OBSTETRICS

Is amniotic fluid of women with uncomplicated term pregnancies free of bacteria?

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BACKGROUND: The "sterile womb" paradigm is debated. Recent evidence suggests that the offspring's first microbial encounter is before birth in term uncomplicated pregnancies. The establishment of a healthy microbiota early in life might be crucial for reducing the burden of diseases later in life.
OBJECTIVE: We aimed to investigate the presence of a microbiota in sterilely collected amniotic fluid in uncomplicated pregnancies at term in the Preventing Atopic Dermatitis and Allergies in children (PreventADALL) study cohort.
STUDY DESIGN: Amniotic fluid was randomly sampled at cesarean deliveries in pregnant women in 1 out of 3 study sites included in the PreventADALL study. From 65 pregnancies at term, where amniotic fluid was successfully sampled, we selected 10 from elective (planned, without ongoing labor) cesarean deliveries with intact amniotic membranes and all 14 with prior rupture of membranes were included as positive controls. Amniotic fluid was analyzed by culture-independent and culture-dependent techniques.
RESULTS: The median (min-max) concentration of prokaryotic DNA (16S rRNA gene copies/mL; digital droplet polymerase chain reaction) was low for the group with intact membranes [664 (544—748)]—corresponding to the negative controls [596 (461—679)], while the rupture of amniotic membranes group had >10-fold higher levels [7700 (1066—251,430)] ($P = .0001$, by Mann-Whitney U test). Furthermore, bacteria were detected in 50% of the rupture of amniotic membranes samples by anaerobic culturing, while none of the intact membranes samples showed bacterial growth. Sanger sequencing of the rupture of amniotic membrane samples identified bacterial strains that are commonly part of the vaginal flora and/or associated with intrauterine infections.
CONCLUSION: We conclude that fetal development in uncomplicated pregnancies occurs in the absence of an amniotic fluid microbiota and that the offspring microbial colonization starts after uterine contractions and rupture of amniotic membrane.

Key words: amniotic fluid, bacteria, fetus, microbiome, microbiota, placenta, sterile

Introduction

The human microbiome discovery has developed quickly over the last decades with culture-independent techniques and unique microbial communities being identified in various body sites.[1,2] A diverse and well-balanced maternal and infant microbiome seems important for normal development of the child's immune system, and a dysbiotic maternal gut microbiome has been associated with offspring allergic disease development, as well as other immune-mediated diseases.[3—5] Identifying the timing of the initial microbial colonization of the offspring could therefore be helpful in further understanding the developmental origin of health and disease.[6]

It has recently been suggested, by the use of 16S rRNA sequencing, that amniotic fluid has a microbiome of its own in term uncomplicated pregnancies.[7] These findings are challenging earlier studies, where cultures from amniotic fluid were negative in term uncomplicated pregnancies with intact membranes.[8—10] The emerging evidence of a unique placental microbiome[11,12] are also questioning the "sterile womb" hypothesis.

Although sensitive molecular techniques are suggesting an intrauterine microbiota, the arguments for a sterile womb, including germ-free mice and contamination bias in molecular studies, are still strong.[13—15] However, the current evidence for a sterile intrauterine environment is inconclusive and to what extent, if, and how maternal microbiome influences the fetal immunological development and the shaping of the infant microbiome is not settled.[4,5]

The aim of our study was to investigate the presence of a microbiota in amniotic fluid in term uncomplicated pregnancies. We therefore combined sampling under strictly sterile and DNA-free conditions with highly sensitive techniques to determine the amniotic fluid bacterial load.

Materials and Methods

Study population

Within 22 months from December 2014, 2701 pregnant women were enrolled in the Preventing Atopic Dermatitis and Allergies in children (PreventADALL) study[16] in Norway and Sweden at the 18-week gestational age (GA) ultrasound screening.[16] Investigations included fetal ultrasound and maternal weight, length, and blood pressure on inclusion, with electronic questionnaires completed at 18- and 34-week GA to assess maternal health, family, sociodemographic, and lifestyle factors. The healthy newborn babies of at least GA 35 weeks were included for the mother-child cohort. All mothers consented to amniotic fluid sampling, in case of delivery by cesarean delivery at the Oslo University Hospital location, by signing the study consent form. From the PreventADALL cohort,[16] 65 women at Oslo University Hospital

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AJOG at a Glance

Why was this study conducted?

It is unclear if the amniotic fluid prior to delivery is sterile or not, the latter possibly influencing offspring health programming through in utero microbiota exposure.

Key findings

We found that prior to uterine contractions and rupture of amniotic membranes, amniotic fluid is sterile in uncomplicated term pregnancies.

What does this add to what is known?

