Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns

Maria G. Dominguez-Bello[a,1,2], Elizabeth K. Costello[b,1,3], Monica Contreras[c], Magda Magris[d], Glida Hidalgo[d], Noah Fierer[e,f], and Rob Knight[b,g]

[a]Department of Biology, University of Puerto Rico, San Juan, Puerto Rico 00931; [b]Department of Chemistry and Biochemistry, [e]Department of Ecology and Evolutionary Biology, and [f]Cooperative Institute for Research in Environmental Sciences, University of Colorado, Boulder, CO 80305; [c]Center of Biophysics and Biochemistry, Venezuelan Institute for Scientific Research, Caracas 1020A, Venezuela; [d]Amazonic Center for Research and Control of Tropical Diseases, Puerto Ayacucho 7101, Amazonas, Venezuela; and [g]The Howard Hughes Medical Institute, University of Colorado, Boulder, CO 80305

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Upon delivery, the neonate is exposed for the first time to a wide array of microbes from a variety of sources, including maternal bacteria. Although prior studies have suggested that delivery mode shapes the microbiota's establishment and, subsequently, its role in child health, most researchers have focused on specific bacterial taxa or on a single body habitat, the gut. Thus, the initiation stage of human microbiome development remains obscure. The goal of the present study was to obtain a community-wide perspective on the influence of delivery mode and body habitat on the neonate's first microbiota. We used multiplexed 16S rRNA gene pyrosequencing to characterize bacterial communities from mothers and their newborn babies, four born vaginally and six born via Cesarean section. Mothers' skin, oral mucosa, and vagina were sampled 1 h before delivery, and neonates' skin, oral mucosa, and nasopharyngeal aspirate were sampled <5 min, and meconium <24 h, after delivery. We found that in direct contrast to the highly differentiated communities of their mothers, neonates harbored bacterial communities that were undifferentiated across multiple body habitats, regardless of delivery mode. Our results also show that vaginally delivered infants acquired bacterial communities resembling their own mother's vaginal microbiota, dominated by *Lactobacillus*, *Prevotella,* or *Sneathia* spp., and C-section infants harbored bacterial communities similar to those found on the skin surface, dominated by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp. These findings establish an important baseline for studies tracking the human microbiome's successional development in different body habitats following different delivery modes, and their associated effects on infant health.

host–microbe interactions | human microbiome | neonatal bacterial assemblages | pioneer community

The healthy human fetus is thought to develop within a bacteria-free environment. Upon delivery, the neonate is exposed to a wide variety of microbes, many of which are provided by the mother during and after the passage through the birth canal, an ecosystem heavily colonized by a relatively limited set of bacterial taxa (1, 2). Babies are born with immunological tolerance that is instructed by the mother by preferential induction of regulatory T lymphocytes (3), which might allow the baby to become colonized by this first inoculum. However, only a subset (if any) of the microbes to which the newborn is initially exposed will permanently colonize available niches and contribute to the distinctive microbiotas harbored by the body habitats of adults (4–7).

Many modern human babies are not exposed to vaginal microbes at birth. In the United States, for example, more than 30% of all live births in 2007 were Cesarean section (C-section) deliveries (http://www.cdc.gov/nchs/births.htm), and differences in delivery mode have been linked with differences in the intestinal microbiota of babies (8–11). Mutualistic relationships with intestinal bacteria are known to influence energy balance (12–15), metabolism of xenobiotics (16, 17), pathogen colonization resistance (18, 19), and the maturation of the intestine and the immune system (20, 21), and similarly important roles are likely played by the microbiotas of nongut body habitats, although the influence of delivery mode on the bacterial communities found in these habitats is unknown. Delivery mode may lead to differences in the microbiota's development, which may then contribute to variations in normal physiology or to disease predisposition.

