last decade that the notion of a sterile womb was challenged,[1] in particular after the introduction of new molecular approaches, such as next-generation sequencing. In nonpregnant women, there is evidence of bacteria not only in the vagina[2] but also in the endometrial cavity, without signs of inflammation,[3] and a microbiome of the endometrium has been described.[4–6] In general, intrauterine pathogens during pregnancy are suggested to be detrimental to pregnancy outcomes[7–9] because they may cause clinical chorioamnionitis, preterm premature rupture of fetal membranes, and preterm labor with intact membranes.[10–12]

The microbiome has been increasingly included in efforts to understand the mechanisms that interact to maintain a healthy immune system and pregnancy.[13] Microbial colonization of the skin, gut, and other mucosal surfaces of the newborn is essential for the development of host metabolism, immunity, and resistance to pathogens.[14,15] However, as molecules from the maternal microbiome are transported in the blood and infiltrate every organ of the mother, those maternal microbial molecules influence the fetus long before the newborn acquires its own microbiota.[16]

The placenta is a barrier against infections and plays an essential role in the modulation of pregnancy immunity.[17] Placental dysfunctions are linked to complications, such as preeclampsia, intrauterine growth restriction, and stillbirth.[18–20] In 2014, the sterile womb hypothesis was challenged by Aagaard et al,[21] who reported the detection of bacterial DNA sequences of multiple taxa in placental samples. A comparison of community patterns suggested the oral microbiome as the body site of origin. In addition, several studies have claimed to detect a distinct placental microbiome.[21–32] In contrast, others

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AJOG at a Glance

Why was this study conducted?

The existence of a placental microbiome is debated. This study compared samples taken from women not in labor with those taken from women who underwent cesarean and vaginal deliveries at term to investigate the existence and origin of a microbiome in the placenta.

Key findings

Only sporadic bacteria, not representing a microbiome per se, were detected in placental tissue. Placental tissue samples from vaginal deliveries produced more live bacteria in culture experiments than those from cesarean deliveries.

What does this add to what is known?

The data are in line with previous studies that indicated lack of evidence for a placental microbiome and suggest that the growth of bacteria entering the uterine cavity is inhibited in normal pregnancies.

have not confirmed these findings and have attributed observed bacterial signals to background contamination arising from reagents used during sample analysis or from the delivery method (in this case, vaginal delivery).[33–39]

Such contradictions are expected, because samples from tissue with bacterial load close to the limit of detection, even with modern detection techniques, are particularly prone to revealing background signals, such as unspecific binding of probes or residual molecules left in the analytical apparatus after disinfection.[40] Furthermore, many of the studies claiming that microbiota are present in the placenta have included results from preterm or other complicated pregnancies.[24,28,31] In addition, studies that have reported mainly negative findings and a small number of microbial species have included placentas from healthy pregnancies, and the load has generally been considered too low to be characterized as a microbial community.[30,34,37] In summary, there is currently no consensus regarding the existence of a placental microbiome in healthy full-term pregnancies.

Amniotic fluid (AF) is another essential factor in the maintenance of intrauterine homeostatic conditions that has been considered sterile,[41] but recent studies have had conflicting results.[23,42] Both the AF and vernix caseosa, covering the fetal skin during the last trimester of pregnancy, contain potent substances with broad-spectrum antimicrobial effect.[43]

The aim of the current study was to investigate the existence and origin of a placental microbiome. Comprehensive samples were collected from the amniotic sac and placenta to determine potential bacterial content. In addition, samples from the oral cavity, gut, and vagina were collected from each woman to identify a possible source of the bacteria. We compared the specimens from cesarean and vaginal deliveries using polymerase chain reaction (PCR) amplification and sequencing (quantitative PCR [qPCR] of the 16S rRNA gene, bacterial taxa-specific qPCR, metabarcoding based on 16S rRNA gene sequencing) and bacterial culture experiments, and we collected detailed information about the mothers' clinical backgrounds to avoid possible confounding factors in our results.

Materials and Methods

Study design and participants

This study used a cross-sectional design to investigate whether there is a placental microbiome in human pregnancies at term and the potential reasons for the placenta being sterile or not by comparing samples from 2 groups of term births: women not in labor with elective cesarean deliveries and women with vaginal deliveries. The inclusion criteria for both groups were as follows: full-term pregnancies, maternal age more than 18 years, Swedish or English speaking. The exclusion criteria were as follows: knowledge of fetal pathology and acute cesarean delivery (Figure 1). All the participants were attending the maternity clinic at Södersjukhuset, Stockholm, Sweden, between March 2017 and October 2017 and were included in the study after receiving information and signing a consent form on the day of the delivery or the day earlier. Data on background and health status were collected from the participants' medical records. The study was approved by the independent regional Research Ethics Committee, Karolinska Institutet, Stockholm, Sweden (2015/2043-31/2) and complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects. All the study participants received oral and written information about the study and the sample protocol or analysis and signed a written consent form.

Sample collection

An overview of the sample collection, storage, and analyses performed is shown in Figure 1. All samples were collected by 2 obstetricians (I.S. and E.W.I.). The samples were collected in the exact order described below.

Cesarean delivery group

In the elective cesarean delivery group, saliva samples were collected before the surgery using a SalivaGene Collector (STRATEC Molecular GmbH, Birkenfeld, Germany) containing lyophilized DNA stabilization buffer according to the manufacturer's instructions. The vaginal and rectal swab samples were taken in the operating theater, before the start of surgery, by inserting the swab (FLOQSwabs, Copan Flock Technologies, Brescia, Italy) 2 to 3 cm into the vagina or anus and swirling the swab for 10 seconds. Swabs were directly put into FluidX tubes (Brooks Life Sciences, Chelmsford, MA) containing 0.8 mL DNA/RNA Shield (Zymo Research, Irvine, CA). Approximately 1.5 mL of AF was collected by aspirating with a sterile syringe inserted at the incision

site directly following the uterotomy. AF was then immediately placed into empty, prebarcoded FluidX tubes (Brooks Life Sciences) and frozen at −80°C.

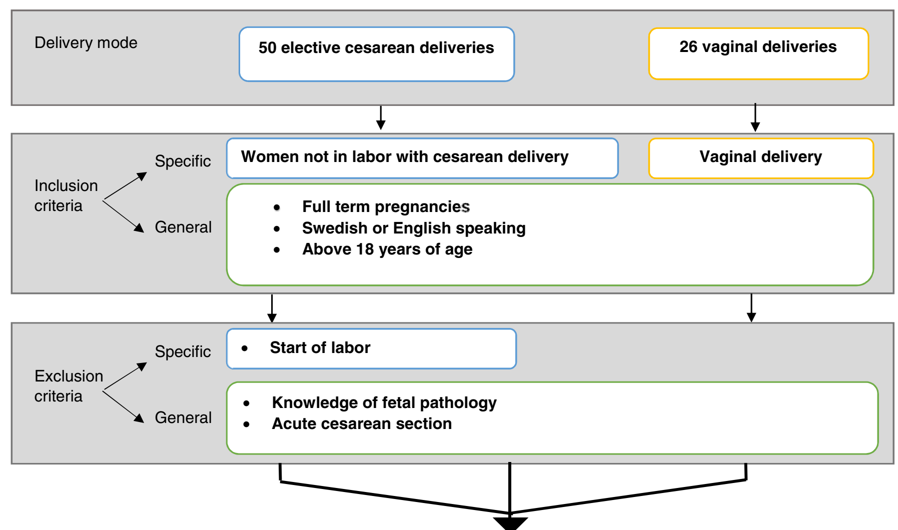
Placenta samplings were performed by 2 experienced obstetricians according to a previously used protocol that was slightly adapted to match the purpose of our study.[21,44] The placentas were collected in sterile containers using sterile gloves and instruments for the dissections, which took place in a room (open room environment) adjacent to the operating theater. Three 1×1−cm cross-sectional tissue samples were circumferentially excised, each at about 4 cm from the cord insertion site. Each sample was then dissected into 3 layers: maternal, middle, and fetal. Each layer was cut into 1 set of 3 biopsies, corresponding to 1 cross-section, and immediately stored in 1 of the following conditions to allow potential detection of bacteria in placental tissue: (1) 0.8 mL DNA/RNA Shield for microbiome analyses (Zymo Research), (2) freezing medium for sensitive bacteria MIK1106 (Karolinska University Hospital substrate unit; containing albumin, bovine serum albumin 25 g, sucrose 74 g, potassium dihydrogen phosphate 0.5 g, potassium hydrogen phosphate 1.2 g, sodium glutamate hydrate 0.6 g, in 1 L with a pH of 7.1). Culture experiments are shown in Figure 1.

The vernix caseosa samples were obtained by rubbing a FLOQSwab (Copan Flock Technologies) in the armpit or groin of the newborns within the first 10 minutes after delivery, in the operating theater. The swab was immediately placed in a FluidX tube (Brooks Life Sciences) with 0.8 mL DNA/RNA Shield and frozen at −80°C.

Vaginal delivery group

The placentas were delivered vaginally and collected in sterile containers and covered with a sterile cloth. The placentas were then directly transported to an adjacent room. All dissections were performed using sterile gloves and surgical instruments in the manner described above. AF was collected vaginally with a sterile syringe after rupture of the amniotic membranes, before the baby was born. Samples of AF that were mixed with blood were excluded (n=6). All other samples (vernix caseosa, saliva, vaginal, and rectal samples) were collected during delivery as described above. Samples were kept at −80°C until analysis.

FIGURE 1
Study design and workflow for sample collection and analyses



Samples		N[a]	Collection tube: FluidX tubes, With FLOQSwabs: DNA/RNA shield	FluidX tubes, With FLOQSwabs: Medium MIK1106	FluidX tubes, Without FLOQSwabs: Empty tubes	FluidX tubes, Without FLOQSwabs: DNA/RNA shield	FluidX tubes, Without FLOQSwabs: Medium MIK1106	Saliva Gene collector (with SLK buffer)	Performed analysis
Saliva		76						-80°C[b]	Metagenomics
Vaginal swabs		76	-80°C	-80°C					Metagenomics
Rectal swabs		76	-80°C	-80°C					Metagenomics
Amniotic fluid		77			-80°C				qPCR
Vernix		77	-80°C						qPCR
Placenta Layers	maternal	77				-80°C	-80°C		qPCR; 16S; Bacterial culture; Bacterial growth inhibition
	middle	77				-80°C	-80°C		qPCR; Bacterial culture
	fetal	77				-80°C	-80°C		qPCR; Bacterial culture; Bacterial growth inhibition

Inclusion and exclusion criteria for the participants are indicated in the upper panel. The number of samples collected from each fluid or tissue source, how they were preserved, and the analysis performed are listed in the lower panel. [a]One of the mothers had a dichorionic diamniotic twin delivery; [b]Storage temperature.

qPCR, quantitative polymerase chain reaction.

Sterpu et al. No evidence for a placental microbiome in full-term pregnancies. Am J Obstet Gynecol 2021.

