

RESEARCH ARTICLE

Bacterial DNA is present in the fetal intestine and overlaps with that in the placenta in mice

Keith A. Martinez, II^{1,2}, Joann Romano-Keeler³, Joseph P. Zackular⁴, Daniel J. Moore^{3,4}, Robert M. Brucker⁵, Christopher Hooper³, Shufang Meng³, Naoko Brown³, Simon Mallal^{4,6,7}, Jeff Reese^{3,8}, David M. Aronoff^{4,6}, Hakdong Shin², Maria Gloria Dominguez-Bello¹, Jörn-Hendrik Weitzkamp³*

1 Department of Microbiology, Sackler Institute, New York, New York, United States of America, **2** Department of Translational Medicine, NYU School of Medicine, New York, New York, United States of America, **3** Department of Pediatrics, Vanderbilt University Medical Center, Nashville, Tennessee, United States of America, **4** Department of Pathology, Microbiology & Immunology, Vanderbilt University Medical Center, Nashville, Tennessee, United States of America, **5** Department of Biological Sciences, Vanderbilt University, Nashville, Tennessee, United States of America, **6** Division of Infectious Diseases Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee, United States of America, **7** Institute for Immunology and Infectious Diseases, Murdoch University, Murdoch, Western Australia, **8** Department of Cell and Developmental Biology, Vanderbilt University, Nashville, Tennessee, United States of America

* These authors contributed equally to this work.

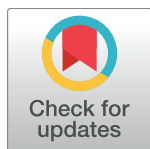
^{‡a} Current address: Mednax Corporation, St. Thomas Midtown, Division of Neonatology Nashville, Tennessee, United States of America

^{‡b} Current address: The Rowland Institute at Harvard, Harvard University, Cambridge, Massachusetts, United States of America

^{‡c} Current address: Department of Food Science and Biotechnology, College of Life Science, Sejong University, Seoul, Republic of Korea

^{‡d} Current address: Department of Biochemistry and Microbiology, Rutgers University of New Jersey, New Brunswick, New Jersey, United States of America

* hendrik.weitzkamp@vanderbilt.edu (JHW); mg.dominguez-bello@rutgers.edu (MGDB)



OPEN ACCESS

Citation: Martinez KA, II, Romano-Keeler J, Zackular JP, Moore DJ, Brucker RM, Hooper C, et al. (2018) Bacterial DNA is present in the fetal intestine and overlaps with that in the placenta in mice. PLoS ONE 13(5): e0197439. <https://doi.org/10.1371/journal.pone.0197439>

Editor: Jefferson Terry, BC Children's Hospital, CANADA

Received: December 16, 2017

Accepted: May 2, 2018

Published: May 17, 2018

Copyright: © 2018 Martinez et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: Sequences have been uploaded to the Qiita servers (qiita.ucsd.edu) under study ID 10453.

Funding: This project was supported by the National Institutes of Health (NIH) Loan Repayment Program and the Eunice Kennedy Shriver National Institute Of Child Health & Human Development (NICHD) T32HD068256 (to JRK), the National Institute Of Diabetes and Digestive Kidney Diseases (NIDDK) T32DK007673, the National Institute of Allergy and Infectious Diseases (NIAID)

Abstract

Bacterial DNA has been reported in the placenta and amniotic fluid by several independent groups of investigators. However, its taxonomic overlap with fetal and maternal bacterial DNA in different sites has been poorly characterized. Here, we determined the presence of bacterial DNA in the intestines and placentas of fetal mice at gestational day 17 (n = 13). These were compared to newborn intestines (n = 15), maternal sites (mouth, n = 6; vagina, n = 6; colon, n = 7; feces, n = 8), and negative controls to rule out contamination. The V4 region of the bacterial 16S rRNA gene indicated a pattern of bacterial DNA in fetal intestine similar to placenta but with higher phylogenetic diversity than placenta or newborn intestine. Firmicutes were the most frequently assignable phylum. SourceTracker analysis suggested the placenta as the most commonly identifiable origin for fetal bacterial DNA, but also over 75% of fetal gut genera overlapped with maternal oral and vaginal taxa but not with maternal or newborn feces. These data provide evidence for the presence of bacterial DNA in the mouse fetus.

F32FAI120553 (to JPZ), the NIDDK K08DK090146 and the Vanderbilt Diabetes Center P30DK20593 (to DM), NICHD R01 HD081121 (to JR), An Investigators in the Pathogenesis of Infectious Disease grant from the Burroughs Wellcome Fund and a grant from the Global Alliance to Prevent Prematurity and Stillbirth (GAPPS) (to DA), the Emch and C&D Foundations (to MGDB), the NICHD K08HD061607 (to JHW), and an Institutional Research Training Award T32 AI007180 (to KAMII). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIAID, NICHD, NIDDK, NSF, or NIH.

Competing interests: The authors have declared that no competing interests exist.

Introduction

While bacterial colonization of the *in utero* environment is well recognized during preterm labor and preterm rupture of membranes [1–4] a long-standing dogma establishes a sterile *in utero* environment during undisturbed term healthy pregnancy [5]. However, recent studies incorporating culture independent techniques have found bacterial DNA in the placenta, amniotic fluid, and meconium [2,6–13]. In addition, we discovered higher bacterial DNA diversity in the presumably sterile small intestinal tissue samples surgically resected shortly after birth for congenital intestinal obstruction compared to fecal samples from the same infants [14]. Shared features between bacterial DNA in placenta and amniotic fluid with infant meconium and the influence of maternal diet on the newborn microbiome suggest microbial transfer at the feto-maternal interface [10,15]. However, presence of bacterial DNA in amniotic fluid, placenta and postnatal meconium is not direct evidence for its existence in the fetal intestine. Therefore, we aimed to detect bacterial DNA in the fetal gut and to study its likely origin. While bacterial DNA does not infer viable bacteria, its presence may be critical for the developing immune system.

Because contaminating DNA has been detected in DNA extraction kits and other laboratory reagents, caution is required in studies of low biomass samples, such as the feto-maternal unit [16]. Here we carefully controlled for exogenous contamination, and provide community membership estimates, their overlap at multiple maternal-fetal body sites and attempted to source-track these taxonomic members using computational methods.