The age-related successional mechanisms involved in the differentiation of the human microbiota across body habitats are only beginning to be understood (6), and defining the pioneer colonizers is a first step toward elucidating the initial stages of microbiota development. It is thought that the initial microbial exposure is important in defining the successional trajectories leading to more complex and stable adult ecosystems (10, 22), and additionally, initial communities may serve as a direct source of protective or pathogenic bacteria very early in life. Here, we use cultivation-independent, molecular-phylogenetic techniques to characterize the first bacterial assemblages associated with full-term babies born vaginally or by C-section, and the assemblages associated with their mothers, across multiple body habitats near the time of delivery.

Results and Discussion

Sampling for this study was performed over 4 d at the obstetrics unit of the Puerto Ayacucho hospital, Amazonas State, Venezuela. A total of nine women, aged 21 to 33 y, and their 10 newborns participated in the study. Four women (two Mestizo and two Amerindians) delivered vaginally, giving birth to three males and one female. Five women (four Mestizo and one Amerindian) delivered via C-section, giving birth to three females and three males, including male dizygotic twins. Mothers who delivered vaginally were not given antibiotics and had not consumed antibiotics during pregnancy, except for one Mestizo women, who declared having taken antibiotics in the seventh month of pregnancy. Women who delivered via C-section were

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[1]M.G.D.-B. and E.K.C. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: maria.dominguez1@upr.edu.

[3]Present address: Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305.

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administered cephalosporin several hours before the C-section. The mothers' skin (ventral forearms), oral mucosa, and vagina were sampled 1 h before delivery, and their babies' skin (before removing the vernix caseosa), oral mucosa, and nasopharyngeal aspirate were sampled <5 min, and meconium <24 h, after delivery. For each sample, variable region 2 (V2) of the bacterial 16S rRNA gene was PCR-amplified using a primer-set with a unique error-correcting barcode (23, 24). Multiplexed pyrosequencing of 34 maternal and 46 infant samples yielded 157,915 partial (~250 bp) 16S rRNA gene sequences, with ~2,000 sequences per sample. We did not compare bacterial loads in the collected samples, as we could not effectively control for differences in the sampled area or volume; instead, we focus here on shifts in bacterial community structure and diversity.

In the mothers, bacterial communities were structured primarily by body habitat, with distinct oral, skin, and vaginal assemblages (Fig. 1*A* and Table S1), as has been reported in previous studies (4, 5). Accordingly, the mothers' aggregate bacterial communities were dominated by taxa typical of these habitats; for example, *Streptococcus* spp. in the oral cavity, *Staphylococcus*, *Corynebacterium*, or *Propionibacterium* spp. on the skin, and *Lactobacillus* or *Prevotella* spp. in the vagina (Fig. 1 *B* and *C* and Table S2). The cephalosporin administered before C-section had no apparent effect on the bacterial community structure at the time of sampling (Table S1).

The dominant vaginal taxa varied from mother to mother (Fig. 1*C* and Table S2). Three women had uneven vaginal communities dominated by *Lactobacillus* spp. (Firmicutes; 88–94% of the sequences) and low representation of *Prevotella* spp. (Bacteroidetes) (Table S2). Other women had more even community structures in which *Prevotella* spp. (15–57% of the sequences in four women, including the two Amerindians), Coriobacterineae (Actinobacteria; mainly the genus *Atopobium*), *Sneathia* spp. (Fusobacteria), or other taxa were significantly represented (Table S2). To our knowledge, this molecular study of the vaginal composition at the time of labor is unique. In other studies in nonpregnant women (1, 2, 6), women typically had vaginal communities dominated by *Lactobacillus.* In nonpregnant United States women, lack of lactobacilli dominance has been related to bacterial vaginosis (25), which was not evident in any of the women in this study, although the possibility of underdiagnosis cannot be ruled out. Future studies are needed to determine the relationship between lactobacilli abundance and vaginal health in both pregnant and nonpregnant women.