Deoxyribonucleic acid extraction, amplification, and sequencing

DNA extraction was performed using the Quick DNA Magbead Plus Kit (Zymo Research, Irvine, CA) according to manufacturer's instructions, with the following modifications: placental specimens were homogenized with lysing matrix A from MP Biomedicals, LLC (Valiant, China), 3 times for 2 minutes. Vernix caseosa and AF were homogenized with ZR BashingBeads (lysis matrix used to lyse the bacteria cell wall; Zymo Research, Irvine, CA) for 1 minute. Furthermore, 200 μL of the pretreated placental specimens were incubated with 20 μL of lysozyme (100 mg/mL) at 37°C for 60 minutes. For the vernix caseosa and AF, 650-μL samples were incubated with 40 μL of lysozyme solution. Samples were then incubated with 40 μL proteinase K (20 mg/mL), using double the amount of solid tissue buffer II (advised by the manufacturer) at 55°C for 90 minutes (placenta) or

double the amount of biofluid and cell buffer II at 55° for 30 minutes (vernix caseosa and AF). DNA purification was performed in Freedom EVO (Tecan, Männedorf, Switzerland) according to the Zymo protocol, and samples were eluted in an EB buffer (Qiagen, Hilden, Germany). Positive controls (ZymoBIOMICS Microbial Community Standard, D6300, Zymo Research, Irvine, CA; a commercial mixture of bacterial colonies containing gram-positive and gram-negative species) and negative controls (DNA/RNA Shield only) were processed with each extraction plate. The resulting sequencing data have been submitted to the European Nucleotide Archive under project PRJEB38528, accession numbers ERX4191918 to ERX4192266.

Quantitative analysis of bacterial communities by quantitative polymerase chain reaction and 16S ribosomal ribonucleic acid gene sequencing

Universal 16S rRNA gene primers covering the V6-V8 region (described by Huys et al[45]; Integrated DNA Technologies [IDT], Coralville, IA) were used because of their good characteristics when amplifying samples with very low microbial content and high human background (V6-V8 primers are more specific to bacterial DNA, whereas V3-V4 primers can amplify human mitochondrial DNA). Therefore, when aiming to quantify a total bacterial load in a sample rich in human content, the V6-V8 primer shows more specificity and should be the primer of choice (Supplemental Figure 1). In addition to the extraction controls, PCR-positive controls (ZymoBIOMICS Microbial Community DNA Standard, D6305, Zymo Research, Irvine, CA; a commercial mixture of DNA from different bacterial colonies) and PCR-negative controls (PCR-grade water, W4502, Sigma-Aldrich, St. Louis, MO) were amplified and sequenced together with the samples. Real-time PCR experiments were performed in a LightCycler 480 using the SYBR Green assay from Bio-Rad (1725270, Bio-Rad Laboratories, Hercules, CA). The qPCR settings for the V6-V8 amplification were as follows: 98°C preincubation for 3 minutes, 98°C melting for 10 seconds, 57°C annealing for 15 seconds, 72°C extension for 40 seconds, and a total of 35 cycles. As a positive control and for quantification of bacterial content, the DNA standard from Zymo (ZymoBIOMICS Microbial Community DNA Standard, D6305, Zymo Research; a commercial mixture of DNA from different bacterial colonies) was expressed in a plasmid using the TOPO TA Cloning Kit (Invitrogen, K457501, Carlsbad, CA), purified using the Plasmid Miniprep Kit I (VWR, 732-2780, Radnor, PA), and quantified using the Qubit system (dsDNA high sensitive, Q32854, Thermo Fisher Scientific, Waltham, MA). After quantification, the DNA was normalized and used as a standard for the quantification of the samples. As a negative control for the reaction, PCR-grade water (W4502, Sigma-Aldrich) and pure human DNA (Sigma 11691112001, Sigma-Aldrich, St. Louis, MO) were used. For background subtraction (because reagents used in the extraction may contain trace amounts of bacterial DNA), negative DNA extraction controls were also submitted to qPCR.

To further investigate the bacterial content representing the positive results from the qPCR of the 16S rRNA gene, selected taxa were amplified using hydrolysis probes designed for specific bacterial targets. The experiments were conducted using the same equipment as the total 16S rRNA sequencing experiments, and more information regarding the probes and PCR conditions can be found in Supplemental Table 1. For this experiment, we used a gBlock (IDT) sequence specific to each bacterium as the standard for quantification. Sequences and references are presented in Supplemental Tables 2 and 3. The probe selection was limited by the amount of remaining DNA and by the characteristics of the 16S rRNA gene for each taxon (specificity, size of the amplified fragment, guanine-cytosine (GC) content, etc.).

For the sequencing procedure, we applied a 2-step PCR approach. We selected the maternal side of the placenta for sequencing because it had the highest sequencing signal during qPCR analysis (Figure 2). Women given antibiotics during delivery were excluded from this analysis (n=6). The input DNA used was 75 ng, and the first PCR was performed under conditions similar to those used for qPCR, except for the polymerase used (High-Fidelity Master Mix, F-565L, Thermo Fisher Scientific, Stockholm, Sweden) and the number of cycles (25 cycles). The samples were then barcoded using the Nextera XT Kit following Illumina's standard protocol (12 PCR cycles; Illumina, 15052163, San Diego, CA) and sequenced on an Illumina MiSeq (Illumina, San Diego, CA) with V3 chemistry and 2×300 bp reads. Samples generated 1020 to 207,009 reads per sample (median, 23,303). Sequenced placental samples are available from the European Nucleotide Archive under project number PRJEB38528, accession numbers ERX4191918-ERX4192266.

Microbial profiling of maternal saliva, vaginal, and fecal samples using shallow shotgun sequencing

DNA extraction was performed using the Quick DNA Magbead Plus Kit (Zymo Research) according to manufacturer's instructions, with few modifications. Each sample type was bead-beaten with a different matrix: saliva (600 μL) was homogenized with lysing matrix B (MP Biomedicals, LLC, Valiant, China); vaginal swabs with ZR BashingBead Lysis matrix (Zymo Research); and rectal swabs with matrix E (Nordic Biolabs, Täby, Sweden). Each sample was bead-beaten at 1600 revolutions per minute for 1 minute before DNA extraction using a FastPrep-96 homogenizer (SKU 116010500, MP Biomedicals, Santa Ana, CA). After bead beating, samples were treated with lysozyme (100 mg/mL, at 37°C for 60 minutes) and proteinase K (20 mg/mL, at 55°C for 30 minutes) previous to extraction using Freedom EVO (Tecan). Extracted DNA was shipped to CoreBiome (OraSure, Bethlehem, PA) and processed with their BoosterShot shallow shotgun-sequencing technology.

The resulting sequencing data have been submitted to the European Nucleotide Archive under project PRJEB38528, accession numbers ERX4192267 to ERX4192489.

Bioinformatics

Cutadapt (version 1.4)[46] was used to trim 3'-bases with a Phred score of <15, remove primer sequences, and discard reads not containing the primers. The resulting quality-trimmed reads were processed with the R package DADA2 (version 1.11.3)[47] to correct errors, remove chimeras, and produce amplified sequence variants (ASVs). The resulting ASV data were assessed for contamination using the Decontam package (version 1.1.2)[48] using the function *isNotContaminant*, which is suitable for samples with low bacterial load, and the prevalence method.

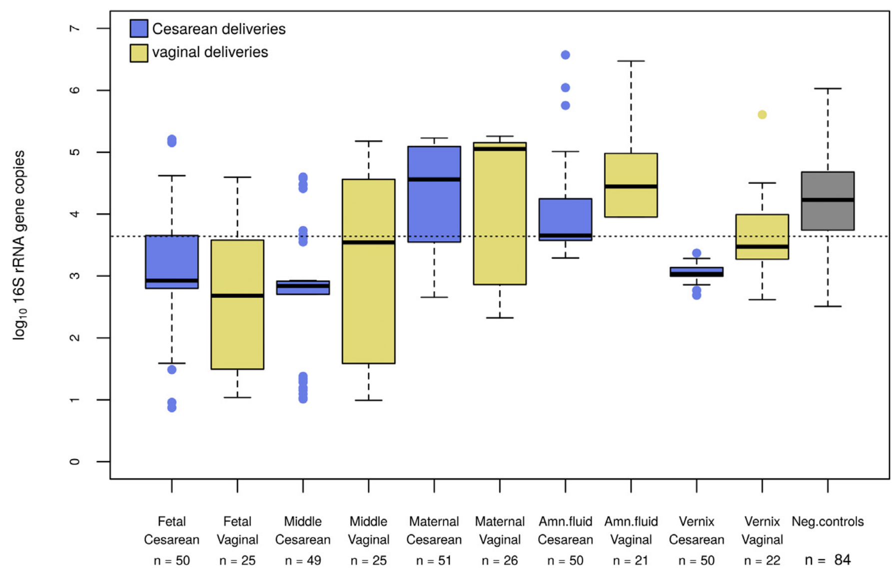
Placental tissue culture and bacterial deoxyribonucleic acid extraction

Here, 3 pieces of the placental tissue (around 1×1 cm, maternal, middle, and fetal) from each participant (n=76) collected in freezing medium for sensitive bacteria MIK1106 (Karolinska University Hospital substrate unit) were inoculated by smearing the tissue on GC agar plates (Karolinska Hospital, MIK0346, Sweden) under a sterile cell culture biosafety cabinet. The plates were then incubated anaerobically at 37°C for 48 hours. GC plates with open lids during the inoculation period were used as negative controls. After incubation, present colonies were enumerated and collected for DNA extraction, using the same DNA extraction protocol applied for saliva. The DNA material from the colonies was sequenced using 16S rRNA gene sequencing, as described above.

Bacterial growth inhibition assay

Escherichia coli (ATCC 25922) was used in the study of growth inhibition. In total, 12 frozen placental specimens (maternal and fetal sides from 6 cesarean deliveries) were selected on the basis of the qPCR results (3 high vs 3 low bacterial content in each side). *E coli* was cultured overnight aerobically in 2 mL Luria-Bertani (LB) broth (Sigma L3522) at 37°C. Furthermore, the *E coli* culture was diluted to 100 colony-forming units (CFUs)/mL on the basis of their optical density measurement to mimic the low bacterial load in the uterine cavity. From the diluted cultures, 10 CFUs in 100 μL were added into each well of the 96-well plate (Thermo Fisher Scientific, 167008). Subsequently, a piece of frozen placental tissue (around 5 mm in diameter) was added into each well for the inhibition experiment. At the same time, 100 μL culture media and placental tissues without bacteria were used as negative controls. In addition, LB broth and LB broth inoculated with 10 CFUs of *E coli* with and without 10 μg/mL gentamycin (Sigma G1272) were also carried out as controls. All these conditions, including each of the 12 frozen placentas and corresponding controls, were performed in triplicate. After 24 hours' coculture according to the bacteria growth condition described above, CFUs were enumerated by serial dilution as described previously.[49]

FIGURE 2
AF and placental specimens may contain bacterial DNA, at concentrations undistinguishable from negative controls



Quantification of total bacterial abundance by 16S rRNA gene copies by qPCR (log 10) on placental, AF, and vernix caseosa samples. The *boxes* represent median and interquartile range; *blue boxes* represent cesarean deliveries, and *yellow boxes* represent vaginal deliveries. Because samples were randomized for extraction, the negative extraction controls (*gray*) cannot be separated by delivery type. The *thin dotted horizontal line* shows the level of bacterial DNA detected in pure commercial human DNA.

AF, amniotic fluid; *qPCR*, quantitative polymerase chain reaction; *rRNA*, ribosomal ribonucleic acid.

Sterpu et al. No evidence for a placental microbiome in full-term pregnancies. Am J Obstet Gynecol 2021.