Results

Fetal intestines harbor bacterial DNA with rich diversity

In this study, we used a mouse model of normal pregnancy to determine the presence of 16S rRNA gene sequences in fetal and newborn intestines, and in placental and maternal (oral, vaginal, colon, feces) samples. The number of samples analyzed for each body site is shown in Fig 1. We collected maternal, placental, fetal samples from 4 individual dams and their pregnancy products after sacrifice for sterile C-section and maternal and neonatal samples from 4 individual dams and their pups delivered by vaginal birth. Dams/litters were housed individually.

We used real time PCR to validate 16S rRNA load across samples with presumed low bacterial biomass. As reflected by cycle threshold (C_t), the amount of relative bacterial biomass detected in the fetal intestines at gestational day 17 (E17) was higher than placental samples at the same time point ($C_t = 25.95$ versus mean $C_t = 28.63$). Assuming perfect PCR efficiency, these differences equate to eight-fold higher bacterial DNA content in the fetal intestine compared to placenta. As expected, the greatest relative bacterial biomass was detected in maternal stool samples on E17 (mean $C_t = 20.73$) and postnatal day 1 (P1, mean $C_t = 19.84$).

After subtracting the OTUs found in blank samples and negative controls, we found bacterial DNA in fetal and newborn intestines, placenta, and all maternal samples (oral, vaginal, feces). PERMANOVA analysis showed a statistical difference between the bacterial DNA found in negative controls when compared to samples from fetal intestines and placentas (p-value < 0.001, data not shown). Bacterial DNA in the fetal intestine showed higher richness (100 ± 47 OTUs) than in the placenta (75 ± 31 ; p-value < 0.05), and newborn intestine (75 ± 38 , p-value < 0.05; Table 1). Maternal sites (vaginal and fecal) had the highest richness (Fig 1A) and phylogenetic diversity (Fig 1B).

Fetal intestines had higher proportions of *Enterococcus* (3.2%) and lower proportions of *Streptococcus* and *Staphylococcus* in relation to the newborn intestine, maternal mouth or

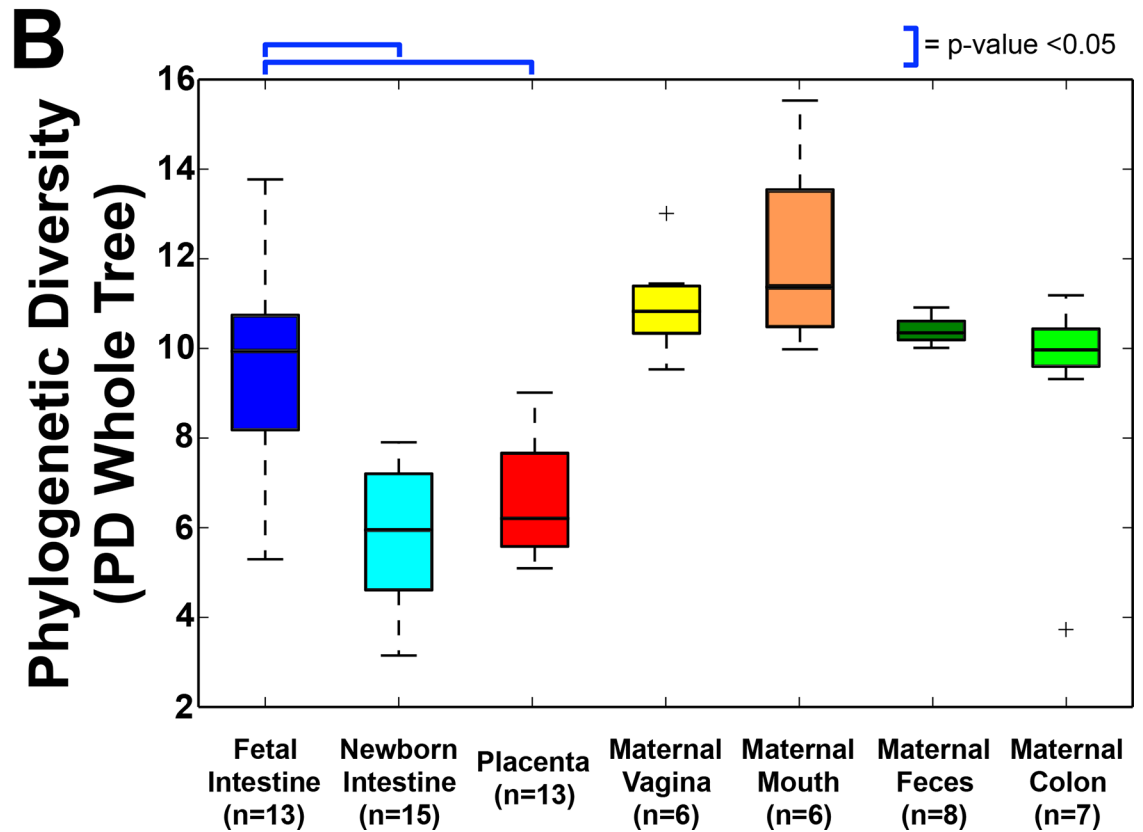
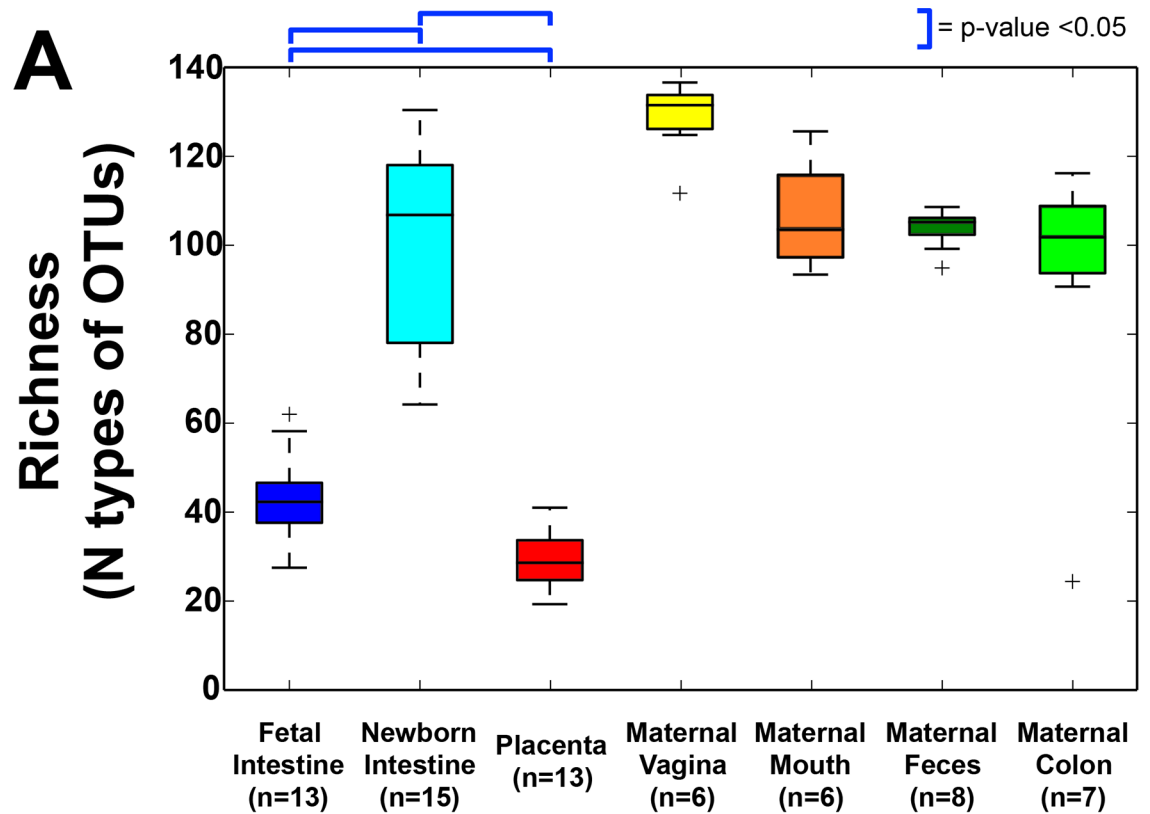