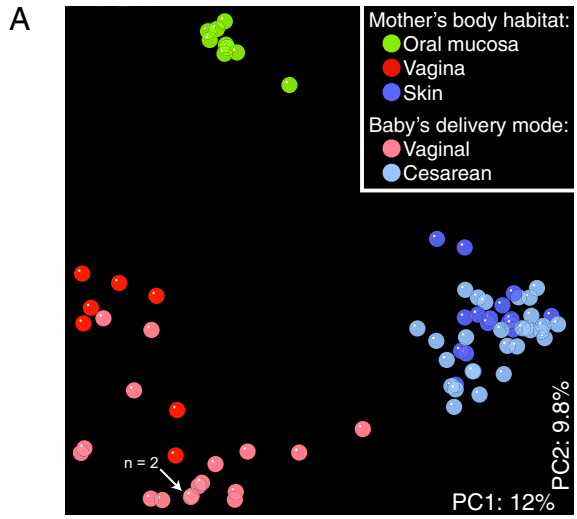
In contrast to their mothers, the newborns harbored bacterial communities that were essentially undifferentiated across skin, oral, nasopharyngeal, and gut habitats regardless of delivery mode (Table S1), showing that in its earliest stage of community development, the human microbiota is homogeneously distributed across the body. Moreover, we found that the primary determinant of a newborn's bacterial community composition was his or her mode of delivery (Fig. 1*A* and Table S1). Vaginally delivered infants harbored bacterial communities (in all body habitats) that were most similar in composition to the vaginal communities of the mothers; as expected, C-section babies lacked bacteria from the vaginal community (Fig. 1*A* and Table S1). On the other hand, infants delivered via C-section harbored bacterial communities (across all body habitats) that were most similar to the skin communities of the mothers (Fig. 1*A* and Table S1). Accordingly, the dominant taxa found in infant communities were reflective of delivery mode: *Lactobacillus*, *Prevotella*, *Atopobium*, or *Sneathia* spp. were abundant in aggregate samples from vaginally delivered babies, and typical skin taxa, including *Staphylococcus* spp., appeared in samples from C-section infants (Fig. 1*B*). The mothers' oral bacterial communities were distinct from infant communities (Fig. 1 *A* and *B*), and did not seem to contribute substantially to the baby's initial bacterial community.

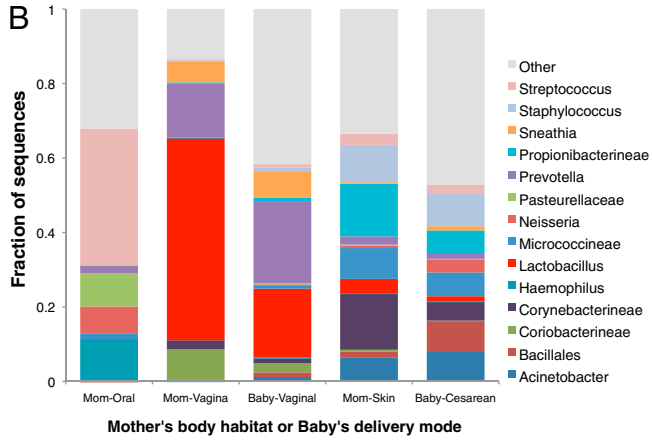
We found evidence that infant delivery mode also affected the direct transmission of initial bacteria from mother to newborn. Using the weighted UniFrac distance metric, which takes into account both membership and the relative abundance of lineages (26), we calculated the overall difference between bacterial communities from mothers and (*i*) their own babies, (*ii*) other babies with the same delivery mode, and (*iii*) other babies with a different delivery mode. For vaginally delivering mothers, comparisons were made to their vaginal microbiota, and for C-section mothers, comparisons were made to their skin microbiota. In three of four vaginal deliveries, the mother's vaginal bacterial community was significantly more similar to her own baby's microbiota than to the microbiota of other vaginally delivered babies (Student's *t* test, $P < 0.01$) (Fig. 2*A*), suggesting that the vaginal community [which is unique to each mother (Fig. 1*C* and Table S2)] is vertically transmitted to the baby. In contrast, skin bacterial communities of C-section mothers were no more similar to their own babies than to the other babies born via C-section (Fig. 2*B*), even though previous work has demonstrated that adult skin communities are highly personalized and personally identifying (27), even when bacteria are transferred to touched objects (28). These patterns are supported by the results of an independent analysis of the number of nearly identical sequences [i.e., operational taxonomic units (OTUs) defined at 100% sequence identity] shared between mothers and babies (Fig. S1). These results suggest that incidental exposures to skin bacteria in the hospital environment could contribute to the microbiota of C-section delivered babies. Direct transmission from nonmaternal sources may be enhanced in C-section babies, but our data do not address specific sources, such as fathers or doctors. Notably, the microbiota of dizygotic male twins born via C-section were significantly more similar to each other than either was to any other C-section baby (Student's *t* test with Monte Carlo, $P < 0.05$), likely because of their shared history of exposures.