Statistical analyses and figures

For the descriptive data, chi-squared tests were used to compare categorical variables between the study groups, and Mann-Whitney tests were applied for the continuous variables, using Statistical Product and Service Solutions (SPSS) (version 25.0, IBM, Armonk, NY). R libraries RColorBrewer (version 1.1-2), vioplot (version 0.2), and treemap (version 2.4-2) were used to create the figures. For qPCR, a 1-sided Kruskal-Wallis test with alternative "greater" was applied using R (version 3.5.2). Bacterial culture comparisons between samples from cesarean and vaginal deliveries in each CFU range group were computed using Fisher's exact test. The Mann-Whitney test was performed for comparison between all placenta groups

(regardless of maternal or fetal side and of high or low bacterial content in the qPCR) with *E coli* LB control in the bacterial growth inhibition assay using SPSS (version 23.0; IBM). The level of statistical significance was set at $P<.05$. When triplicates were used, the average value of each triplicate was inserted. In addition, the Kruskal-Wallis H test, followed by posthoc tests using the Dunn multiple comparisons test, was carried out for pairwise comparison between the groups, with the significance level set at $P<.05$.

Results

Characteristics of participants

Maternal and infant characteristics are presented in Table 1. In total, 76 women with normal pregnancies and 77 infants were included. There were no significant differences regarding background characteristics between the 2 groups (elective cesarean delivery and vaginal delivery) except regarding parity ($P=.04$). All newborns had normal birthweights, except 1 baby from the twin pregnancy, who was small for gestational age. None of the newborns needed neonatal care. As expected, onset of labor, mode of delivery, and gestational length differed between the groups.

16S ribosomal ribonucleic acid gene quantitative amplification and sequencing could not conclusively distinguish any microbial content of placenta from background signals

There was a high degree of variability in the gene counts observed by qPCR from the negative extraction controls (Figure 2). No significant difference in copy number was observed between any of the sample types from the placenta, AF, or vernix caseosa and the negative controls. The median gene counts were highest for the maternal side of the placenta and for AF, regardless of the mode of delivery.

To further characterize the bacterial signal observed in the maternal side of the placenta, samples from women who had not taken antibiotics during pregnancy or delivery (n=45 cesarean-delivered infants; n=19 vaginally delivered infants) were used for 16S rRNA gene sequencing. Of 964 observed ASVs, 835 were flagged as contaminants by the Decontam software, corresponding to 3.7% to 67% of each sample (median, 57%) (Figure 3). A large fraction of the remaining tags came from the genus *Massilia* (0%−45% of total sample counts; median, 35%), also a known laboratory contaminant.[40] Another common laboratory contaminant observed in our samples was the genus *Escherichia or Shigella*.[50] Finally, regardless of the mode of delivery, we observed a variety of typical vaginal bacteria and opportunistic pathogens (Supplemental Figure 2).

To further validate these findings, selected taxa were amplified by qPCR with taxon-specific primers and probes. Because the maternal side of the placenta gave the strongest signal with universal primers, the same samples were used for the amplification of 10 specific bacterial taxa suspected to be present in the placenta on the basis of sequencing data and previous studies (Supplemental Tables 2 and 3). In total, 21 of 48 cesarean-delivered and 11 of 22 vaginally delivered placentas were positive for at least 1 probe, yielding no statistical difference in the rate of positive samples (Fischer's exact test; odds ration=0.78; $P=.8$) (Figure 4). There was also no difference between cesarean-delivered and vaginally delivered samples with regard to the number of positive signals per positive sample nor on the strength of these signals (Figure 4).

The lack of a specific placental microbiome did not allow for a comparison of the bacterial content of saliva, vaginal, and fecal samples

Vaginal swabs, rectal swabs, and saliva samples collected from women at the time of delivery were analyzed to identify the origin of the placental microbiome. Because we did not detect a specific placental microbiome, no meaningful comparison of these samples could be made. However, the bacterial composition of saliva, vaginal, and fecal samples is presented in Supplemental Figure 3. We also display the overlap between these body sites and bacteria detected in the placenta by at least 2 independent methods in Supplemental Table 4.

Bacterial culture of placental specimens yielded typical vaginal and skin bacteria

As shown in Figure 5, A and B, after culturing placental tissue on rich medium plates, all the plates were classified into 4 groups according to the number of bacterial colonies (CFUs): no bacteria, 1 to 5 CFUs, 6 to 30 CFUs, and >30 CFUs. Most placental specimens from the cesarean deliveries did not show any CFUs (n=87 of 152 [57.2%]), with only a small portion of tissues containing more than 30 colonies (n=10 of 152 [6.6%]) (Table 2). In contrast, a quarter of the placental tissues from vaginal delivery presented more than 30 CFUs (n=21 of 78 [26.9%]) (Table 2). In 3 of the placenta sections, there were significantly more bacteria observed from vaginally delivered samples than from cesarean-delivered samples (maternal side, $P=.007$; middle side, $P=.041$; fetal side, $P<.001$). Overall, significantly more placental specimens from the cesarean delivery mode had no bacterial colonies ($P\leq.001$), whereas more specimens from the vaginal delivery mode had bacterial colonies over 30 CFUs (Table 2). For both modes of deliveries, no significant correlation was observed between the estimated bacterial load by qPCR and the number of colonies found in culture, except for a weak correlation on the fetal side (Pearson's correlation; r=0.290; $P=.041$).

The 16S rRNA sequencing data on the collected colonies revealed that most of the bacteria that grew on the plates from cesarean-delivered placentas were genera typically found on human skin, such as *Propionibacterium*, *Streptococcus*, and *Staphylococcus* (Figure 5, C), except for *Gardnerella*, which was retrieved in 1 fetal side tissue, and *Bifidobacterium*, which was found in 1 piece from the maternal side. In contrast, placentas delivered vaginally had a high prevalence of *Lactobacillus* and *Gardnerella*, in addition to the same genera presented in cesarean-delivered samples (Figure 5, C;

TABLE 1
Demographics of the 76 participants and their 77 infants

Variables	Elective cesarean delivery (n=50)	Vaginal delivery (n=26)	*P* value[a]
Maternal characteristics			
Age (y)	34.5 (23.0–47.0)	31.0 (21.0–49.0)	.4
BMI in early pregnancy	23.1 (19.1–45.2)	23.3 (18.5–36.8)	.8
Smoking in early pregnancy	1 (2.0)	0 (0)	1.0
History of psychiatric disease	7 (14.0)	1 (3.8)	.2
IVF	5 (10.0)	1 (3.8)	.6
Parity			.04
Nulliparous	17 (34.0)	18 (69.2)	
Multiparous	33 (66.0)	8 (38.2)	
Twins[b]	1 (2.0)	0 (0)	
Complications of pregnancy[c]	2 (4.0)	3 (11.5)	.3
GBS in pregnancy	2 (4.0)	1 (3.8)	1.0
Preexisting comorbidities[d]	10 (20)	4 (15.4)	1.0
Antibiotics during pregnancy[e]			.2
Total	9 (18.0)	3 (11.5)	
First trimester	2	1	
Second trimester	4	0	
Third trimester	3	2	
Antibiotics during delivery[f]	3 (6.0)	3 (11.5)	.4
Onset of labor			<.01
Spontaneous	0 (0)	18 (69.2)	
Induced	0 (0)	8 (30.8)	
Planned cesarean delivery	50 (100.0)	0 (0)	
Mode of delivery			<.01
Spontaneous vaginal	0 (0)	23 (88.5)	
Vacuum	0 (0)	3 (11.5)	
Cesarean	50 (100.0)	0 (0)	
Fetal characteristics			
Gestational age (d)[g]	272 (262–282)	283 (262–296)	<.01
Birthweight (g)	3680 (2225–4450)	3620 (3140–5282)	.9
Female sex	20 (39.2)	15 (57.7)	.1

Continuous variables are presented as median (minimum-maximum); categorical variables are presented as number of participants (percentages).

BMI, body mass index; *GBS*, group B *Streptococcus*; *IVF*, in vitro fertilization.

[a] Chi-squared tests were used for categorical variables and Mann-Whitney tests were applied for the continuous variables; [b] Dichorionic diamniotic pregnancy; [c] Diagnoses included preeclampsia (n=1), hypertension (n=2), and cholestasis of pregnancy (n=2); [d] Diagnoses included asthma, hypothyroidism, inflammatory bowel disease, and diabetes; [e] Treatment with penicillin in all cases except 3 that received broad-spectrum antibiotics; [f] Elective cesarean deliveries treated with broad-spectrum antibiotic (cefuroxime) and vaginal deliveries treated with benzylpenicillin; [g] Planned cesarean deliveries were performed around 273 days of gestation.

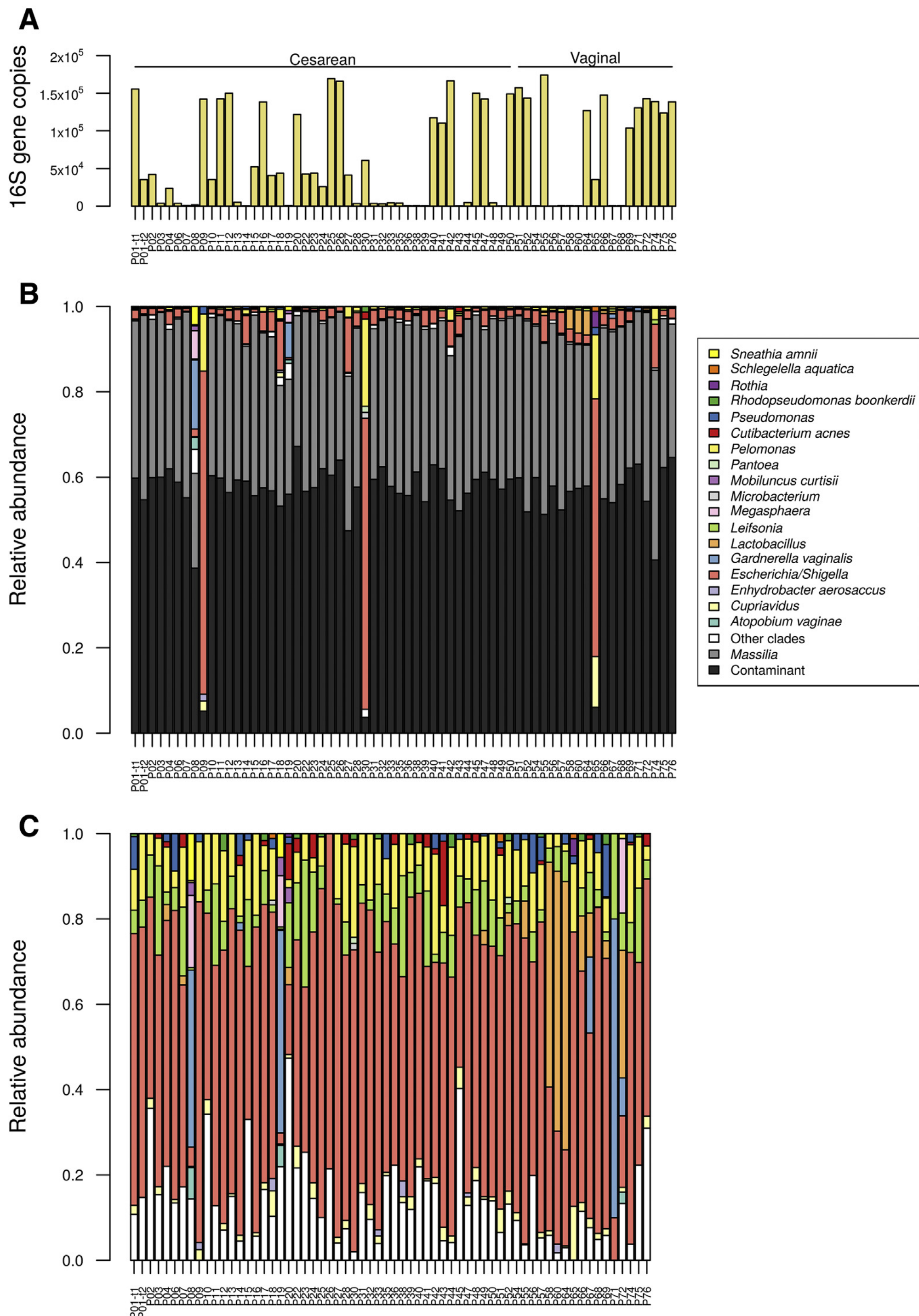
Sterpu et al. No evidence for a placental microbiome in full-term pregnancies. Am J Obstet Gynecol 2021.