Fig 1. Alpha diversity of bacterial DNA from fetal, newborn and maternal samples. (A) Richness (observed species metric) (B) Phylogenetic diversity, accounting for richness, evenness, and phylogeny. Figure generated in QIIME. Blue brackets indicate comparisons with p-values <0.05, paired sample t-test.

<https://doi.org/10.1371/journal.pone.0197439.g001>

vagina (Fig 2). The bacterial DNA composition in the fetal intestine was highly even, with 90% of taxa represented at <2% relative abundance (Table 2). Conversely, most taxa found in the maternal mouth and vagina were found at >2% relative abundance (73% and 56%, respectively). Interestingly, 22% of the genera found in the fetal intestine were unique to that site, while only 14% were unique to the placenta. Phylotype analysis using Ribosomal Database Project classification (RDP) [17] confirmed the results of Greengenes analyses (S1 Fig). The most abundant fetal intestine bacterial signature was from *Lachnospiraceae*, a common gut bacterium in mammals. In addition, fetal intestines and placenta had the greatest abundance of *Burkholderiaceae* DNA. Postnatally, the intestine became dominated by *Streptococcus* and *Staphylococcus* (S2 Fig).

The majority of fetal intestinal bacterial DNA was of placental origin

SourceTracker analysis revealed the placenta as the most commonly identifiable origin of fetal intestinal bacterial DNA (approximately 10% of fetal intestine OTUs, Fig 3A) while maternal mouth and vagina were the most commonly identified sources for bacterial DNA in the placenta (S3 Fig) and the second most common sources for bacterial DNA in the fetal intestine (Fig 3A). However, many OTUs were found to be common between different sites (Fig 3B). Principal coordinate analysis (PCoA) of unweighted and weighted UniFrac distances, as well as Bray-Curtis similarity, revealed that although OTUs from fetal intestines and placentas segregate apart from all maternal and newborn samples (PERMANOVA p-values 0.001), they also segregate apart from each other (PERMANOVA p-value 0.01) (Fig 4 and S4 Fig) independently of the mother they were sampled from (data not shown). However, linear discriminant analysis effect size (LEfSe) analysis detected no significant differences in genus level taxa level between fetal intestine and placental samples. Litter effect did not account for similarities in bacterial composition between fetal intestines and placentas (S5 Fig).

Discussion

In this study, we describe the first account of bacterial signatures in mammalian fetal intestinal tissue. The fetal intestinal microbial signature during normal murine pregnancy was unique yet most similar to the placental signature, and after birth, the newborn intestine resembled maternal vaginal and oral microbiomes. Mice tend to lick their newborn pups immediately

Table 1. Sequencing depth and OTU counts for maternal, fetal, placental and newborn samples.

Sample Type	Maternal				Fetal		Newborn	Controls		Total
	Feces	Colon	Oral	Vagina	Placenta	Intestine	Intestine	PCR Control	DNA Ext. Control	
N samples	8	7	6	6	13	13	15	2	7	136
Total N sequences to pick OTUs	47,271	9,151	46,625	35,662	21,480	23,482	5,764	6,811	557,518	1,116,193
Mean N of sequences ±SD	5,909 ±2,409	1,307 ±675	7,771 ±1,107	5,944 ±3,703	1,652 ±953	1,806 ±1,031	384±208	3,406 ±4,523	79,645 ±60,626	
N of OTUs represented	3,074	1,009	5,344	2,915	863	1,172	379	326	4,094	17,484
Mean N OTUs ±SD	789 ±178	238 ±83	1,688 ±255	714 ±415	75 ±31	100 ±47	75 ±38	171 ±91	816 ±509	