Our results demonstrate that the mother's vaginal microbiota provides a natural first microbial exposure to newborn body habitats. In C-section babies, the lack of a vaginal exposure leads to first microbial communities resembling the human skin microbiota, with an abundance of *Staphylococcus* spp. This finding may, in part, explain why susceptibility to certain pathogens is often higher in C-section than in vaginally delivered infants. For example, 64 to 82% of reported cases of methicillin-resistant *Staphylococcus aureus* (MRSA) skin infections in newborns occurred in Cesarean-delivered infants (29). The direct transmission of the vaginal microbiota to the baby may serve a defensive role, occupying niches and reducing colonization by MRSA and other pathogens as site-specific communities develop.

Differences in the initial communities (e.g., exposure to a nonlactobacillus-dominated vaginal community or a lack of vaginal exposure in C-section delivered babies) may lead to differences in the microbial succession patterns in the gut and other body habitats that persist over time. For example, culture-based studies have shown that the intestinal colonization by *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* in infants born by C-section is delayed (8, 30). Likewise, the composition of the initial microbiota may have implications for nutritional and immune functions associated with the developing microbiota. For example, recent studies suggest that Cesarean-delivered babies may be more susceptible to allergies and asthma (31, 32), and the administration of probiotics (including lactobacilli) from birth until age 6 mo reduced the incidence of allergy at age 5 y in C-section but not vaginally delivered children (33). Breastfeeding has been suggested to enrich vaginally acquired lactic acid-producing bacteria in the baby's intestine (34), although it is not clear that the predominant lactobacilli in the baby's intestine are the same as those acquired at birth from the mother's vagina (35).

Finally, as we have found that the neonatal microbiota is essentially undifferentiated across body habitats, it will be important to determine the timeline over which the distinctive microbiotas