Supplemental Figure 4). In general, cultures obtained from vaginal deliveries had higher bacterial richness (number of unique ASVs; median 10 [interquartile range (IQR), 6–33] vs median 3 [IQR, 0–9] unique sequences or plate; Kruskal-Wallis, $P=10^{-5}$) (Supplemental Figure 4).

Lack of agreement between sequencing and culturing data

Very few genera detected by 16S sequencing could be confirmed by

FIGURE 3
Most of the detected taxa in placenta are likely contaminants



The maternal side of the placentas from women who had not taken antibiotics during pregnancy or delivery (n=45 for cesarean delivery; n=19 for vaginal delivery) was taxonomically profiled by sequencing of the V6-V8 region of the 16S rRNA gene. Samples from each individual are placed in the same order in each panel. "P" stands for participant and "t" for twin. **A,** Total 16S rRNA gene copies for each sample. **B,** Taxonomic profile of the placentas, expressed as proportion of total bacteria. No correlation is observed between the total bacterial quantification and the observed microbial profile. C is the same as B but excluding the most likely contaminants.

rRNA, ribosomal ribonucleic acid.

Sterpu et al. No evidence for a placental microbiome in full-term pregnancies. Am J Obstet Gynecol 2021.

culturing. These are *Pelomonas*, which could be found by 16S sequencing in 7 of 8 samples where it could be cultured, *Massilia* (10 of 11), *Leifsonia* (5 of 5), and *Escherichia or Shigella* (12 of 13). All of these were detected by sequencing in >40 samples each, and confirmed by culture in a much smaller set. The overlap between clades detected by qPCR and sequencing is typically 1 sample per genus investigated. In particular, *Escherichia of Shigella,* although abundant in sequencing data and cultures, was detected in very few samples by qPCR.

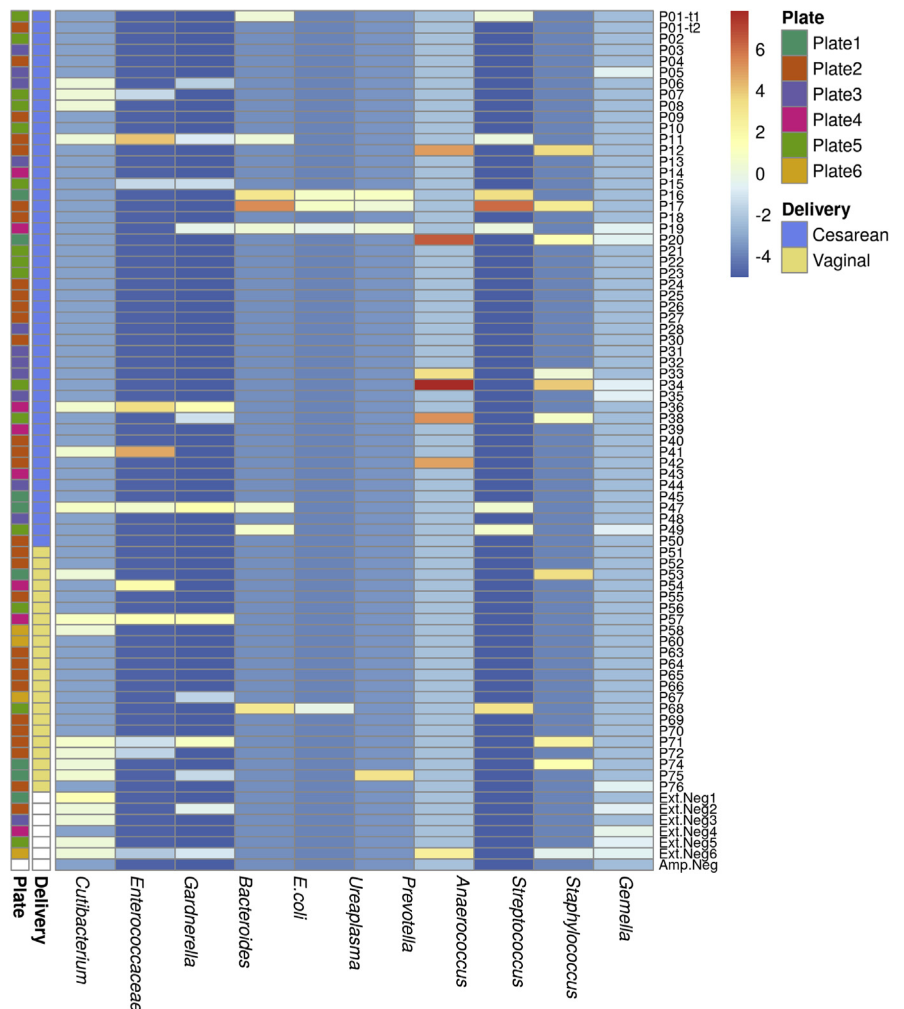
A direct comparison of the 16S gene sequencing data from cesarean-delivered placentas to the metagenomic sequencing data of saliva, feces, and vaginal samples is not possible. However, it is noteworthy that there are a few signals that were confirmed by at least 2 methods and not recovered in any other body site, such as *Acinetobacter* (n=2), *Cupriavidus* (n=3), *Gemmatirosa* (n=1), and *Pelomonas* (n=7). All of these are typically free-living bacteria.

Bacterial growth inhibition was observed with placental tissue

We did not find a strong bacterial signal in cesarean-delivered samples, but we observed a large variation of qPCR quantification in placental tissues, some of which had a much higher bacterial load than others. Thus, we tested whether the placenta could inhibit bacterial growth and whether this effect is influenced by the bacterial load according to our qPCR analysis (Figure 6, A). We selected frozen placental tissues (both maternal [PM] and fetal [PF] side) from 3 cesarean deliveries with relatively high 16S rRNA gene load (PM high and PF high) according to our qPCR results and 3 deliveries with low 16S rRNA gene load (PM low and PF low). Growth inhibition analysis showed that co-culture with placental tissues resulted in significantly less *E coli* growth than bacteria that grew without placental tissue (P<.01) (Figure 6, B).

When comparing the *E coli* growth between each group (PM high, PF high, PM low, PF low, and no placenta control), using a Kruskal-Wallis H test, an overall significant difference was observed (P=.033). In addition, posthoc tests comparing each placenta group with the control group showed that only placental tissues with high 16S rRNA gene load had a significant inhibition effect compared with *E coli* in LB broth (*E coli* in LB vs PM high; P=.042; *E coli* in

FIGURE 4
Selected bacterial taxa could be detected in low numbers in the maternal side of a few placentas



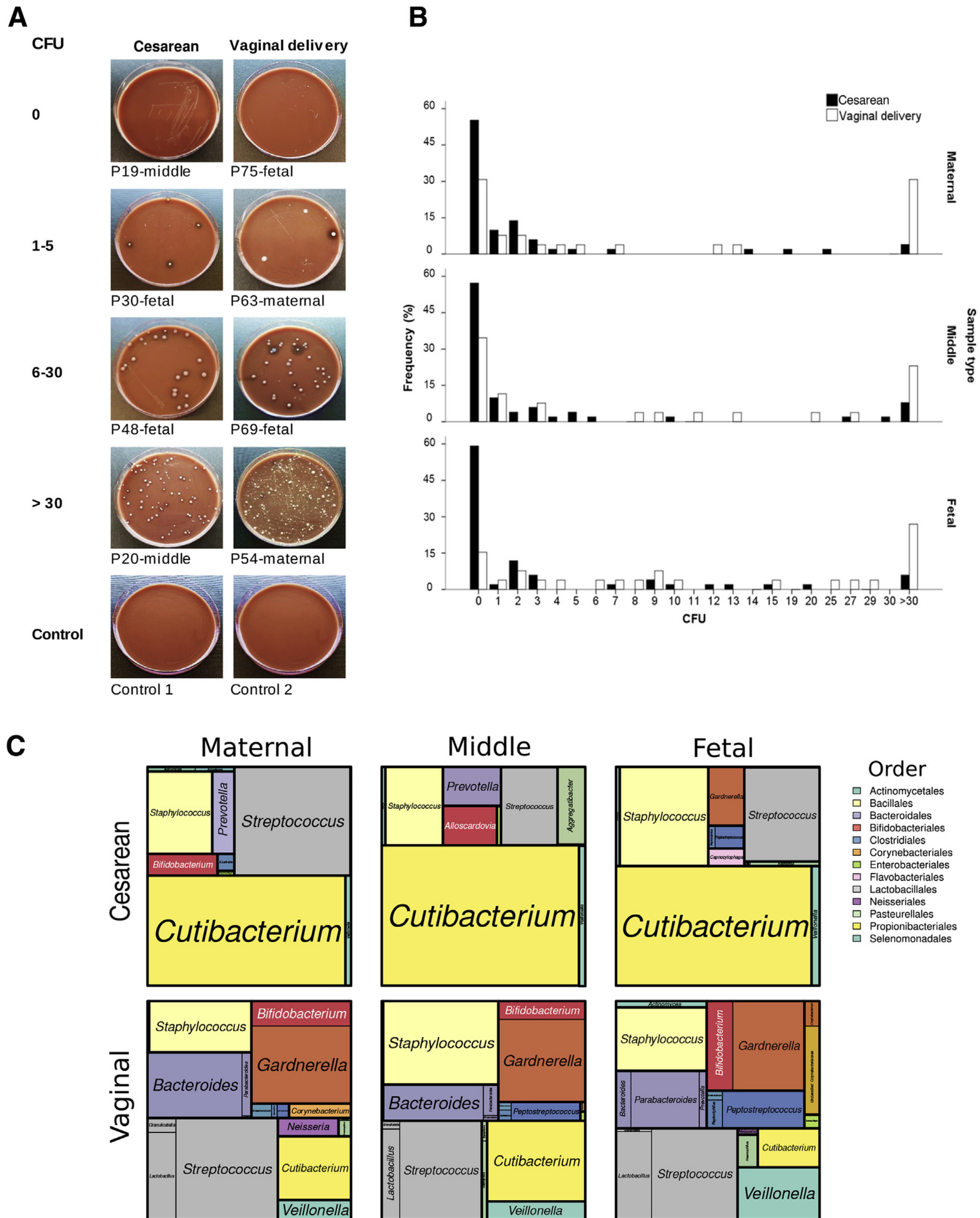
Probe-based, taxon-specific qPCR was used to detect DNA from bacterial taxa suspected to be present in the placenta on the basis of sequencing data and previous studies. Supplemental Tables 1 and 2 provide a list of probe-based qPCR primers, probes, and gBlocks. Bacterial concentration is expressed as $\log_{10}$ of 16S copy numbers per nanogram of total DNA, from 10^{-4} (blue) to 10^{6} (red). "P" stands for participant and "t" for twin. The varying estimated copy numbers for each probe in negative samples depend on that probe's specific binding characteristics and limits of detection. In addition to placental samples, a randomly selected negative extraction control from each plate was amplified (marked "Neg.Ext"). A negative amplification control is marked "Neg.Amp." The 2 columns on the left depict the delivery type and extraction plate for each sample. Although the negative amplification control presents no signals, all the negative extraction controls have at least a few signals, mostly associated with typical skin bacteria. These weak signals correlate poorly to the signals detected in placentas extracted in the same plate, highlighting the high degree of variability observed when working in this concentration range.

qPCR, quantitative polymerase chain reaction.