<https://doi.org/10.1371/journal.pone.0197439.t001>

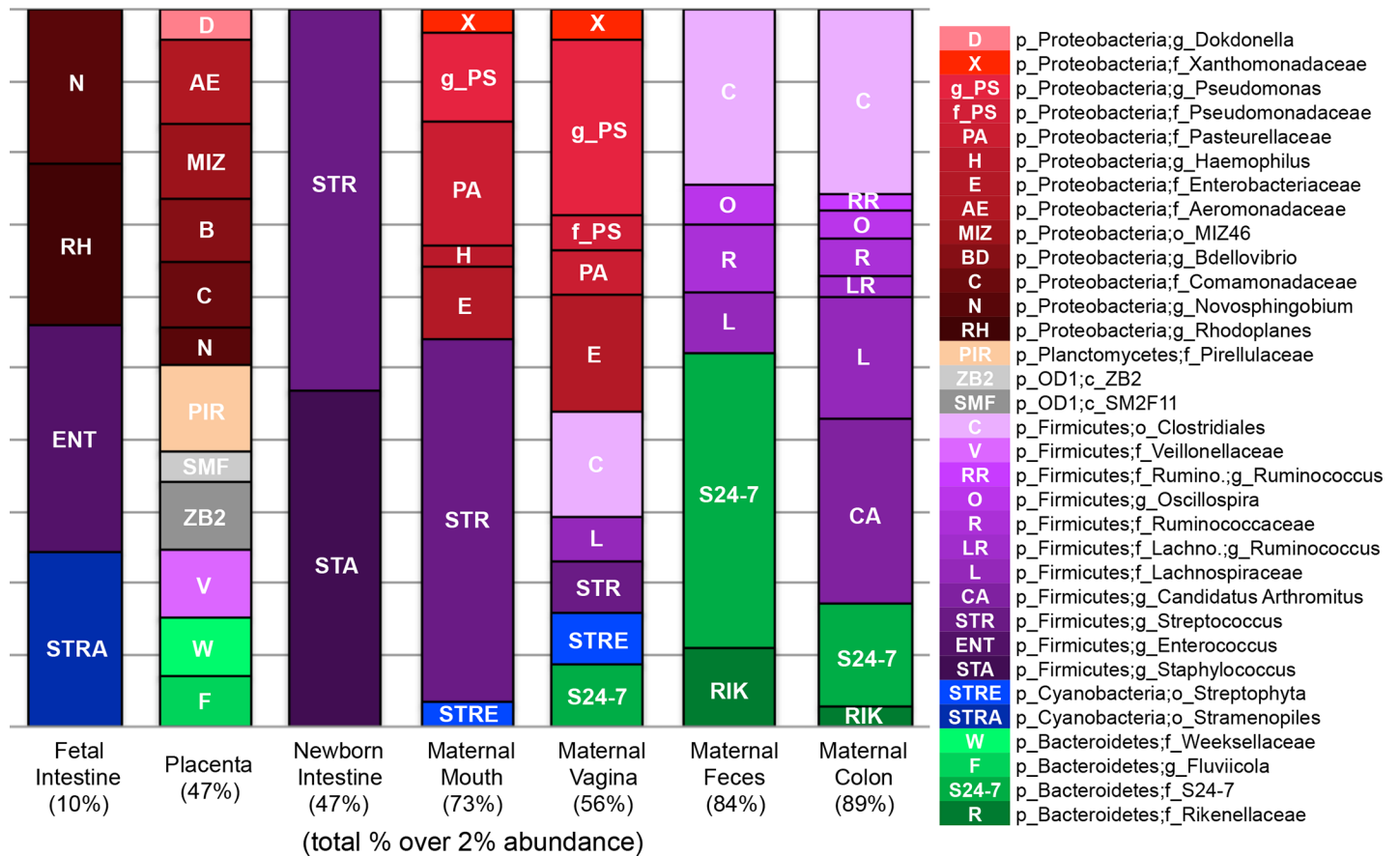


Fig 2. Major bacterial taxa to which fetal, newborn and maternal bacterial DNA were assigned. Taxa present at ≥ 2% relative abundance. Taxonomies are the lowest level of identification provided by OTU classification. Figure generated in QIIME.

<https://doi.org/10.1371/journal.pone.0197439.g002>

after birth and we speculate this behavior as a possible rationale for our findings. In addition, the fact that the newborn intestine undergoes a reduction in the diversity of bacterial taxa has been previously reported and is attributable to the selective effect of maternal milk [18].

Low biomass samples, such as the fetal intestine and placenta, require a careful design to minimize contamination [16]. While we cannot completely rule out DNA contamination completely, we exhibited the following cautionary steps: First, we applied stringent experimental practices to reduce contaminants, including UV irradiation of reagents and surfaces,

Table 2. Percentage of taxa with relative abundance in each percentile by sample type.

Sample Type	Percent Taxa with Relative Abundance in each Percentile		
	Major Taxa ≥ 2%	Minor Taxa 2%–0.1%	Sub-taxa ≤ 0.1%
Fetal Intestine	10	47	43
Placenta	47	46	7
Newborn Intestine	47	13	40
Maternal Mouth	73	20	7
Maternal Vagina	56	37	7
Maternal Feces	84	15	1
Maternal Colon	89	9	2