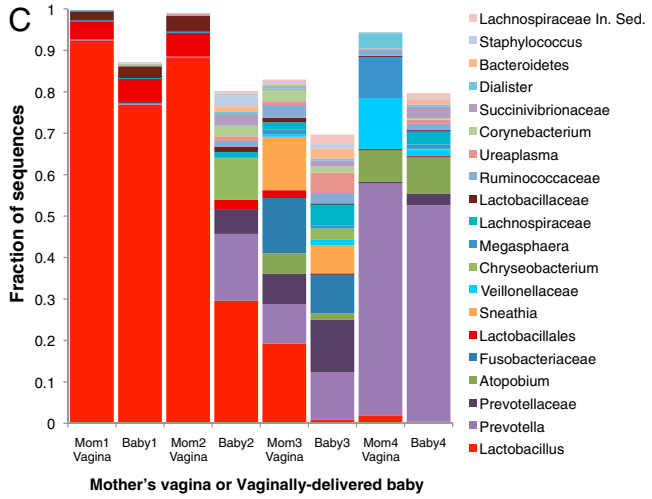


Fig. 1. Bacterial 16S rRNA gene surveys reveal that the first microbiotas of human newborns are primarily structured by delivery mode. (*A*) Communities clustered using principal coordinates analysis of the unweighted UniFrac distance matrix. PC1 and PC2 are plotted on *x*- and *y*-axes. Each point corresponds to a community colored according to the mother's body habitat or the newborn's delivery mode. All newborn body habitats are shown. The percentage of variation explained by the plotted principal coordinates is indicated on the axes. The white arrow indicates a pair of superimposed points. Vaginal samples were not obtained from two of the mothers who delivered by C-section. (*B*) Average relative abundances of the dominant taxa found in this study in aggregated samples. (*C*) Relative abundances of the 20 most abundant taxa in mothers' vaginal communities and in the babies they delivered vaginally. Sequences were classified to highest taxonomic level to which they could be confidently assigned.

found in adult body habitats establish. Our findings emphasize the need to design prospective studies tracking the successional development of the baby's microbiome in different body habitats and after different modes of delivery, and the effects that any associated microbial community shifts may have on infant health.

Methods

Subjects. The Amerindian mothers who participated in this study live in small rural communities of people of a single ethnic group, and the Mestizo mothers live in Puerto Ayacucho, the capital of Amazonas State in Venezuela. We did not perform genetic confirmation of the patient's ethnicity (because that would have required special permits), which was based on the patient's self-description, and the reported information was consistent with phenotype and language spoken by the mothers. Mothers were made aware of the nature of the study, specifically consented to give their personal information, and gave written informed consent for their and their child's participation. The sampling protocol was approved by the Venezuelan Institute of Scientific Research Institutional Review Board (DIR 0229/10) and samples were managed without identifiers in accordance with protocols approved by the University of Puerto Rico Institutional Review Board (0809-51).

Sample Collection. The newborn's skin (right and left ventral forearms and forehead) and oral mucosa swabs were taken within seconds of delivery, before the umbilical cord was cut (with the exception of the nasopharyngeal aspirate, which was taken a few minutes later). Rectal swabs were taken after the babies passed meconium and were collected within 24 h of delivery. Swabs were also taken from the mother's skin (right and left ventral forearms), oral mucosa, and vagina 1 h before delivery. After collection, swab samples were immediately placed on dry ice, and then frozen in liquid N_2 within the following 5 h. All mothers had healthy pregnancies and all babies were born at term, without complications. Babies weighed between 2 and 5.2 kg (the smallest baby was the twin in second order of birth, after his 3-kg brother). Vaginal deliveries occurred during the morning or afternoon, and C-sections were performed in the mornings.

DNA Extraction and Purification. Genomic DNA was extracted from the swabs using the MO BIO PowerSoil DNA Isolation kit with the following modifications. The cotton tips of frozen swabs were broken off directly into bead tubes to which 60 μL of Solution C1 had been added. Tubes were incubated at 65 °C for 10 min and then shaken horizontally at maximum speed for 2 min using the MO BIO vortex adapter. The remaining steps were performed as directed by the manufacturer. Extracted DNA was stored at −20 °C.