Sterpu et al. No evidence for a placental microbiome in full-term pregnancies. Am J Obstet Gynecol 2021.

FIGURE 5

Bacteria grown from placentas are predominantly typical skin and vaginal taxa



A, GC agar plates showing the bacterial growth from placental tissues after 48 hours. The placenta cultures are represented in ranges according to the number of CFUs. "P" stands for participant. **B,** Histogram showing frequency (percentage) distributions of CFUs according to the placental sample type and the delivery mode. Chi-squared tests (Fisher exact tests) were performed with significance level at $P=.05$. The comparison was between vaginal delivery (*white bars*) and cesarean delivery (*black bars*) in each CFU range group. **C,** Treemaps showing the relative proportion of the taxa that grew in culture by location in the tissue and mode of delivery. Each area is colored according to the bacterial order, as shown in the legend, and the genus is overlaid on the *boxes* themselves.

CFU, colony-forming unit; *GC*, guanine-cytosine.

Sterpu et al. No evidence for a placental microbiome in full-term pregnancies. Am J Obstet Gynecol 2021.

TABLE 2
Bacterial growth of placental tissues according to the delivery mode

	Delivery mode						Cesarean delivery vs vaginal delivery
	Cesarean delivery (N=152)[a]			Vaginal delivery (N=78)			
CFU range	Maternal (n=51)	Middle (n=50)	Fetal (n=51)	Maternal (n=26)	Middle (n=26)	Fetal (n=26)	*P* value
0	28 (54.9)	29 (58.0)	30 (58.8)	8 (30.8)	9 (34.6)	4 (15.4)	<.001
1–5	17 (33.4)	13 (26.0)	10 (19.6)	7 (26.9)	5 (19.2)	5 (19.2)	.787
6–30	4 (7.8)	3 (6.0)	8 (15.7)	3 (11.5)	6 (23.1)	10 (38.5)	.996
>30	2 (3.9)	5 (10.0)	3 (5.9)	8 (30.8)	6 (23.1)	7 (26.9)	<.001

Data are expressed as absolute and relative values for total plate count number (percentages). *P* value shows the differences between delivery groups at the level of the CFU range. The significance level was set at $P<.05$. The Fisher exact test was used for comparisons.

CFU, colony-forming unit.

[a] One mother delivered after a dichorionic diamniotic pregnancy.

Sterpu et al. No evidence for a placental microbiome in full-term pregnancies. Am J Obstet Gynecol 2021.

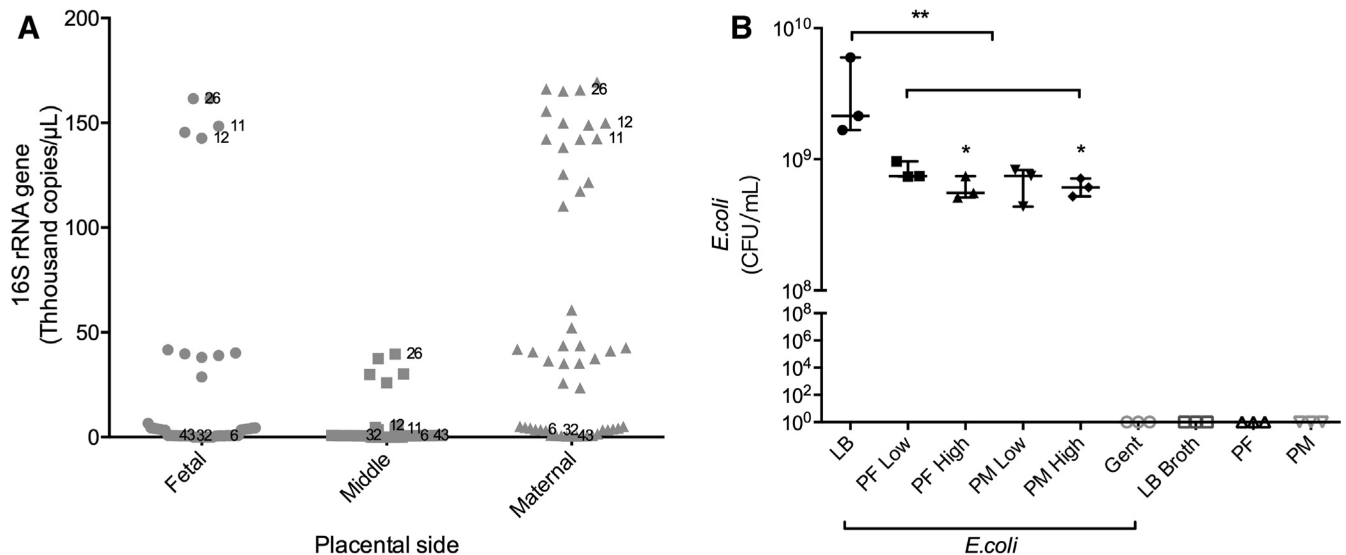
LB vs PF high; P=.048). Other comparisons did not reach statistical significance but showed the following trend: the placental tissues (both maternal and fetal sides) with high bacterial gene load had a stronger inhibition effect than the tissues (both maternal and fetal sides) with low bacterial gene load.

Comment

Principal findings

This study was designed to investigate the potential presence of a placental

FIGURE 6
The placenta presents antimicrobial activity



A, Total 16S rRNA gene counts of specimens from the fetal, middle, or maternal side of each individual placental sample (51 maternal, 50 middle, and 49 fetal), as determined by qPCR. Each *dot* represents 1 sample. The individuals whose samples were later used for inhibition experiments in (B) are labeled with their participant number. **B,** Twelve frozen placental specimens from 6 cesarean deliveries were selected on the basis of the qPCR results; 3 high vs 3 low bacterial content in the fetal and maternal sides (as shown in A). The inhibition effect on the proliferation of *E coli* cultured in LB alone was assessed by co-culturing the selected placental tissues with 10 CFUs of *E coli*/well in a 96-well plate. Controls were LB broth inoculated with 10 CFUs of *E coli* and 10 μg/mL gentamycin, LB broth alone, and placental tissues alone. All culture conditions were performed in triplicate. After incubation for 24 hours at 37°C, *E coli* CFUs from each well were enumerated. Data are presented as median±interquartile range in a log 10 scale, and each dot represents the average of the triplicates. Statistical comparisons were performed between *E coli* grown in LB and each placenta group and between *E coli* grown in LB and all placenta groups together. The *asterisk* indicates $P<.05$, whereas the *double asterisk* indicates $P<.01$.

CFU, colony-forming unit; *LB*, Luria-Bertani broth; *PF*, the fetal side of placenta; *PM*, the maternal side of placenta; *qPCR*, quantitative polymerase chain reaction.

Sterpu et al. No evidence for a placental microbiome in full-term pregnancies. Am J Obstet Gynecol 2021.

microbiome in samples from women not in labor with elective cesarean delivery vs samples from women who underwent vaginal deliveries at term. Using qPCR and 16S rRNA gene sequencing, we found that bacterial signals from placental tissue were not distinguishable from background signals, except in some individual cases. Samples with detectable bacterial signals were present in both cesarean and vaginal deliveries and were not associated with any specific health conditions. The apparently random nature of the bacterial signal between different methods (culturing, qPCR, 16S rRNA gene sequencing), combined with the weakness of these signals, suggests that any bacteria present in the human placenta are at the limit of detection of most available technologies. Other investigations of the same placental tissue, using probe-based qPCR targeting commonly reported bacteria from the reproductive tract[2] and commonly reported contaminants,[40,50] did not provide clear evidence of a group difference or of a specific microbiome.

To obtain a comprehensive picture of potential bacterial colonization in utero, we also performed bacterial gene sequencing of the AF and vernix caseosa samples collected from the newborn. As for the placental samples, 16S rRNA gene quantitative amplification sequencing could not conclusively distinguish any microbial content of the AF and vernix caseosa from background signals. Thus, our conclusion is that bacterial presence in pregnancy at term is low and cannot be considered proof of the presence of a microbiome.

Results: in the context of what is known

Our findings are supported by several previous reports that were unable to distinguish between sample signals and contamination introduced during DNA purification.[35,36,38] A recent study of 500 placentas from human pregnancies concluded that the placenta has no microbiome but can contain pathogens.[34]

Although there are studies that corroborate our findings, the literature regarding the existence of a microbiome in the placenta is controversial, and other studies in the field have presented opposite conclusions. Aagaard et al[21] reported the detection of bacterial DNA sequences in placental samples from multiple taxa that were suggested to originate from the oral cavity. A possible reason for the contradictory findings is that some studies have included samples from preterm or other complicated pregnancies.[25,28,31] Other circumstances that might differ between studies are the time between the delivery of the placenta and the sampling. One study investigated the effects of the start of the ischemic process (after the placenta has been parted from the maternal circulation) on the presence of bacteria in the placenta and concluded that placental samples should be processed within 10 minutes of the delivery to minimize the impact of the degradation process in the tissue.[51] In summary, the sampling procedure, the time between delivery and sample conservation in the medium, and the type of preservation medium can impact the results.[52]

In bacterial cultures from placental tissues examined in this study, some samples produced live bacterial colonies. However, only a few samples resulted in cultures with a high number of colonies. Notably, substantially fewer colonies were found in cesarean-delivered placental tissue cultures. In fact, most live colonies observed in cultures of placental tissue from cesarean deliveries were common skin bacteria (*Propionibacterium*, *Streptococcus*, and *Staphylococcus*),[53] which likely represent contaminations that occurred during surgery. Placental tissue cultures from vaginally delivered placentas also contained common vaginal microbiota species (*Lactobacillus* spp., *Gardnerella*, and *Bifidobacterium*),[54] a plausible sign of contamination from vaginal delivery. This is supported by other studies that have reported data on culture or in situ hybridization experiments on placenta.[29,30]

The placental tissue has no lumen, and colonizing bacteria would be subject to elimination by circulating immune cells and other immune effectors, such as antimicrobial peptides and immunoglobulins from the blood.[15,16] Our findings indicate that placental tissue has the capacity to inhibit bacterial growth. This effect was more pronounced in the tissue specimen with a higher 16S rRNA gene signal. It can be speculated that a higher number of bacteria in the tissue may also trigger a stronger immune reaction, resulting in a higher antibacterial inhibition effect. Thus, our findings reinforce the notion that the uterus during healthy pregnancy is sterile or at least a low bacterial milieu.