<https://doi.org/10.1371/journal.pone.0197439.t002>

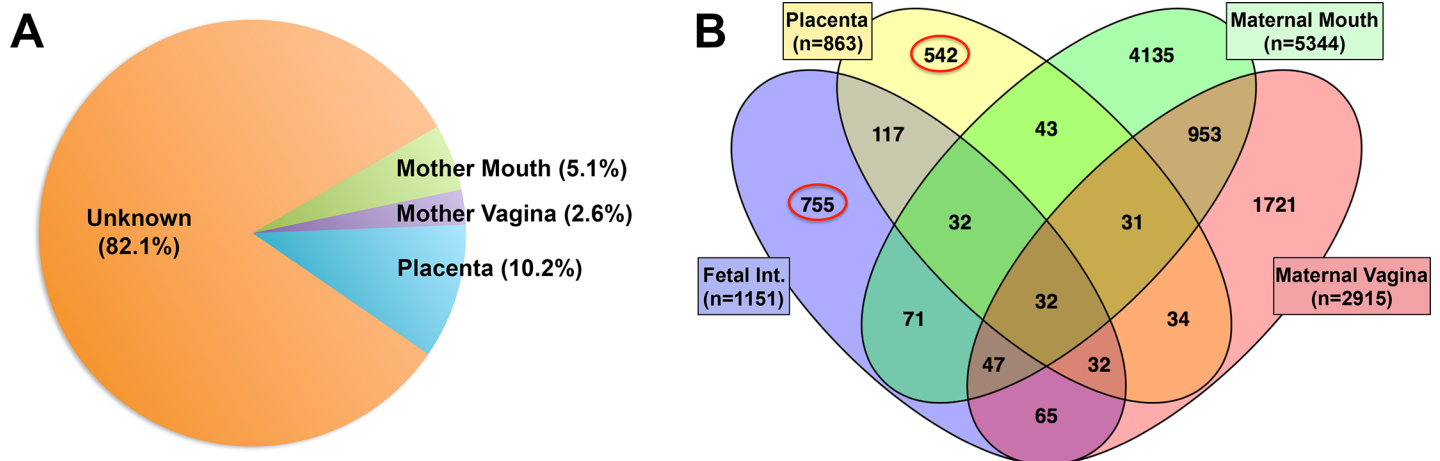


Fig 3. Sources of bacterial DNA-OTUs in the fetal intestine. (A) SourceTracker analysis showing predicted origin of OTUs in placenta, mouth and vagina. (B) Venn diagram showing that 2/3 of the fetal intestine OTUs were not found in placenta, maternal mouth or vagina.

<https://doi.org/10.1371/journal.pone.0197439.g003>

replacement of autoclaved tools between extraction steps (opening of abdominal cavity, extraction of uteri, extraction of fetus), experimental replicates, and maximal surgical barrier precautions for all protocols related to sample preparation. Second, during dissections, we confirmed the absence of environmental contaminants with surface cultures from pregnant dams prepped sterilely and from all fetal samples. Third, we processed negative ‘blank’ controls collected in the same type tubes as tissue samples each time we extracted bacterial DNA from samples and subsequently sequenced these negative controls. Fourth, as is recommended in studies of low bacterial biomass [16], we removed known contaminants detected in sequenced negative controls and reagents from all of our results, including biologically anomalous taxa.

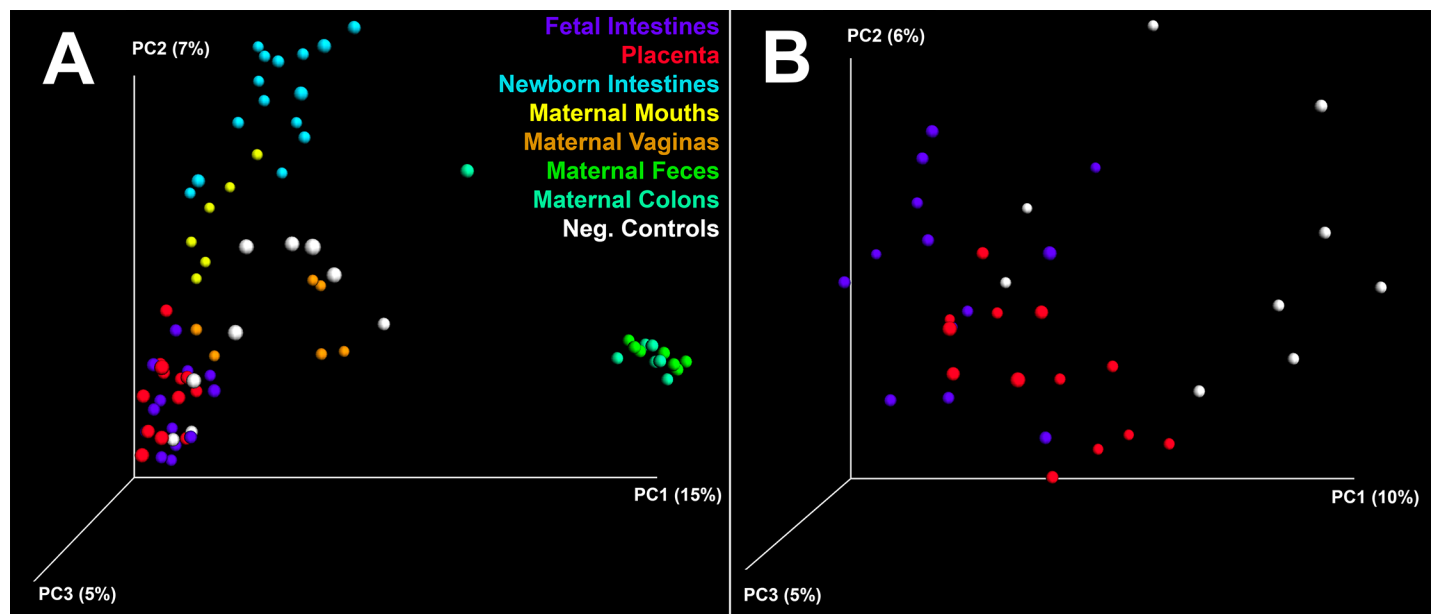


Fig 4. Beta diversity in bacterial DNA from fetal, newborn and maternal samples. (A) Fetal intestine and placenta cluster away from newborn intestines and maternal sites (all PERMANOVA p-values = 0.001). (B) Fetal intestine, placenta and negative controls are significantly different from each other (PERMANOVA p-values = 0.001). Figure generated in QIIME.

<https://doi.org/10.1371/journal.pone.0197439.g004>

Finally, we analyzed sequences independently using two different microbiome analysis software platforms, mothur and QIIME. With these approaches, a diverse fetal microbial DNA signal remained as early as embryonic day 17 (E17) in mice.