This study resolves the uncertainty about a sterile intrauterine environment in uncomplicated pregnancies at term, due to stringent amniotic fluid sampling procedures, together with accurate and high-sensitivity microbiota analyses.

had amniotic fluid sampled during term cesarean delivery by dedicated health personnel in 3 different operating rooms. Out of these 65 women, 51 had intact amniotic membranes and 14 had prior rupture of amniotic membranes (ROM). For the no prior ROM group, we selected 10 amniotic fluid samples, all from elective term cesarean deliveries, none of these having started labor and all sampled in the same operating room. We included all 14 samples with prior ROM (ROM group) as positive controls for the non-ROM group (see Figure 1 for a detailed description on how the study population was selected). The study was approved by the Regional Committee for Medical and Health Research Ethics in South-Eastern Norway (2014/518) as well as registered at clinicaltrial.gov (NCT02449850).

Sampling

Amniotic fluid was collected in a sterile manner during elective (planned, with no ongoing labor) or acute (labor already started) cesarean delivery, after uterotomy, by aspiration of amniotic fluid through intact amniotic membranes using a sterile 19G needle and 10-mL syringe. The amniotic fluid samples were left at 4°C for maximum 24 hours and subsequently aliquoted into 1-2 sterile Cryotubes 4.5 mL SI 363452 (Millipore Sigma, Damstadt, Germany) and 0.5 mL into 1 sterile tube containing 1 mL Aimes medium (ESwab Copan 490CE; Thermo Fischer Scientific). These vials were stored at −80°C until further analysis. Negative controls were sampled from 2 different operating rooms using sterile containers with NaCl (9 mg/mL, 100 mL intravenous infusion; B. Braun), using the same sampling and aliquoting procedure as the amniotic fluid samples. In addition, 2 negative controls from the polymerase chain reaction (PCR) water used in the laboratory were included.

Initial handling and DNA extraction

Amniotic fluid (1 mL) was pulse centrifuged at 1200 rpm × 3 to remove large particles before it was centrifuged at 13,000 rpm for 10 minutes. We included negative controls in all steps, both sterile NaCl from the operating theater and PCR water from the laboratory. Pellet was washed twice in PBS suspended in 100 μL PBS, 50 μL was used for the DNA extraction, done manually by mag midi kit (LGC Genomics, United Kingdom) following the manufacturer's recommendations.