Previous studies have used placental extracts and proven its anti-inflammatory capacity.[55,56] Moreover, antimicrobial peptides, like human β-defensins, have been detected in AF.[57–59] Similarly, fetal membranes have shown antimicrobial properties[60,61] and the capacity to suppress group B *Streptococcus*.[62] Furthermore, vernix caseosa possesses anti-inflammatory properties in vivo.[43,63]

Taken together, the physiology of the placenta and that of the uterus seem to discourage bacterial colonization—unlike tissues that are known to be colonized (such as the gut, skin, mouth, and vagina). In our study, we used multiple methods, including various molecular methods (total qPCR of the 16S rRNA gene, probe-specific qPCR for selected taxa, and 16S rRNA gene sequencing), to investigate the placental tissue and bacterial cultures derived from the tissue. Comparing the results obtained from qPCR with those obtained from 16S rRNA gene sequencing or culturing, the overlap among the taxa was very low. These highly diverse findings may have resulted from the extremely low signals, which were close to the limit of detection, creating stochastic observations of specific bacteria in samples. Some of our results, such as the *Massilia* findings, were not always algorithmically flagged as a contaminant in this study. However, *Massilia* is a well-known laboratory contaminant,[40] which is often observed in our negative extraction controls. Many species in this genus were initially isolated from air samples, suggesting that they easily spread by this route and can therefore be difficult to completely eliminate. These previous observations,

combined with the even distribution of *Massilia* spp. across almost all samples, make it highly unlikely that it is a true signal. *Massilia* spp. are found in soils or fresh water. However, various species have been isolated from patient material, such as blood, bone, cerebrospinal fluid, and intraocular fluid.[64] *Massilia* are not typically reported as a human commensal bacterium.

Clinical implications

It can be hypothesized that bacteria that enter the placenta are killed or inhibited during normal healthy gestation and that this function is strengthened during the course of gestation, because the placenta serves as a tool with which the immune system protects the fetus from microbes.[65] In an experimental study using *Salmonella enterica* serovar Typhimurium to infect human placentas, viable bacteria were recovered from 100% of the placental explants exposed to the strain, but the bacterial numbers obtained from first trimester of pregnancy tissues were markedly higher than those obtained from second trimester and term placental of pregnancy tissues,[66] indicating an evolving immune function of the placenta during the course of pregnancy.[67] Moreover, a recent study suggested that conditions in the fetal gut strongly limit bacteria.[68] A possible explanation is the continuous exposure of the fetal gut to AF. Another important piece of evidence pointing toward a bacterial colonization of the infant only after birth is the difference in early microbial colonization between infants born vaginally and those born through cesarean delivery.[69] Finally, the ability to breed germ-free mammals is strong evidence that the fetus is sterile. Germ-free neonates have been generated for mice, rats, rabbits, guinea pigs, cats, dogs, lambs, pigs, calves, goats, baboons, chimpanzees, marmosets, and humans (reviewed in Perez-Muñoz et al[1]). There are cases in which, because of the expected severe immune deficiencies, human infants have been delivered in sterile conditions and kept in isolators where they remained sterile.[70,71] These facts are convincing evidence, if not for the sterile womb hypothesis, for the low bacterial presence in the womb. Thus, if the physiology of the placenta does not encourage bacterial colonization, it might be possible for bacteria to enter the uterine cavity but not to colonize it. This could explain the random presence of bacteria in a few samples during analysis, but it should not be considered a placental microbiome.

In summary, despite the presence of a small number of bacteria in some samples, our interpretation of the results is that the placenta does not harbor a distinct microbiome. In accordance with the literature,[33–39] our data indicate that healthy placental tissue facilitates the inhibition of bacterial growth. However, because bacteria are present everywhere in the environment, it is very difficult to prove the absolute absence of bacteria in the samples.

Strengths and limitations

The most important strength of the study is the comprehensive and controlled sample collection from women not in labor undergoing cesarean delivery at term and from women delivering vaginally, in which case contaminations from the passage through the birth channel could be expected. Sampling was performed exclusively by the 2 clinicians in the group and included placental tissue from 3 parts of the placenta divided into 3 layers and AF and infant vernix caseosa. Samples from the oral cavity, gut, and vagina were collected to allow for the comparison with potential findings from the placental tissue. Furthermore, detailed clinical data were obtained from each study subject and reported to allow for a careful evaluation of each case. Multiple methods for the analysis of bacterial DNA and bacterial culture and growth inhibition were combined in this study to investigate whether the amniotic cavity contains bacteria in normal pregnancies at term.

Limitations of our study include the fact that some of the mothers had been treated with antibiotics during pregnancy or at delivery, which may have influenced the results. However, only 18% of the women who underwent cesarean deliveries and 11% of the women who delivered vaginally received antibiotics, and no differences in copy number or bacterial growth pattern could be seen for those cases. Another limitation of the study is that the AF was collected in very different ways from women undergoing cesarean or vaginal deliveries. Therefore, the bacterial signals from these 2 sample types could not be effectively compared. Furthermore, in the culture experiments of placental tissue, freezing of biologic samples before culture may have influenced the recovery of some bacterial clones. In addition, for the culture experiments, only 1 growth medium and anaerobic culture conditions were used. Thus, this may not be the optimal culture conditions for all bacterial species. However, our study used multiple methods, and results are therefore not only dependent on 1 experimental condition.

The technical limitations of our study are related to the difficulty of accurately characterizing microbial communities in low microbial biomass niches, especially because there is no clear understanding of how many bacteria constitute a tissue microbiome. The inclusion of controls with placental tissue samples that have been spiked with known numbers of bacteria would help to define a limit of DNA detection in our study. Moreover, controls of human tissue with very low confirmed biomass, such as muscle, could have been used as controls but were not available to us.

Conclusions

Our data, together with recent reports and current immunologic understanding, do not support the existence of a placental microbiome. We conclude that the healthy fetus develops in utero in an environment enclosed by the amniotic membrane that is free of bacterial colonization. ■

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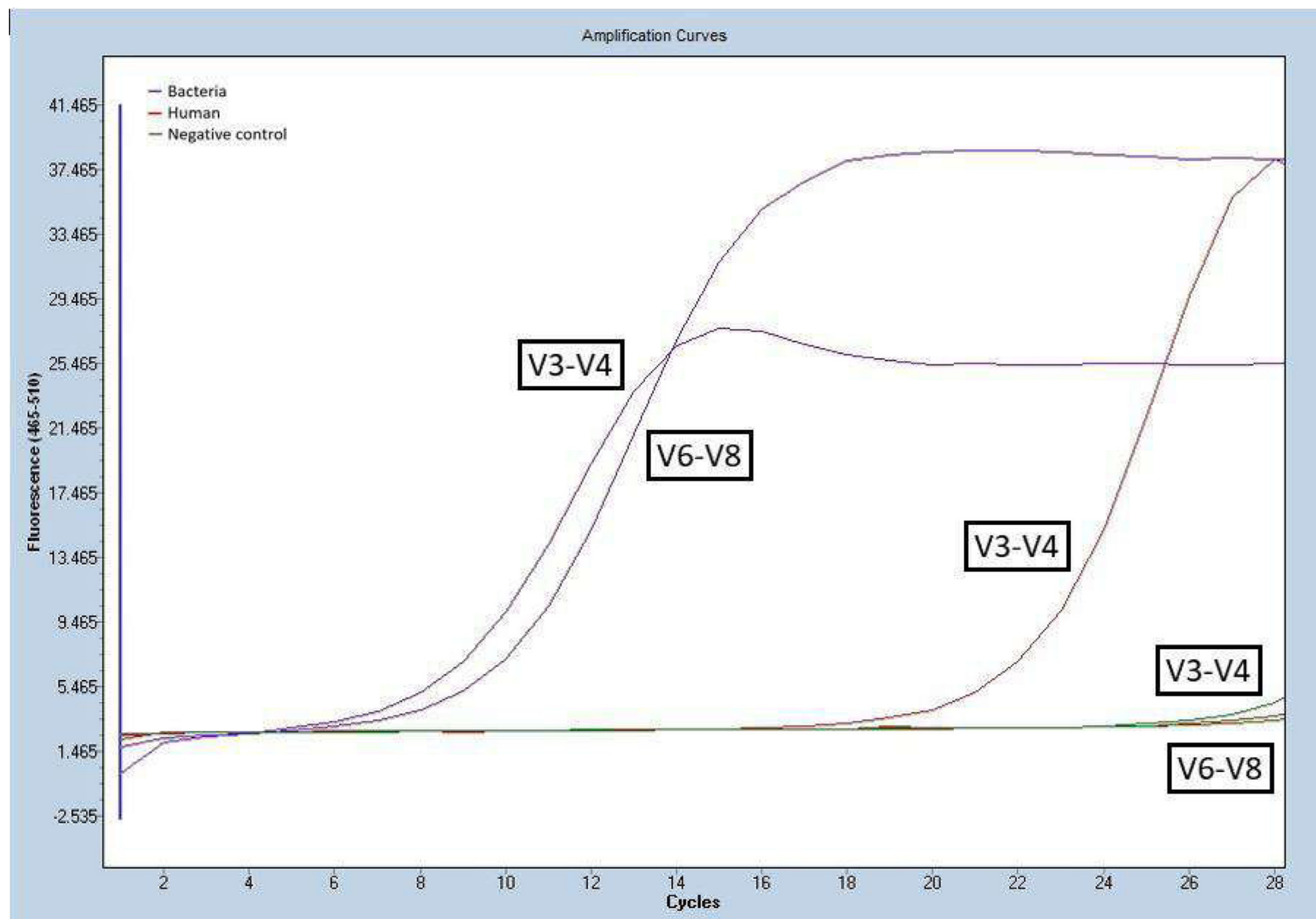
SUPPLEMENTAL FIGURE 1

V3-V4 universal bacterial primers also amplify human DNA

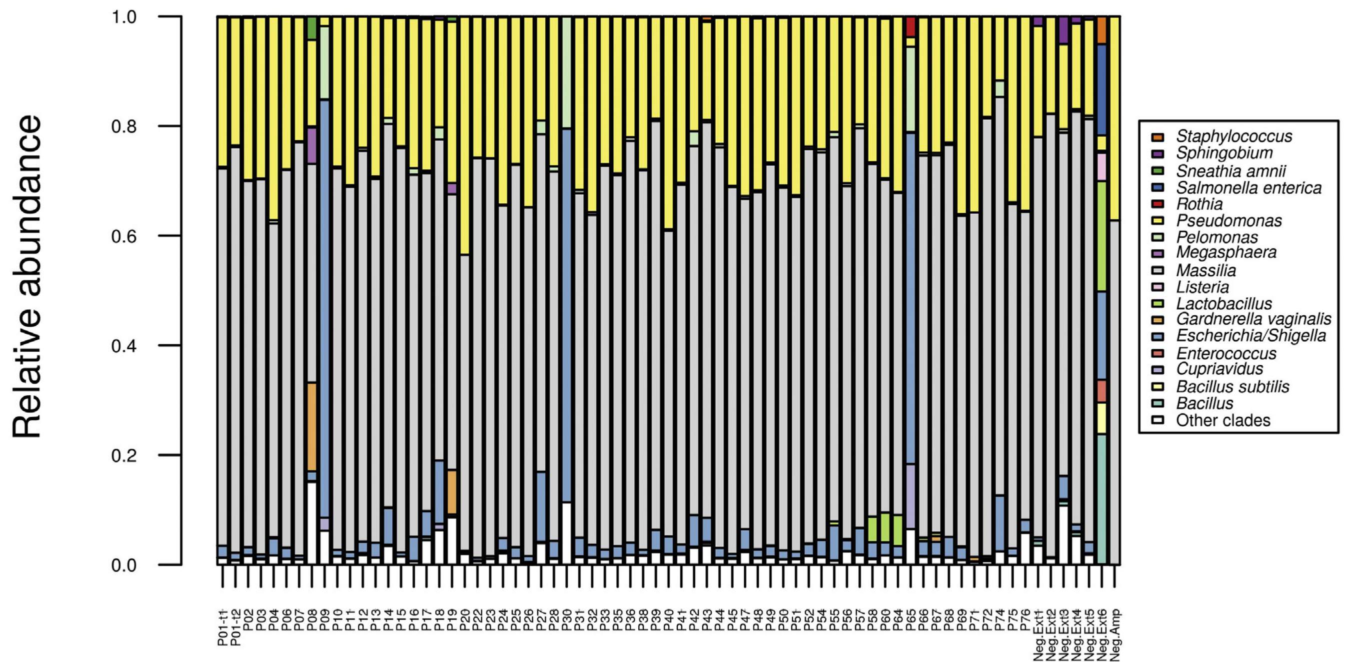


Amplification of mostly bacterial vs pure human DNA by universal 16S primers V3-V4 or V6-V8. *Purple lines* depict ZymoBIOMICS Microbial Community DNA Standard (artificial microbial consortium). *Blue lines* depict pure human DNA. *Green lines* depict negative template control.