Our study has several limitations. First, we did not process collection material, surfaces, or collection tubes in parallel with tissue samples. However, by subtracting control DNAs, we believe we have controlled for contamination issues. Yet, we observe DNA from environmental bacteria such as *Rhodoplanes* (phototrophic bacteria), *Novosphingobium* (water, soil, sediment-dwelling bacteria) and *Stramenopile* chloroplasts (eukaryotic organisms—algae, diatoms) in the fetal intestines (Fig 2). These species may be originating from the diet or drinking water and their DNA might be circulating in the maternal bloodstream. Future studies should include maternal blood samples as controls. Second, we used V4 primers, rather than V1-V3, which may have limited the taxa detected [19]. Other studies with similar objective to ours also targeted the V4 region [1] while others used the V1-V3 sites [10]. A recent metaanalysis in pre-term infants found that *Proteobacteria* were more abundant and *Firmicutes* less abundant in studies targeting V3-V5 compared to V1-V3 [20]. However, in this study we compared different body sites with each other and do not emphasize the genera detected. We do not believe that the choice of primers would have altered the principle finding of bacterial DNA detection in the fetal gut.

In this study, we show maternal oral, placental, and fetal bacterial DNA preceding delivery in a murine model. This is consistent with findings in humans, where bacterial DNA has also been reported in placenta, amniotic fluid and meconium [2,9]. Our data also support resemblance between bacterial DNA in the placenta and fetal intestine. The results do not challenge the idea of a sterile fetus, but rather suggest translocation of bacterial DNA from the mother's microbiome into the fetal intestine. If bacterial DNA is translocated to the fetal intestine through the placenta, its role is unknown. During pregnancy, bacterial components and products are likely disseminated by the maternal vascular supply, as has been shown with regulatory RNAs that cross the placenta [21–23] and may reach the growing fetus. Bacterial DNA transferred from the mother to the fetal gut may be a critical stimulus for normal mucosal immune development [10], but this has not been clearly demonstrated.

Mode of delivery shapes the neonatal microbiome structured by live communities [24], type of feeding (formula versus breast milk), and antibiotic exposure and body site-specific environments largely determine the infant microbiome later in life [21,22], but the maternal microbiome may initiate priming of the fetus immune system, which in turn will modulate the microbiota structure after birth. Further research is needed to elucidate which prenatal interventions could change the fetal microbiome with the goal of improving neonatal outcomes.

Materials and methods

Mice

All animal experiments were conducted in accordance with the National Institutes of Health (NIH) animal care standards and were approved by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC). Timed matings of adult C57BL6 mice at 7–10 weeks of age were performed to obtain samples at embryonic day 17 (E17) and post-partum day 1 (P1).

Maternal sample collection

Following euthanasia with isoflurane, topical calcium hydroxide and potassium thioglycolate was applied to the dams' abdomen for hair removal prior to disinfection. The abdomen was serially prepped with betadine and isopropyl alcohol. Prepped dams were given to trained

laboratory personnel, who resected all maternal samples in a ventilated hood with sterile instruments. All instruments were replaced between mice. In addition, laboratory personnel donned facial masks, surgical caps, sterile surgical gowns, and sterile gloves. Excised gravid uteri were placed inside sterile petri dishes and given to a second trained lab member, who performed all fetal dissections in a separate laminar flow hood as described below. To minimize the possibility of DNA contamination, both hoods were treated with UV light and surfaces wiped with ethanol prior to tissue extractions. Maternal fecal samples from proximal to the colonic lumen were collected in sterile sample vials and immediately snap frozen and stored at -80°C until bacterial DNA extraction.

Fetal sample collection

To minimize contamination, all fetal dissections were conducted in a sterile, ventilated hood geographically separated from the hood used for maternal dissections. In addition, the dedicated lab person for fetal dissections was never the same person completing maternal dissections. This individual also dressed in sterile surgical garb as described above. New sterile instruments and petri dishes were used to: 1) remove each fetus from the amniotic sac; 2) dissect the placenta from the amniotic tissue; and 3) resect the intestines from the abdominal cavity. For each fetus, placental and intestinal samples were resected, snap frozen, and stored at -80°C .

Confirming sterile dissection technique

To confirm sterile technique, we performed bacterial cultures of: a) dam's abdomen before and after sterile surgical preparation; b) oropharynx; c) vagina; and d) peritoneal cavity prior to uterine excision. We collected bacterial cultures from the fetal surface immediately after excision from the amniotic sac and prior to abdominal incision. A bacterial culture was also obtained from the fetal intestinal lumen after resection from the peritoneum. All bacterial cultures were enriched in Luria broth (LB) and incubated at 37°C for 48 hours. Vaginal cultures were also collected in anaerobic transport media and grown for 48 hours in an anaerobic chamber. Bacterial growth was assessed at 24-hour intervals. A positive control, which was a mixture of several non-fastidious bacterial strains, was included with each dissection's set of bacterial cultures to ensure maintenance of normal bacterial growth conditions. Cultures of placentas, fetal surfaces, and fetal intestines at E17 had no bacterial growth after 48 hours. However, after birth, newborn (P1) intestinal cultures were positive at 24 hours. Cultures of the maternal peritoneum after surgical excision were negative while external maternal abdominal wall cultures were also positive 24 hours prior to, but not after disinfection for surgery. As expected, maternal mouth and vaginal cultures were culture-positive.

Real-time PCR of fetal and pup intestinal samples

Real-time quantitative (q) PCR amplification was performed in triplicate for all fetal and postnatal pup intestinal samples on an ABI 7900 TaqMan Real Time PCR System (Applied Biosystems, NY) to quantify fetal intestinal bacterial load. We used the conserved eubacterial (EUB) 1114 forward (CGGCAACGAGCGCAACCC) and 1221 reverse (CCATTGTAGCACGTGTGTGCC) 16S ribosomal primers to detect total bacteria [25]. Reaction mixtures consisted of the 10ng of DNA template, 10 μM concentration of each primer, 0.625 μL 1X Omni KlenTaq (DNA Polymerase technology, cat no. 350), 25mM dNTP (Enzymatics, cat no. N2050L), and 1.25 μL EvaGreen (Biotium, CA) in a final reaction volume of 25 μL . Cycling conditions were as follows: initial incubation of 95°C for 3 min, denaturing at 95° for 10 sec, then 58 for 30 sec then 72 for 30 sec, for 40 cycles. We used cycle threshold (C_t) as an indirect indicator for biomass as

suggested by others [26]. We normalized C_t data by grams of tissue weight and arbitrarily considered a mean $C_t > 20$ low relative biomass.