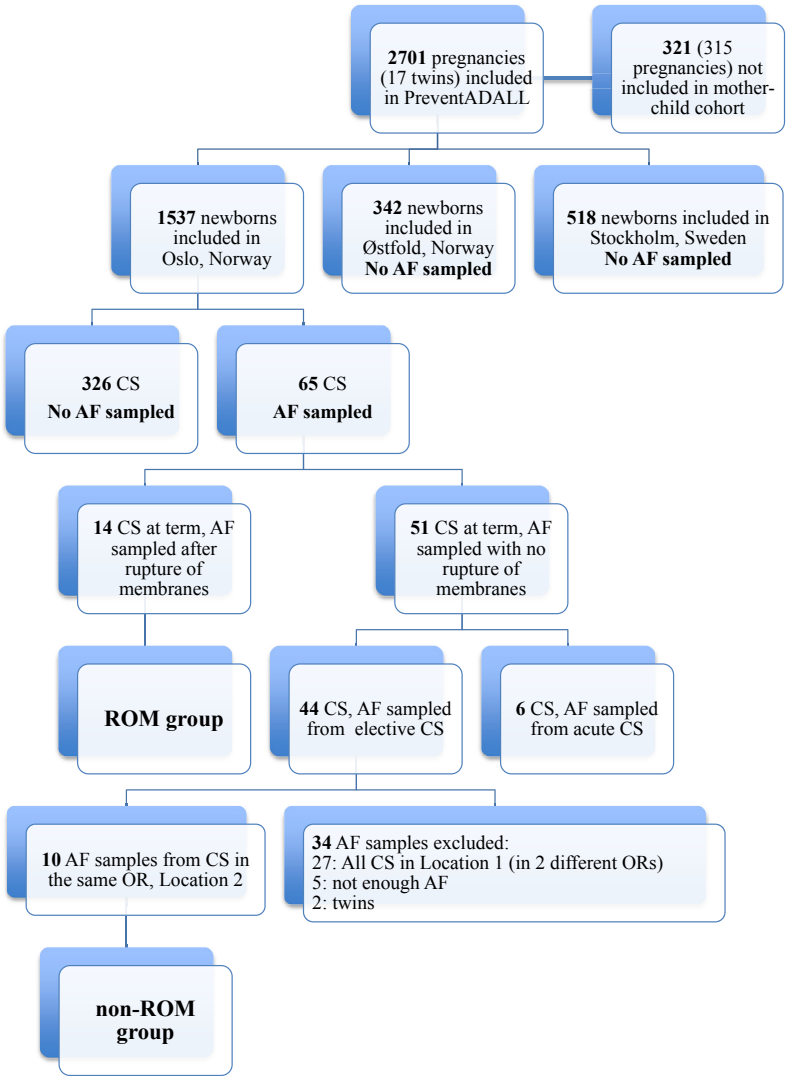
Quantification by digital droplet PCR

Quantification of prokaryotic 16S rRNA gene copies in the amniotic fluid samples was done using digital droplet PCR (ddPCR) (Bio-Rad, Hercules, CA).[17] Droplet generation, droplet transfer, and plate sealing was done according to the manufacturer's instructions. DNA was amplified by PCR using reaction mixes containing 1x QX 200 ddPCR EvaGreen Supermix (Bio-Rad), 0.2 μmol/L of each primers PRK341F (5'-CCTAC GGGRB GCASC AG-3') and PRK806R (5'-GGACT ACYVG GGTAT CTAAT-3') (Thermo Fisher Scientific),[18] and 2 μL DNA. Thermal cycles involved initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 45 seconds, <1 cycle at 4°C for 5 minutes and finally 1 cycle at 90°C for 5 minutes. All reactions were performed on a 2720 Thermal Cycler (Applied BioSystems, Waltham, MA). The droplets were quantified using software (Quantisoft; Bio-Rad). The baseline was set manually with a fluorescence threshold of 15,000 relative fluorescence units. Both the interassay and intraassay variability of ddPCR was validated by *Escherichia coli* spiking of non-ROM amniotic fluid (30,000 and 3000 colony-forming unit/mL) with 3 interassay replicates for each dilution, and duplicates analyses for each interassay replicate. In all cases the coefficient of variation was <15%, with the DNA recovery being ~100%.

Culturing, DNA extraction, and PCR

In all, 150 μL of amniotic fluid in Aimes medium was suspended in 1350 μL of liquid brain heart infusion (BHI) medium, making a 10^{-1} dilution and further diluted to a 10^{-2} dilution, for both aerobic and anaerobic culturing. Tubes for anaerobic culturing were prepared in a closed jar using Oxoid AnaeroGen 3.5-L sachets (Thermo Fisher Scientific) for 48 hours; the closed jar and new sachets were used for the anaerobic culturing both in liquid BHI medium and on the BHI agars. The samples in liquid BHI medium were incubated at 37°C for 48 hours and 10 μL from each sample was plated out on BHI agar for aerobe (48 hours) and anaerobe (120 hours) incubation at 37°C. DNA was extracted manually by mag midi kit (LGC Genomics, United Kingdom) following the manufacturer's recommendations from all the cultures in liquid BHI 10^{-1} dilutions, as well as from the bacterial colonies found on the BHI plates after incubation. Amplification by PCR was performed on DNA from all the liquid culture samples, using 1xHotFirePol DNA polymerase ready to load (Solis BioDyne, Estonia), 0.2 μmol/L of the same PRK primers used in ddPCR, and 2 μL template DNA. Thermal cycles involved initial denaturation at 95°C for

FIGURE 1
Selection of study population for amniotic fluid analysis in the PreventADALL study



In the PreventADALL study, amniotic fluid (AF) was only sampled from cesarean delivery (CS) performed in Oslo, in location 1 (2 operating rooms [ORs]) and location 2 (1 OR). AF was randomly sampled in 65/326 CS (20%), where main indication for sampling was no prior rupture of membranes (ROM), but 14/65 samples were from CS with prior ROM in both locations.

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15 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C, and elongation at 72°C for 45 seconds. A final elongation at 72°C for 7 minutes was included.

Gel electrophoresis

The size of the PCR products was determined using gel electrophoresis with a 1.5% agarose (Sigma Aldrich). The electrophoresis ran at 80 V for 30 minutes. A 100-base pair DNA ladder (Solis BioDyne, Tartu, Estonia) was used as size marker for the DNA fragments. The fragments were visualized using the Molecular Imager Gel Doc XR Imaging system with Quantity One 1-D analysis software v.4.6.7 (Bio-Rad), using ultraviolet light.

Measuring DNA concentration by Qubit

DNA concentrations were measured on the Qubit fluorometer (Life Technologies, Waltham, MA), by using the double-stranded DNA high-sensitivity assay kit (Life Technologies). The measurements were done following the kit protocol, mixing 198 μL of working solution (Quant-iT reagent diluted 1:200 in Quant-iT buffer) with 2 μL sample. Calibration of the instrument was performed before the measurements as recommended by manufacturer.

Sanger sequencing

DNA of the isolates from culturing were amplified using 1xHotFirePol DNA polymerase ready to load (Solis BioDyne), 0.2 μmol/L of each of the primers, GA-map CoverAll primer pair (Genetic Analysis AS, Oslo, Norway), and 2 μL template DNA. Thermal conditions involved initial denaturation at 95°C for 15 minutes, followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. A final elongation at 72°C for 7 minutes was included. PCR products were purified using 0.8x AMPure XP beads (Beckman Coulter, Brea, CA) before measuring DNA concentration using a Qubit fluorometer (Life Technologies). GATC BioTech, Norway, sequenced the resulting purified PCR products.