Sterpu et al. No evidence for a placental microbiome in full-term pregnancies. Am J Obstet Gynecol 2021.

SUPPLEMENTAL FIGURE 2

Abundant taxa detected in placenta and negative controls

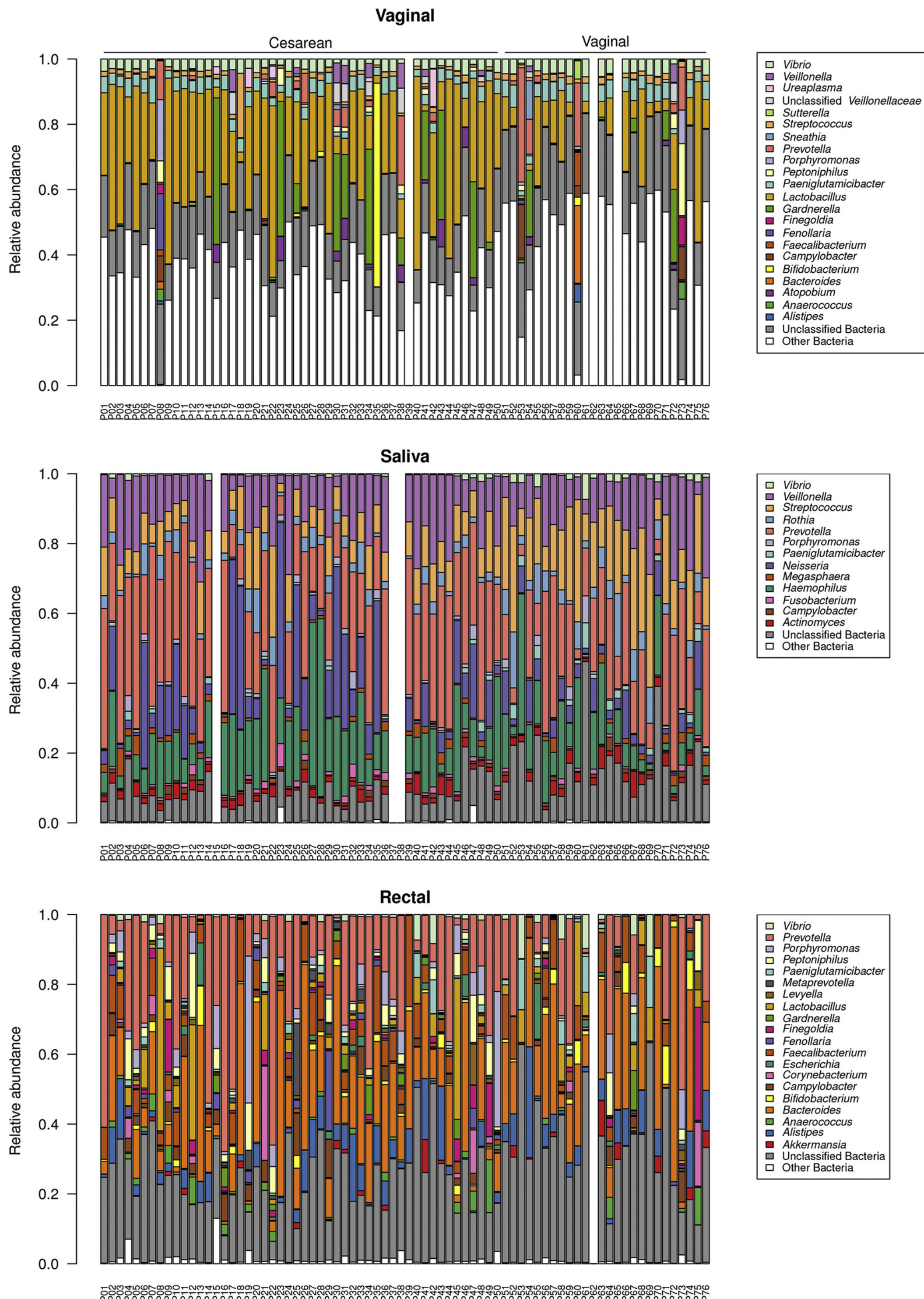


The maternal side of the placentas from women who had not taken antibiotics during pregnancy or delivery (n=45 for cesarean delivery; n=19 for vaginal delivery) was profiled by sequencing of the V6-V8 region of the 16S rRNA gene. “P” stands for participant and “t” for twin. The Neg.Ext labels are negative controls from each of the extraction plates, whereas the Neg.Amp label is a negative amplification (PCR) control, as described in the methods.

PCR, polymerase chain reaction; *rRNA*, ribosomal ribonucleic acid.

Sterpu et al. No evidence for a placental microbiome in full-term pregnancies. Am J Obstet Gynecol 2021.

SUPPLEMENTAL FIGURE 3
Bacterial profile of vaginal swabs, saliva, and rectal swabs at the time of delivery

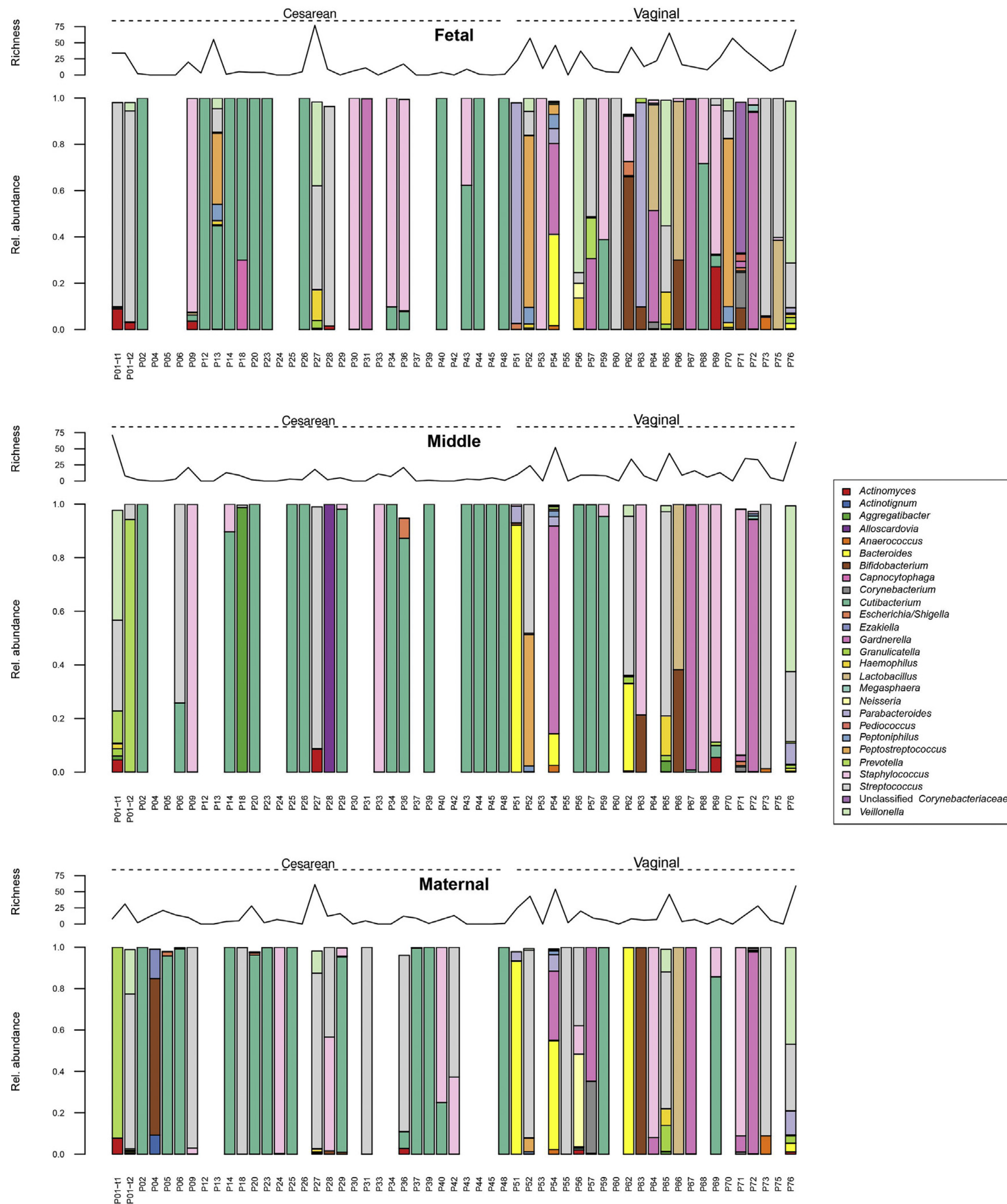


Bar plots depict the relative abundance of each taxon in each sample, taken at the time of delivery from each participant and analyzed by shallow shotgun sequencing, as described in the methods. The taxa that appear in more than 1 dataset have consistent colors. Each participant's samples are stacked in each dataset. "P" stands for participant. Bacterial DNA from vaginal and rectal samples and saliva were characterized by metagenomic sequencing.

Sterpu et al. No evidence for a placental microbiome in full-term pregnancies. Am J Obstet Gynecol 2021.

SUPPLEMENTAL FIGURE 4

Bacteria from cesarean-delivered placentas are similar to skin, whereas vaginal origin is common for normal deliveries



Richness (number of identified amplified sequence variants) and taxonomic composition of bacterial isolates grown from placental specimens, based on sequencing of the V6-V8 region of the 16S rRNA gene. For each set of panels, richness is represented at the top and composition at the bottom. Study participants are aligned in all plots, and their study number is depicted under each *bar plot*. Where a column is left *blank*, no bacterial colonies were obtained. The colored legend is common to all 3 panels. Top indicates the fetal side of the placenta, middle indicates the middle part, and the bottom indicates the maternal side.

rRNA, ribosomal ribonucleic acid.

Sterpu et al. No evidence for a placental microbiome in full-term pregnancies. Am J Obstet Gynecol 2021.