DNA extraction and 16S rRNA gene sequencing

DNA was extracted from fecal and tissue samples using a modified Qiagen protocol that included pretreatment for lysis of Gram-positive bacteria with 20 mg/ml lysozyme in Tris-HCl and EDTA buffer (Qiagen, DNeasy Blood & Tissue Kit, Hilden, Germany) without additional bead beating, as previously reported [14]. The remainder of the DNA extraction protocol proceeded per the manufacturer's instructions. The V4 region of the 16S rRNA gene from each sample was amplified and sequenced using the Illumina MiSeq Personal Sequencing platforms as described previously [25]. Briefly, 1 μ l of DNA template from each sample was added to all reactions and 30 PCR cycles were performed to minimize contaminant amplification. DNA template-free controls (negative controls) were processed concurrently with samples using the same DNA extraction and PCR amplification kits. Negative controls as well as a mixture of bacterial plasmids developed in-house at the Center for Microbial Systems at the University of Michigan for positive PCR control, were sequenced in parallel with study samples. We performed amplifications with barcoded primers, and amplicons from samples and controls were pooled, leaving none excluded.

Microbiome analysis

The 16S rRNA sequence analyses were performed initially with the mothur software package [25,27,28]. For data confirmation and additional illustration, sequences were subsequently analyzed using the QIIME suite of software tools (v1.8) [29]. The sequence reads were used to pick operational taxonomic units (OTUs), with an open-reference OTU picking method based on 97% identity to entries in the Greengenes database (v13_8). Eleven DNA extraction reagent negative controls and 2 PCR reagent negative controls were used. PCR reagent negative controls collected during sampling in same type tubes as study samples were included in the amplification and sequencing procedures to determine background noise and/or contamination, during the processing of the sampling. OTUs from blanks were subtracted from study samples. Of the 11 DNA extractions, 2 had negative controls with extremely high (~2500 each) OTUs and 2 DNA extractions had low efficiency. The negative controls and samples from these DNA extractions were removed from final analysis (analyzed samples and negative controls for each DNA extraction listed in [S1 Table](#) and unprocessed sequence information is listed in [S2 Table](#)). The negative-control-derived OTUs from the remaining 7 DNA extraction controls were discarded from the OTU table using a filtration script (`filter_otus_from_otu_table.py`) in QIIME (OTUs found in negative controls are listed in [S3 Table](#)). Chimeric sequences were removed using UCHIME [30] prior to analysis. All communities were rarefied to 200 reads per sample because we assumed that low biomass is the cause for low PCR efficiency, resulting in low sequencing yield not surpassing 200 reads per sample in some cases. For comparison of levels of beta diversity between communities, the unweighted/weighted UniFrac distances [31] and Bray-Curtis dissimilarities [32] were calculated and PERMANOVA [33] was used to test significance. We applied linear discriminant analysis effect size (LEfSe) [34] to detect unique biomarkers by determinations of the relative abundances of the members of the bacterial taxonomies.

Supporting information

S1 Fig. Heat maps generated in mothur showing E17 based on operational taxonomic units (OTUs), clustered at 0.03 and classified with RDP classifier.
(TIFF)

S2 Fig. Heat maps generated in mothur showing P1 based on operational taxonomic units (OTUs), clustered at 0.03 and classified with RDP classifier.

(TIFF)

S3 Fig. Bacterial OTUs in placental samples. SourceTracker analysis shows maternal mouth and vaginal sources.

(TIF)

S4 Fig. Beta diversity in bacterial DNA from fetal, newborn and maternal samples. (A) Weighted UniFrac PCoA generated in QIIME shows fetal intestine (dark blue) and placenta (red) (cluster away from newborn intestines (light blue) and maternal sites (mouth = yellow, vagina = orange, feces = dark green, colon = light green) as well as negative controls (white) and the mock community (gray) (PERMANOVA p-values < 0.05). (B) Bray-Curtis PCoA generated in QIIME shows fetal intestine and placenta cluster away from newborn intestines and maternal sites (PERMANOVA p-values < 0.05).

(TIF)

S5 Fig. Litter effect does not account for similarities in bacterial composition between fetal intestines and placentas. NMDS plots generated in mothur comparing the fetal microbiome with those of matched placentas from the same litter for each time point, including E17 (A) and P1 (B). For any given time point, each individual mother-fetus or mother-pup unit is reflected as triangles, circles, or diamonds. Color key for samples is as follows, red: fetal intestines; blue: placenta; green: maternal vagina; orange: maternal mouth; black: maternal stool.

(TIFF)

S1 Table. Negative controls and samples processed and analyzed in each DNA extraction.

(DOC)

S2 Table. Sequencing depth and OTU counts for unfiltered maternal, fetal, placental and newborn samples.

(DOC)

S3 Table. Number of sequences found in OTUs from negative controls.

(DOC)

Acknowledgments

We thank Dr. Martin Blaser for review of the manuscript and helpful discussion.

Author Contributions

Conceptualization: Joann Romano-Keeler, Robert M. Brucker, Simon Mallal, Jörn-Hendrik Weitekamp.

Data curation: Keith A. Martinez, II, Joseph P. Zackular.

Formal analysis: Keith A. Martinez, II, Joann Romano-Keeler, Joseph P. Zackular, Daniel J. Moore, Hakdong Shin, Maria Gloria Dominguez-Bello, Jörn-Hendrik Weitekamp.

Funding acquisition: Jeff Reese, Jörn-Hendrik Weitekamp.

Investigation: Joann Romano-Keeler, Christopher Hooper, Shufang Meng, Naoko Brown, Jörn-Hendrik Weitekamp.