Illumina sequencing

The taxonomic composition of the microbiota in the samples with a DNA concentration >1000 16S rRNA gene copies/μL was determined by sequencing the resulting amplicons from a 2-step PCR using the same primers as used in ddPCR. The 2 negative controls (1 from the hospital operating room and 1 from the laboratory) were also included. Amplification was performed in 25 μL volumes containing 1x HotFirePol blend master mix ready to load (Solis BioDyne), 0.2 μmol/L of both primers (Thermo Fisher Scientific), and 2 μL (0.4–60 ng) genomic DNA. First PCR was performed with initial denaturation at 95°C for 15 minutes, followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. A final elongation at 72°C for 7 minutes was included. Resulting amplicons were purified with AMPure XP beads (Beckman-Coulter), following the manufacturer's instructions. For attachment of dual indices and Illumina sequencing adapters, a second PCR was

performed with Illumina-modified primers following the same conditions as before, only with 10 cycles and an increased annealing step to 1 minute. Amplicon libraries were quantified by Qubit double-stranded DNA HS assay kit and normalized to a sequencing pool before purification by AMPure XP beads. Final library was quantified in a QX200 Droplet Digital PCR System (Bio-Rad) using primers targeting Illumina adaptors, following the manufacturer's recommendations. The library was loaded on a MiSeq platform (Illumina) following manufacturer's recommendations.

Analysis of Illumina data

Resulting sequences were analyzed using the open-source Quantitative Insights Into Microbial Ecology (QIIME) bioinformatics pipeline,[19] implementing Ultrafast Sequence analysis (USEARCHs)[20] High-accuracy, high-throughput operational taxonomic unit (UPARSE-OTU) algorithm[21] for OTU clustering. OTUs were defined at 97% similarity and taxonomy was assigned based on >97% identity using the High quality ribosomal RNA (SILVA) databases.[22]

Statistical analysis

The nonparametric data (ddPCR results) were calculated using independent samples Mann-Whitney *U* test. The significance level was set to 5%. The statistical analysis including the descriptive statistics was performed in software (SPSS Statistics, Version 24; IBM Corp, Armonk, NY).

Results

Study population characteristics

From the 65 amniotic fluid samples, collected at cesarean delivery from the PreventADALL cohort,[16] we analyzed 10 with intact amniotic membranes (non-ROM group) and all 14 samples with prior ROM (ROM group). The women in both groups were similar in age, while GA and weight at birth was slightly higher in the ROM group, as shown in Table 1. None of the newborns had low Apgar score, and none needed intensive care. The median (min-max) time of ROM until cesarean delivery was 14 (2−36) hours in the ROM group.

TABLE 1
Baseline characteristics in group with intact amniotic membranes and rupture of amniotic membrane group

Characteristics	non-ROM n = 10	ROM N = 14
Mothers		
Age, y, mean (SD)	34.4 (3.6)	33.1 (3.6)
Pregnancy complications		
Clinical chorioamnionitis	0	4
GBS in urine	0	1
Antibiotics antepartum	0	5
Antibiotics intrapartum	0	14
Indications for CS		
Maternal request	6	
Heart disease mother	1	
2 Previous CS	1	
Breech and/or large for GA	1	1
Breech and fetal growth restriction	1	
Slow progression of birth		7
Fetal distress		2
Chorioamnionitis		4
ROM, h, median (min−max)	−	14 (2−36)
GA at CS, wk, mean (min−max)	39.1 (37.9−40.0)	40.5 (37.7−42.3)
Birthweight, g, mean (SD)	3548.6 (546.4)	3749.0 (578.7)

CS, cesarean delivery; *GA*, gestational age; *GBS*, group B streptococcus; *ROM*, rupture of amniotic membranes.
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Digital droplet PCR

The amniotic fluid in the non-ROM group contained very low numbers of bacterial DNA, with a median (min-max) of 664 (544−748) 16S rRNA gene copies/mL. This was comparable to our 4 negative controls (2 sterile NaCl samples from 2 different operating rooms and 2 sterile PCR water samples from the laboratory) where 596 (461−679) copies were detected. In contrast, the ROM group had significantly higher bacterial DNA levels of 7700 (1066−251,430) 16S rRNA gene copies/mL ($P = .0001$, by Mann-Whitney *U* test). The difference between non-ROM and ROM groups remained significant ($P = .0001$) also after exclusion of the 4 women who had a clinical infection and 1 with group B streptococcus in urine at cesarean delivery [median (min-max) of 1462 (1066−6743) 16S rRNA gene copies/mL]. In our samples we did not see any clear relation between time from ROM to cesarean delivery and/or clinical infection and bacterial DNA levels, as depicted in Table 2, however, the sample size in the ROM group is too small to study correlations.