PCR Amplification of the V2 Region of Bacterial 16S rRNA Genes. For each sample, we amplified 16S rRNA genes using a primer set described by Fierer et al. (24) that had been optimized for the phylogenetic analysis of pyrosequencing reads (36). The forward primer (5′-GCC TTG CCA GCC CGC TCA GTC AGA GTT TGA TCC TGG CTC AG-3′) contained the 454 Life Sciences primer B sequence, the broadly conserved bacterial primer 27F, and a two-base linker sequence ("TC"). The reverse primer (5′-GCC TCC CTC GCG CCA TCA GNN NNN NNN NNN NCA TGC TGC CTC CCG TAG GAG T-3′) contained the 454 Life Sciences primer A sequence, a unique 12-nt error-correcting Golay barcode used to tag each PCR product (designated by NNNNNNNNNNNN), the broad-range bacterial primer 338R, and a "CA" linker sequence inserted between the barcode and the rRNA primer. PCR reactions were carried out in triplicate 25-μL reactions with 0.6 μM forward and reverse primers, 3-μL template DNA, and 1× of HotMasterMix (5 PRIME). All dilutions were carried out using certified DNA-free PCR water (MO BIO). PCR reactions were assembled within a PCR hood in which all surfaces and pipettes had been decontaminated with DNA AWAY (Molecular BioProducts) and UV-irradiated for 30 min. Thermal cycling consisted of initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 45 seconds, annealing at 50 °C for 30 seconds, and extension at 72 °C for 90 seconds, with a final extension of 10 min at 72 °C. Replicate amplicons were pooled and visualized on 1.0% agarose gels using SYBR Safe DNA gel stain in 0.5× TBE (Invitrogen). Amplicons were cleaned using the UltraClean-htp 96-well PCR Clean-up kit (MO BIO) according to the manufacturer's instructions.

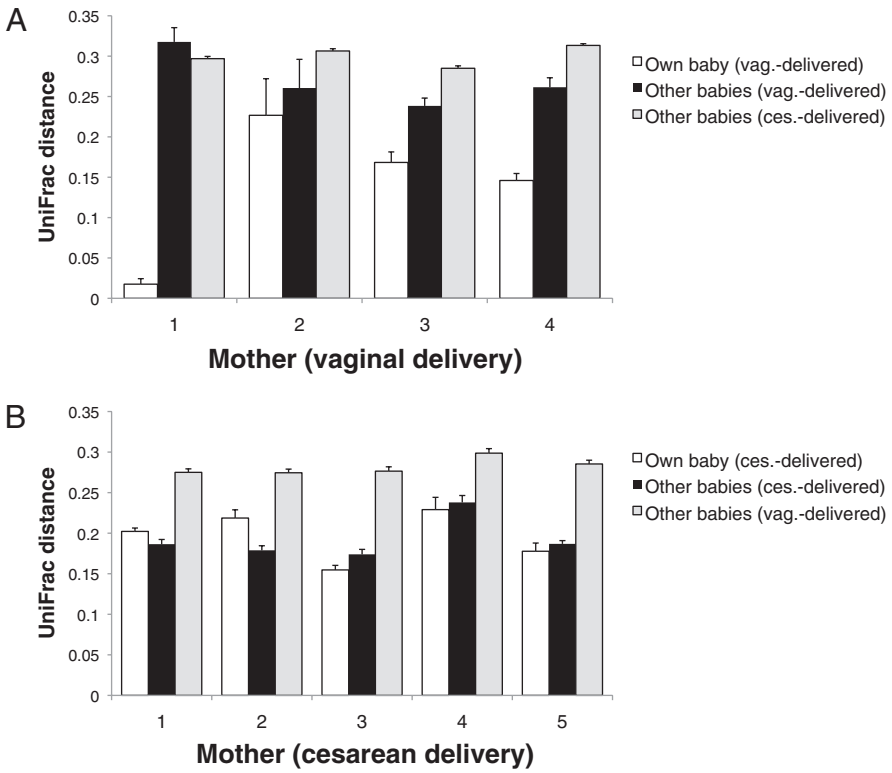


Fig. 2. The effect of delivery mode on the direct transmission of bacteria from mother to newborn. Average (±SEM) weighted UniFrac distance for pairwise comparisons between (*A*) vaginal microbiota of mothers who delivered vaginally, or (*B*) the skin microbiota of mothers who delivered via C-section and the microbiotas of the newborn babies. The maternal microbiota (vagina or skin) was compared with the microbiota of her own baby, or babies of the same or different delivery mode. Baby samples were from the skin (arms and forehead), oral mucosa, nasopharyngeal aspirate, and meconium. The mothers' skin samples were from both arms.