SUPPLEMENTAL TABLE 1

Probe-based qPCR primers and probes used as standard

Clade	Forward	Reverse	Melting temperature	Probe	Tm	Fluorophore
Cutibacterium	GGAAGTGTAATCTTGGGGCTTA	CTGGTGTTCCTCCTGATATCTG	59	CCACCGCTCCACCAGGAATT	65	FAM (498-580)
Enterococcaceae	CGGTTTCTTAAGTCTGATGTGA	TCCTCCATATATCTACGCATTTC	59	ATTCCACTCTCCTCTTCTGC	65	Red_610 (533-610)
Gardnerella	GGTTGTAAACCGCTTTTGATTG	CAAGCTCTTTACGCCCAATAATTC	59	AGTGTACCTTTCGAATAAGC	65	Cy5/Cy5-5 (618-660)
Bacteroides	ATGGGGATGCGTTCCAT	GCACGGAGTTAGCCGAT	58	CTGAACCAGCCAAGTAG	63	FAM (498-580)
Escherichia coli	GAGGAAGGGAGTAAAGTTAATAC	GGGATTTCACATCTGACTTAAC	58	CTCATTGACGTTACCCGCAG	63	Red_610 (533-610)
Ureaplasma	GAACGATGAAGGTCTTATAGATTG	ACGCTTGCATCCTATGTATTAC	58	CATAGTTAGCCGATACTTATTCAA	63	Cy5/Cy5-5 (618-660)
Streptococcus	TAACGCGTAGGTAACCT	TACTGCTGCCTCCCGTAGGA	57	GGACCTGCGTTGTATTA	62	Cy5/Cy5-5 (618-660)
Anaerococcus	AACGCGTGAGTAACCTGCCTT	CACTGCTGCCTCCCGTAGGAGT	57	GTGTACGGCCACATTGGG	62	Red_610 (533-610)
Prevotellaceae	GGCGGGGTAACGGCCCAC	GGAATTAGCCGGTCCTT	57	ACCAGCCAAGTAGCGTG	62	FAM (498-580)
Staphylococcus	GGTACCTAATCAGAAAGCCACG	GCGCGCTTTACGCCCAATAATTC	57	CGGATAACGCTTGCCACCTAC	63	FAM (498-580)

FAM, fluorescein amidite; *qPCR*, quantitative polymerase chain reaction.

Sterpu et al. No evidence for a placental microbiome in full-term pregnancies. Am J Obstet Gynecol 2021.

SUPPLEMENTAL TABLE 2
Bacterial taxa-specific gBlock sequences used as standard

Clade	gBlock sequence
Cutibacterium	CGCATTTATTGGGCGTAAAGGGCTCGTAGGTGGTTGATCGCGTCGGAAGTCTAATCTTGGGGCTTAACCCTGA GCGTGCTTTTGATACGGGTTGACTTGAGGAAGGTAGGGGAGAATGGAATTCCTGGTGGAGCGGTGGA ATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGTTCTTCGGGCCTTTCCTGACGC
Enterococcaceae	CGRCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCATATATCTACGCATTTCACCGCTACACATGGAATTCCACTCT CCTCTTCTGCACTCAAGTTTCTCAGTTTCCAATGACCCTCCCCGGTTAAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGC TCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCACCTACGT
Gardnerella	TTCACACCAGACGCGACGAACCGCCTACAAGCTCTTTACGCCCAATAATTCCGGATAACGCTTGCGCCCTACGTATTACCGCGGC TGCTGGCACGTAGTTAGCCGGCGCTTATTCGAAAGGTACACTCACCCGAAAGCTTGCTCCCAATCAAAAGCGGTTTACAACCCGA AGGCCTTCATCCCGCACGCGGCGTCGCTGCGTCAGGGTTTCCCCCATTGCGCAATATTCCCCACTGCTGCCTCC
Bacteroides	CATCTTGAGAAAGTTAAAGATTTATTGGTTATGGATGGGGATGCGTTCCATTAGATAGTTGGTGAGGTAACGGCTCACCAAGTCTTCG ATGGATAGGGGTTCTGAGAGGAAGGTCCCCCACATTGGTACTGAGACACGGACCAAACTCCTACGGGAGGCAGCAGTGAGGAATATT GGTCAATGGGCGAGAGCCTGAACCAGCCAAGTAGCGTGAAGGATGAAGGTCCTATGGATTGTAAACTTCTTTTATAGTAGAATAAAGT GACCCACGTGTGGGTTTTTGTATGTATACTATGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCGAGC
Escherichia coli	TGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTTGCTCATT GACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAA TTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCT
Ureaplasma	GGGAATTTTTCACAATGGGCGAAAGCCTTATGAAGCAATGCCGCGTGAACGATGAAGGTCTATAAGATTGTAAAGTTCTTTTATTTGGG AAGAACCACTAAAATAGGAAATGATTTTAGTTTGACTGTACCATTTGAATAAGTATCGGCTAACTATGTGCCAGCAGCCGCGGTAATACA TAGGATGCAAGCGTTATCCGGATTTACTGGGCGTAAAACGAG
Streptococcus	CACTCACGCGGCGTTGCTCGGTCAGGGTTCCCCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAG TCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATGTATCGAAGCCTTGGTAAGCCGTTACCTTACCAACTAGCTAATACAACGCAGGT CCATCTTTAAGTGGTGCACTTGCACCTTTTAAGTAGCTGACATGTGTCCGCCACTATTATGCGGTATTAGCTATCGTTTCCA
Anaerococcus	CAGGGTTTCCCCCATTGTGCAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAATGTGGCCGTACACTCT CTCAAGCCGGCTACTGATCGTTGCCTTGGTGAGCTGTTATCTCACCAACTAGCTAATCAGACGCAAGTCCATCTTAGAGCGATAAATCT TTGACCAGCACTTCATGCGAGGTGTTGGTTTCATAGGGTATTATTCTTCGTTTCCAAAGGCTATCCCCTTCTCTAAGGCAGGTTACTCAC GCGTTACTCACCCGTCCGCCACTAATCCATCTAATTTCACTCCGAAGAGATCAATTAGGTTTCATCGTTCGACTTGCATGTGTTATGCA CGCCGCCAGCGT
Prevotellaceae	ATCCGATTTGGACCAAAGGCTTAGCGGTAAAGGATGGGGATGCGTCCGATTAGCTTGACGGCGGGGTAACGGCCCACCGTGGCAACGAT CGGTAGGGGTTCTGAGAGGAAGGTCCCCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTC AATGGGCGTAAGCCTGAACCAGCCAAGTAGCGTGCAGGATGACGGCCCTATGGGTTGTAAACTGCTTTTATGCGGGGATAAAAGAGCCCA CGTGTGGGTTTTTGCAGGTACCGCATGAATAAGGACCGGCTAATTCCGTGCCAGCAGCCGCGG
Staphylococcus	TGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTG ATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTAACTGTGCACGTCTTGACGGTACCTAATCAGAAAGC CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTT CTTAAGTCTGATGTGAAAGCCC

Sterpu et al. No evidence for a placental microbiome in full-term pregnancies. Am J Obstet Gynecol 2021.

SUPPLEMENTAL TABLE 3
A comparison of observed bacterial clades with literature data

Clade	Observed in our study (16S gene sequencing)	Observed in our study (culture)	Previously observed in other studies	Comment
Cutibacterium	Yes	Yes	Aagaard et al[12]	Formerly known as *Propionibacterium*
			Bassols et al[13]	
			Collado et al[14]	
			Zheng et al[23]	
Enterococcaceae	Yes	No	Bassols et al[13]	Genus *Enterococcus* was the intended target, but no genus-level probe with good characteristics could be designed
			Zheng et al[23]	
Gardnerella	Yes	Yes	DiGiulio et al[7]	—
			Bassols et al[13]	
			Doyle et al[16]	
Bacteroides	Yes	Yes	Aagaard et al[12]	—
			Bassols et al[13]	
Escherichia coli	Yes	Yes	Aagaard et al[12]	Genera *Escherichia* and *Shigella* were the desired target, but a species-level assay was chosen to avoid unspecific binding
			Bassols et al[13]	
			Collado et al[14]	
			Zheng et al[23]	
Ureaplasma	No	No	DiGiulio et al[7]	—
Streptococcus	Yes	Yes	DiGiulio et al[7]	—
			Bassols et al[13]	
			Collado et al[14]	
			Gomez-Arango et al[17]	
			Zheng et al[23]	
Anaerococcus	Yes	No	—	—
Prevotellaceae	Yes	Yes	DiGiulio et al[7]	Genus *Prevotella* was the intended target, but no genus-level probe with good characteristics could be designed
			Aagaard et al[12]	
			Gomez-Arango et al[17]	
			Zheng et al[23]	
Staphylococcus	Yes	Yes	DiGiulio et al[7]	—
			Aagaard et al[12]	
			Bassols et al[13]	
			Collado et al[14]	
			Zheng et al[23]	
Gemella	Yes	No	Bassols et al[13]	Excluded because of very high background signal
Lactobacillus	Yes	Yes	Aagaard et al[12]	Excluded because of unspecific binding to human DNA
			Bassols et al[13]	
			Collado et al[14]	
			Zheng et al[23]	

Sterpu et al. No evidence for a placental microbiome in full-term pregnancies. Am J Obstet Gynecol 2021.

SUPPLEMENTAL TABLE 4

Number of specimens from cesarean deliveries with bacteria detection by different methods and in different body sites

Genus	Positive culture samples (n=51)	Postive sequencing samples (n=48)	Positive qPCR samples (n=48)	Overlap culture vs sequencing	Overlap culture vs qPCR	Positive vaginal swabs (n=50)	Positive saliva samples (n=50)	Positive rectal swabs (n=50)
Acinetobacter	5	9	NA	2	NA	0	0	0
Aerococcus	2	2	NA	0	NA	49	47	48
Anaerococcus	4	0	7	0	0	21	0	47
Bacteroides	3	0	7	0	0	49	41	50
Blastococcus	1	4	NA	0	NA	0	0	0
Bradyrhizobium	2	3	NA	0	NA	0	0	0
Burkholderia or Paraburkholderia	1	5	NA	0	NA	49	47	42
Cupriavidus	4	35	NA	3	NA	0	0	0
Cutibacterium	23	0	7	0	2	0	0	3
Dialister	1	2	NA	0	NA	27	46	46
Escherichia or Shigella	13	45	3	12	0	2	0	31
Gardnerella	6	3	7	0	1	28	0	25
Gemella	2	0	3	0	0	7	47	11
Gemmatirosa	2	10	NA	1	NA	0	0	0
Lactobacillus	7	5	NA	1	NA	49	47	50
Lawsonella	1	2	NA	0	NA	9	0	42
Leifsonia	5	42	NA	5	NA	0	0	2
Massilia	11	43	NA	10	NA	20	1	25
Megasphaera	1	2	NA	0	NA	11	46	24
Mesorhizobium	1	16	NA	0	NA	0	0	0
Methylobacterium	1	2	NA	0	NA	0	0	0
Microbacterium	3	2	NA	0	NA	1	0	5
Mycobacterium	1	4	NA	0	NA	48	46	40
Pelomonas	8	43	NA	7	NA	0	0	0
Prevotella	4	0	6	0	1	30	47	50
Pseudomonas	15	13	NA	7	NA	49	31	38
Staphylococcus	15	5	6	2	1	13	1	23
Streptococcus	16	3	6	0	1	49	47	50
Veillonella	4	1	NA	0	NA	24	47	38

Only bacteria detected in a placental biopsy by at least 2 methods are included.

NA, not applicable.

Sterpu et al. No evidence for a placental microbiome in full-term pregnancies. Am J Obstet Gynecol 2021.