Cultures and Sanger sequencing

No bacteria were detected from amniotic fluid in the non-ROM group, nor from the negative controls by culturing (anaerobically and aerobically) and PCR. In the ROM group, bacteria were detected in 50% by performing PCR on the samples cultured in broth under anaerobic conditions, and in 14.3% of the samples cultured in broth under aerobic conditions. In addition, bacterial colonies were detected in 21.4% of the samples grown anaerobically on agar (Tables 2 and 3). These colonies were

TABLE 2
Clinical information on 14 women with cesarean delivery with prior rupture of membranes and results from microbiological amniotic fluid analysis

ROM group	GA (wk + d) at ROM	ROM prior to start of labor	Spontaneous ROM or amniotomy	Regular contractions prior to CS	Time from ROM to cesarean delivery, h	Indication for cesarean delivery	Other information	ddPCR DNA copies/mL	Culture aerobic/ anaerobic	Sanger sequencing species (percentage represents identity to closest match in NCBI database)	Illumina 16S rRNA gene sequencing taxonomy present in ≥1%
1	42+2	Yes	Amniotomy	Yes	11	Slow progression	Meconium-stained amniotic fluid	45,066	Positive		*Lactobacillus* (69.5%) *Caulobacteraceae* (10.6%) *Sphingomonas* (1.5%) *Pseudomonas* (7.6%)
2	39+0	No	Spontaneous	No	2	Breech		1553	Negative		Not sequenced
3	41+6	Yes (PROM)	Spontaneous	Yes	6	Fetal distress	Induction with prostaglandins after external version from breech	6873	Negative		Not sequenced
4	38+2	Yes (PROM)	Spontaneous	Yes	36	Slow progression	GBS	1888	Negative		Not sequenced
5	39+4	Yes	Amniotomy	Yes	4	Slow progression	Pathologic CTG	46,893	Positive	*Streptococcus agalactiae* (99%) *Peptoniphilus harei* (99%) *Peptoniphilus asaccharolyticus* (99%)	*Bifidobacterium* (22.4%) *Olsenella* (38.6%) *Prevotella* (18.7%) *Aerococcus* (4.6%) *Lactobacillus* (6.2%) *Shuttleworthia* (1.2%) *Megasphaera* (1.3%) *Sneathia* (1.9%) *Caulobacteraceae* (1.0%)
6	37+5	Yes	Amniotomy	Yes	17	Slow progression and clinical chorioamnionitis	MCDA twins, induction with balloon catheter and amniotomy	1462	Positive		Not sequenced
7	40+4	Yes (PROM)	Spontaneous	No	31	Slow progression		67,077	Positive	*Lactobacillus reuteri* (98%) *L crispatus* (99%) *L vaginalis* (98%)	Inconclusive results

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(continued)

TABLE 2
Clinical information on 14 women with cesarean delivery with prior rupture of membranes and results from microbiological amniotic fluid analysis *(continued)*

ROM group	GA (wk + d) at ROM	ROM prior to start of labor	Spontaneous ROM or amniotomy	Regular contractions prior to CS	Time from ROM to cesarean delivery, h	Indication for cesarean delivery	Other information	ddPCR DNA copies/mL	Culture aerobic/ anaerobic	Sanger sequencing species (percentage represents identity to closest match in NCBI database)	Illumina 16S rRNA gene sequencing taxonomy present in ≥1%
8	41+1	No	Amniotomy	Yes	18	Slow progression	Induction with balloon catheter and prostaglandins	57,246	Positive	*Prevotella amnii* (99%) *Prevotella bivia* (99%)	*Bifidobacterium* (28.1%) *Olsenella* (8.4%) *Aerococcus* (50.5%) *Sneathia* (2.9%) *Caulobacteraceae* (1.0%)
9	40+5	No	Spontaneous	No	13	Slow progression and clinical chorioamnionitis	Induction with balloon catheter and prostaglandins	1275	Negative		Not sequenced
10	42+1	Yes	Amniotomy	Yes	9	Slow progression and clinical chorioamnionitis	Induction with prostaglandins and amniotomy	6743	Negative		Not sequenced
11	40+3	No	Spontaneous	Yes	22	Slow progression and clinical infection	Pathologic CTG	1066	Positive		Not sequenced
12	41+6	No	Amniotomy	No	20	Slow progression		251,430	Positive		*Sneathia* (98.3%)
13	40+0	No	Spontaneous	Yes	6	Slow progression and fetal distress	Breech	170,520	Negative		*Lactobacillus* (21.1%) *Caulobacteraceae* (27.7%) *Bradyrhizobium* (2.7%) *Sphingomonas* (5.1%) *Halomonas* (1.0%) *Pseudomonas* (17.8%)
14	41+1	Yes	Amniotomy	No	15	Slow progression	Induction with balloon catheter and amniotomy	8526	Negative		Not sequenced

CTG, cardiotocography; *ddPCR*, digital droplet polymerase chain reaction; *GA*, gestational age; *GBS*, group B streptococcus; *MCDA*, monochorionic diamniotic; *NCBI*, National Center for Biotechnology Information; *PROM*, premature rupture of membranes; *ROM*, rupture of amniotic membranes.