Amplicon Quantitation, Pooling, and Pyrosequencing. Amplicon DNA concentrations were measured using the Quant-iT PicoGreen dsDNA reagent and kit (Invitrogen). Assays were carried out using 5 μL of cleaned PCR product in a total reaction volume of 200 μL in black, 96-well microtiter plates. Fluorescence was measured on a BioTek Synergy HT plate reader using the 480/520-nm excitation/emission filter pair. Following quantitation, cleaned amplicons were combined in equimolar ratios into a single tube. The final pool of DNA was precipitated on ice for 45 min following the addition of 5 M NaCl (0.2 M final concentration) and 2 volumes of ice-cold 100% ethanol. The precipitated DNA was centrifuged at 7,800 × *g* for 40 min at 4 °C, and the resulting pellet was washed with an equal volume of ice-cold 70% ethanol and centrifuged again at 7,800 × *g* for 20 min at 4 °C. The supernatant was removed and the pellet was air dried for 10 min at room temperature and then resuspended in nuclease-free water (MO BIO). The final concentration of the pooled DNA was determined using a NanoDrop spectrophotometer (Thermo Fisher). Pyrosequencing was carried out on a 454 Life Sciences Genome Sequencer FLX instrument (Roche) by the Environmental Genomics Core Facility at the University of South Carolina (Columbia, South Carolina).

Sequence Analysis. Sequences were processed and analyzed following the procedure described by Hamady et al. (23). Sequences were removed from the analysis if they were <200 or >300 nt, had a quality score <25, contained ambiguous characters, contained an uncorrectable barcode, or did not contain the primer sequence. Remaining sequences were assigned to samples by examining the 12-nt barcode. Similar sequences were clustered into OTUs using CD-HIT (37) with a minimum coverage of 97% and a minimum identity of 97%. Chimera checking was not performed because we focus on similarities and differences among communities in this work, and these results are not substantially affected by levels of chimeras much higher than those seen in practice (38). A representative sequence was chosen from each OTU by selecting the longest sequence that had the largest number of hits to other sequences in the OTU. Representative sequences were aligned using NAST (39) and the Greengenes database (40), with a minimum alignment length of 150 and a minimum identity of 75%. The PH Lane mask was used to screen out hypervariable regions after alignment. A phylogenetic tree was inferred using FastTree with Kimura's 2-parameter model (41). Taxonomy was assigned using the Ribosomal Database Project (RDP) classifier with a minimum support threshold of 60% (42) and the RDP taxonomic nomenclature. Nearly identical sequences (i.e., OTUs picked at 100% sequence identity) were found using UCLUST and the default settings (http://www.drive5.com/uclust/).

Community Comparisons. To determine the amount of bacterial diversity shared between two communities (beta diversity), we used the UniFrac metric (26, 43). UniFrac distances are based on the fraction of branch length shared between two communities within a phylogenetic tree constructed from the 16S rRNA gene sequences from all communities being compared. A relatively small UniFrac distance implies that two communities are compositionally similar, harboring lineages sharing a common evolutionary history. In unweighted UniFrac, only the presence or absence of lineages is considered (community membership). In weighted UniFrac, branch lengths are weighted based on the relative abundances of lineages within communities (community structure).

Statistics. We used the analysis of similarities (ANOSIM) (44) function in the program PRIMER (45) to test for differences in community composition among various sample groups. ANOSIM is a permutation-based test of the null hypothesis that within-group distances are not significantly smaller than between-group distances. The test statistic (*R*) can range from 1 to −1, with a value of 1 indicating that all samples within groups are more similar to each other than to any other samples from different groups. *R* is ≈0 when the null hypothesis is true, that distances within and between groups are the same on average. Finally, in some cases, to simply determine if UniFrac distances were, on average, significantly different between different types of comparisons (e.g., mothers compared with their own babies vs. mothers compared with other babies), we performed *t* tests.

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