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TABLE 3
Results from digital droplet polymerase chain reaction, gel electrophoresis of polymerase chain reaction products from aerobic and anaerobic cultures, and Sanger sequencing

	ddPCR DNA copies/mL	GE aerobic (band)	GE anaerobic (band)	Aerobic colonies	Anaerobic colonies	Sanger sequencing species (percentage represents identity to closest match in NCBI database)
Non-ROM (n = 10)	Mean: 672 Median: 664 (544–748) SD 65.5	No	No	No	No	
Negative control operating room	679	No	No	No	No	
Negative control laboratory	461	No	No	No	No	
Positive control (*Escherichia coli*) ddPCR	32,190					
Negative control ddPCR	104					
ROM (n = 14)	Mean: 47,687 Median: 7700 (1066–251,430) SD 74,751					
1	45,066	No	Yes	No	No	
2	1553	No	No	No	No	
3	6873	No	No	No	No	
4	1888	No	No	No	No	
5	46,893	Yes	Yes	No	3 Colonies	*Streptococcus agalactiae* (99%) *Peptoniphilus harei* (99%) *Peptoniphilus asaccharolyticus* (99%)
6	1462	No	Yes	No	No	
7	67,077	No	Yes	No	2 Colonies	*Lactobacillus reuteri* (98%) *L crispatus* (99%) *L vaginalis* (98%)
8	57,246	No	Yes	No	1 Colony	*Prevotella amnii* (99%) *Prevotella bivia* (99%)
9	1275	No	No	No	No	
10	6743	No	No	No	No	
11	1066	No	Yes	No	No	
12	251,430	Yes	Yes	No	No	
13	170,520	No	No	No	No	
14	8526	No	No	No	No	
Negative control operating room	618	No	No	No	No	
Negative control laboratory	574	No	No	No	No	
Positive control (*Escherichia coli*) ddPCR	24,012					
Negative control ddPCR	244					

ddPCR, digital droplet polymerase chain reaction; *GE*, gel electrophoresis; *NCBI*, National Center for Biotechnology Information; *ROM*, rupture of amniotic membranes.
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TABLE 4
Illumina 16S rRNA gene sequencing taxonomy in rupture of amniotic membranes group and in negative controls

	Total	1	5	8	12	13	Negative control laboratory	Negative control OR
Taxonomy — genera	%	%	%	%	%	%	%	%
Bifidobacterium	8.4	0.0	22.4	28.1	0.0	0.0	0.0	0.0
Olsenella	7.8	0.0	38.6	8.4	0.0	0.0	0.0	0.0
Bacteroidales uncultured	0.3	0.2	0.0	0.0	0.0	0.1	1.4	0.4
Prevotella	3.2	0.0	18.7	0.3	0.0	0.0	0.0	0.0
Aerococcus	9.2	0.0	4.6	50.5	0.1	0.0	0.0	0.0
Lactobacillus	16.2	69.5	6.1	0.2	0.0	21.0	0.1	0.0
Lachnospiraceae	0.4	0.2	0.0	0.0	0.0	0.5	2.0	0.2
Shuttleworthia	0.2	0.0	1.2	0.0	0.0	0.0	0.0	0.0
Megasphaera	0.2	0.0	1.3	0.0	0.0	0.0	0.0	0.0
Sneathia	17.2	0.0	1.9	2.9	98.3	0.0	0.0	0.0
Caulobacteraceae; other	14.6	10.6	1.0	2.1	0.4	27.7	46.0	65.9
Bradyrhizobium	1.8	0.8	0.1	0.5	0.1	2.7	6.3	3.9
Sphingomonas	2.0	1.5	0.2	0.9	0.2	5.1	4.1	4.2
Ralstonia	0.7	0.3	0.0	0.1	0.0	0.9	2.9	0.2
Delftia	0.3	0.1	0.0	0.1	0.0	0.4	1.1	0.3
Pseudoalteromonas	0.4	0.3	0.1	0.1	0.0	0.3	1.7	1.0
Halomonas	0.7	0.4	0.1	0.1	0.0	1.0	2.8	2.2
Pseudomonas	9.4	7.6	1.1	2.6	0.4	17.8	26.7	19.5
Stenotrophomonas	0.3	0.2	0.0	0.0	0.0	0.1	1.3	0.4
Ureaplasma	1.0	0.0	0.2	0.0	0.0	5.9	0.0	0.0
Other	1.9	1.7	2.3	1.1	0.5	2.4	3.6	1.8
Unassigned; other	3.8	6.6	0.1	2.0	0.0	14.1	0.0	0.0

OR, operating room.
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identified (by Sanger sequencing) as bacterial strains that are commonly part of the vaginal flora and/or associated with intrauterine infections, namely *Streptococcus agalactiae*, *Peptoniphilus harei*, *Peptoniphilus asaccharolyticus*, *Lactobacillus reuteri*, *Lactobacillus crispatus*, *Lactobacillus vaginalis*, *Prevotella amnii*, and *Prevotella bivia*, as seen in Table 2.

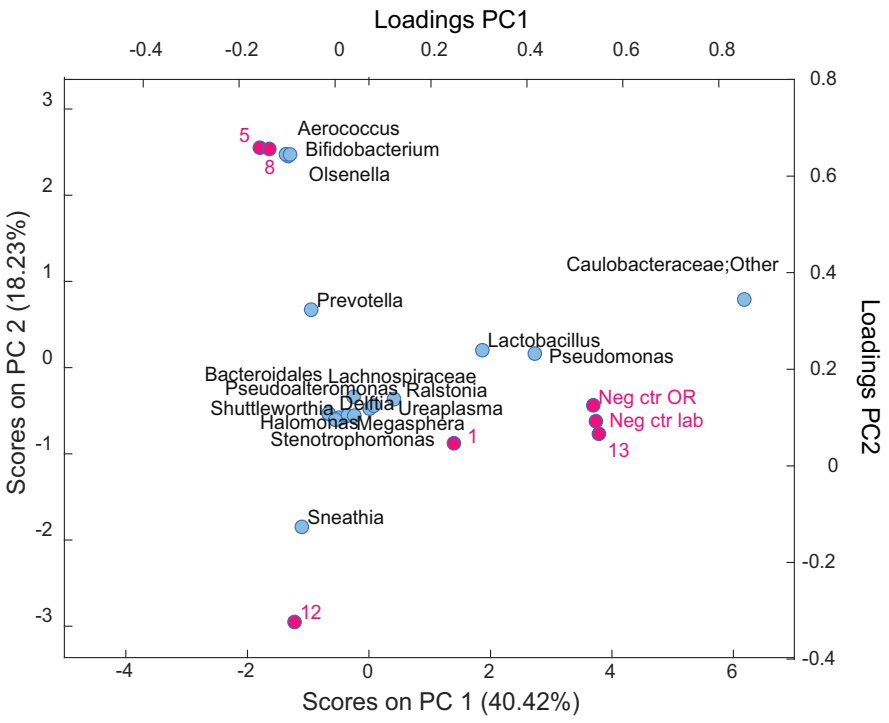
Illumina 16S rRNA gene sequencing

In 5 of the 6 amniotic fluid samples (with >1000 16S rRNA copies/μL) amplicon sequencing of the 16S rRNA gene revealed species belonging to bacterial genera that are part of a normal vaginal flora, namely *Bifidobacterium*, *Olsenella*, *Prevotella*, *Aerococcus*, *Lactobacillus*, *Shuttleworthia*, *Sneathia*, *Caulobacteraceae*, *Pseudomonas*, and *Ureaplasma*, of which some are known to contain species that are associated with bacterial vaginosis and/or infections, as well as possible contamination. In 2 negative controls (1 from operating room and 1 from the laboratory), we found genera associated with reagent and laboratory contamination, namely: *Caulobacteraceae*, *Pseudomonas*, *Sphingomonas*, *Bradyrhizobium*, *Ralstonia*, and *Stenotrophomonas*,[23] as seen in Table 4. Associations of microbiota with the samples analyzed are shown in a principal component plot; these analyses confirmed tight clustering of the negative controls and the relative large diversity in the ROM group (Figure 2).

Comment

Recently, the view that amniotic fluid does not have live bacterial communities present in uncomplicated term pregnancies was challenged by identifying an amniotic fluid microbiota (using 16S rRNA gene sequencing PCR) in 15 uncomplicated term pregnancies, finding a core set of bacterial phylotypes that was overlapping with the microbiota found in placenta and meconium.[7] Our

FIGURE 2
Associations of microbiota with samples analyzed in rupture of amniotic membranes (ROM) group



Taxonomic groups of bacteria were clustered based on principal component (PC) analysis, with corresponding scores for first 2 PC (blue circles) and explained variance (parentheses). Corresponding loadings for samples analyzed (red circles).

ctr, control; *lab*, laboratory; *neg*, negative; *OR*, operating room.

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findings, however, support a sterile amniotic fluid until the start of labor, which are in line with previous studies using cultivation techniques,[8–10,24] as well as a study using both cultivation and 16S rDNA qPCR in term uncomplicated pregnancies.[25] Studies that demonstrate the pioneer microbiota in newborns are also supporting that fetal bacterial colonization in uncomplicated term pregnancies does not start before labor.[9,26–29] In newborns delivered by cesarean delivery, the initial colonization is predominately by skin microbes, not only originating from their mother,[26,27] but also from the operating room.[30] A recent study by Chu et al[28] found that cesarean delivery newborns from mothers having been in labor had similar initial colonization pattern to a vaginal delivery, with both vaginal and skin microbes present, compared to unlabored cesarean delivery infants, with predominantly skin microbes present.

We designed our study to minimize the source of possible contamination in the sampling, aliquoting, and analyzing process. In the 10 subjects selected for the non-ROM group, amniotic fluid was sampled during elective cesarean delivery, in the same operating room by the same health personnel, minimizing variations in case of contamination. As reflected by our sterile controls, avoiding all forms of minor contaminations in a clinical setting is nearly impossible. The bacterial DNA found in studies on low-microbial biomass samples has been criticized to not originate from live bacteria, but as a result of contamination or transport of dead microbial products brought by the bloodstream.[13,14] In a study by Lauder et al,[15] the placental samples were indistinguishable to the negative controls (both in the low number of DNA copies and by sequencing). It is likely that the fetus is exposed to maternal microbial components,[4] but if they have any role in promoting health or disease in the fetal and/or newborn life is unknown.

In the ROM group we found species that are known to be a part of the vaginal flora in women of reproductive age,[31] dominated by lactobacilli species, but we also found genera that can either be part of a normal vaginal flora or be associated with bacterial vaginosis, such as bifidobacteriae, prevotellae, aerococci, peptoniphili, streptococci, ureaplasma, and sneathiae. These findings support an ascending microbial colonization of the intrauterine cavity with term ROM,[24,28,32,33] helped by premature ROM and prolonged labor.[9,32,34] Previous studies also suggest that colonization depends upon the length of the labor and the number of vaginal examinations during labor.[9,29] However, in our study there were too few women with ROM to study potential correlations between the length of labor and bacterial load. In the ROM group samples, we also found bacterial genera that are associated with reagent and laboratory contamination,[23] namely *Caulobacteraceae* and *Pseudomonas*. These genera were also identified in our negative controls, and could therefore be accounted for as contamination, which emphasizes the need for appropriate controls when performing molecular-based studies.

Preterm deliveries and neonatal death are associated with microbial invasion of the intrauterine cavity both in those with preterm premature ROM and with intact membranes,[35] suggesting several routes of microbial spread; either ascending from the vagina or descending from other organs through the maternal bloodstream, from the peritoneal cavity via the fallopian tubes or due to prenatal intrauterine procedures. In several studies analyzing amniotic fluid with molecular techniques, from preterm deliveries, bacteria have been identified that would not have been found by the only use of culturing,[29,36,37] as is also demonstrated in the sequencing results of our study. In contrast to our study

where lactobacilli were dominating in the ROM group, they are rarely found in case of preterm microbial invasion of intrauterine cavity as the bacteria commonly found here are mostly associated with bacterial vaginosis, but periodontal pathogenic bacteria have also been identified.[29,36,37]

With molecular-based studies on amniotic fluid, if appropriate measures for avoiding contamination are considered, it has been possible to get a clearer picture of how microbial invasion of the intrauterine cavity occurs and which microbes are involved. With our study, we believe that we can settle that the first colonization of the fetus normally occurs during labor. If the baby is born by cesarean delivery in an uncomplicated term pregnancy without prior labor it will not be in contact with the vaginal microbiota, which in turn can negatively affect how the child's microbiota and immune system develops.[3–5] We therefore believe that our study adds to the arguments that an indication for an elective (planned) cesarean delivery should be carefully considered in each individual case and that it is not to be taken lightly. Interestingly, preliminary results of swabbing the infant with vaginal microbes from their mother immediately after cesarean delivery has implicated that the pioneer microbiota in these cesarean delivery–born infants resembles that of a vaginally born infant.[38]

Although the amount of DNA in the non-ROM group was too low to identify a bacterial microbiota, the highly sensitive and accurate ddPCR quantification[17] allowed us to identify bacterial DNA at the single copy level. Regular qPCR cannot accurately detect single copies of bacterial DNA, and would therefore be less useful due to the very low bacterial content in amniotic fluid, as shown in a recent study where no 16S rRNA nor 18S rRNA was found in amniotic fluid from amniocentesis in 344 asymptomatic women at midgestation,[39] and a median 16S rRNA gene copy number of 0 in 20 amniotic fluid samples from term gestation in another study.[25]

A limitation of our study is the small number of samples, with a heterogeneous bacterial load in the ROM group, as well as a relatively large time span from ROM until delivery. However, the lack of bacterial detection in the non-ROM group is consistent, and similar to the findings of negative controls and clearly different from the consistent positive bacterial findings (both by highly sensitive DNA quantifications and cultures) in the ROM group.

Despite our lack of identifying a unique amniotic fluid bacterial microbiota in our population of uncomplicated pregnancies, we cannot exclude the existence of a placental microbiota. The evidence of a placental microbiota is conflicting, nonetheless we hypothesize that in pregnancies with a dysfunctional placenta, such as in infections, fetal growth restriction, or preeclampsia, prenatal microbial intraamniotic invasion is possible. This is supported by findings of an altered placental microbiome in preterm births with and without chorioamnioinitis.[11,12,40–42] In a recent study by Doyle et al,[12] a placental microbiome was identified in 50% of the samples (by 16S rRNA sequencing), and specific bacterial communities were found to be associated with chorioamnionitis and low birthweight. These bacteria originated mostly from the vagina, which is in contrast to previous findings of placental microbiome resembling oral bacterial communities.[11] If these findings favor a healthy placental microbiome that could become dysbiotic, or if the bacterial colonization of the placenta only occurs in a diseased state, is still not clear.

We find it reasonable to assume, in the light of our findings, that previous publications of an amniotic fluid microbiome[7] may have been hampered by potential contamination, possibly combined with unrecognized placental dysfunction and/or uterine contractions with prior ROM. Initial colonization of the infant is affected by ROM.[9,28,29,32,33] We speculate that the long-term offspring adverse health effects seen in pregnancies with placental dysfunction[43] may partly be mediated through an early in utero microbial exposure.

We conclude that amniotic fluid is sterile in uncomplicated pregnancies with intact amniotic membranes at term. ■

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Supplementary Material

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