Methodology: Keith A. Martinez, II, Joann Romano-Keeler, Robert M. Brucker, Christopher Hooper, Shufang Meng, Naoko Brown, Jeff Reese, Hakdong Shin, Maria Gloria Dominguez-Bello, Jörn-Hendrik Weitekamp.

Project administration: Jörn-Hendrik Weitekamp.

Resources: Daniel J. Moore, Robert M. Brucker, Simon Mallal, Jeff Reese, David M. Aronoff, Maria Gloria Dominguez-Bello, Jörn-Hendrik Weitekamp.

Supervision: Jeff Reese, David M. Aronoff, Maria Gloria Dominguez-Bello, Jörn-Hendrik Weitekamp.

Validation: Keith A. Martinez, II, Joseph P. Zackular, Hakdong Shin, Maria Gloria Dominguez-Bello.

Visualization: Keith A. Martinez, II, Joseph P. Zackular.

Writing – original draft: Keith A. Martinez, II, Joann Romano-Keeler, Joseph P. Zackular, Daniel J. Moore, Robert M. Brucker, Maria Gloria Dominguez-Bello, Jörn-Hendrik Weitekamp.

Writing – review & editing: Keith A. Martinez, II, Joann Romano-Keeler, Joseph P. Zackular, Simon Mallal, Jeff Reese, David M. Aronoff, Hakdong Shin, Maria Gloria Dominguez-Bello, Jörn-Hendrik Weitekamp.

References

1. Ardisson AN, de la Cruz DM, Davis-Richardson AG, Rechcigl KT, Li N, Drew JC, et al. Meconium microbiome analysis identifies bacteria correlated with premature birth. *PLoS One* 2014; 9: e90784, <https://doi.org/10.1371/journal.pone.0090784> PMID: 24614698
2. DiGiulio DB, Romero R, Amogan HP, Kusanovic JP, Bik EM, Gotsch F, et al. Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. *PLoS One* 2008; 3: e3056, <https://doi.org/10.1371/journal.pone.0003056> PMID: 18725970
3. Han YW, Shen T, Chung P, Buhimschi IA, Buhimschi CS. Uncultivated bacteria as etiologic agents of intra-amniotic inflammation leading to preterm birth. *J Clin Microbiol* 2009; 47: 38–47, <https://doi.org/10.1128/JCM.01206-08> PMID: 18971361
4. Chernikova DA, Koestler DC, Hoen AG, Housman ML, Hibberd PL, Moore JH, et al. Fetal exposures and perinatal influences on the stool microbiota of premature infants. *J Matern Fetal Neonatal Med* 2016; 29: 99–105, <https://doi.org/10.3109/14767058.2014.987748> PMID: 25394613
5. Funkhouser LJ, Bordenstein SR. Mom knows best: the universality of maternal microbial transmission. *PLoS Biol* 2013; 11: e1001631, <https://doi.org/10.1371/journal.pbio.1001631> PMID: 23976878
6. Jiménez E, Marín ML, Martín R, Odriozola JM, Olivares M, Xaus J, et al. Is meconium from healthy newborns actually sterile? *Res Microbiol* 2008; 159: 187–193, <https://doi.org/10.1016/j.resmic.2007.12.007> PMID: 18281199
7. Jiménez E, Fernández L, Marín ML, Martín R, Odriozola JM, Nueno-Palop C, et al. Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. *Curr Microbiol* 2005; 51: 270–274, <https://doi.org/10.1007/s00284-005-0020-3> PMID: 16187156
8. Zheng J, Xiao X, Zhang Q, Mao L, Yu M, Xu J, et al. The Placental Microbiome Varies in Association with Low Birth Weight in Full-Term Neonates. *Nutrients* 2015; 7: 6924–6937, <https://doi.org/10.3390/nu7085315> PMID: 26287241
9. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J, et al. The placenta harbors a unique microbiome. *Sci Transl Med* 2014; 6: 237ra265, <https://doi.org/10.1126/scitranslmed.3008599> PMID: 24848255
10. Collado MC, Rautava S, Aakko J, Isolauri E, Salminen, S. Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. *Sci Rep* 2016; 6: 23129, <https://doi.org/10.1038/srep23129> PMID: 27001291
11. Amarasekara R, Jayasekara RW, Senanayake H, Dissanayake VH. Microbiome of the placenta in pre-eclampsia supports the role of bacteria in the multifactorial cause of pre-eclampsia. *J Obstet Gynaecol Res* 2015; 41, 662–669, <https://doi.org/10.1111/jog.12619> PMID: 25492799

12. Antony KM, Ma J, Mitchell KB, Racusin DA, Versalovic J, Aagaard K et al. The preterm placental microbiome varies in association with excess maternal gestational weight gain. *Am J Obstet Gynecol* 2015; 212: 653 e651-616, <https://doi.org/10.1016/j.ajog.2014.12.041> PMID: 25557210
13. Prince AL, Ma J, Kannan PS, Alvarez M, Gisslen T, Harris RA, et al. The placental membrane microbiome is altered among subjects with spontaneous preterm birth with and without chorioamnionitis. *Am J Obstet Gynecol* 2016; 214: 627 e621-627 e616, <https://doi.org/10.1016/j.ajog.2016.01.193> PMID: 26965447
14. Romano-Keeler J, Moore DJ, Wang C, Brucker RM, Fonnesebeck C, Slaughter JC, et al. Early life establishment of site-specific microbial communities in the gut. *Gut Microbes* 2014; 5: 192–201, <https://doi.org/10.4161/gmic.28442> PMID: 24637795
15. Chu DM, Antony KM, Ma J, Prince AL, Showalter L, Moller M, et al. The early infant gut microbiome varies in association with a maternal high-fat diet. *Genome Med* 2016; 8: 77, <https://doi.org/10.1186/s13073-016-0330-z> PMID: 27503374
16. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, et al. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol* 2014; 12: 87, <https://doi.org/10.1186/s12915-014-0087-z> PMID: 25387460
17. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007; 73: 5261–5267, <https://doi.org/10.1128/AEM.00062-07> PMID: 17586664
18. Pantoja-Feliciano IG, Clemente JC, Costello EK, Perez ME, Blaser MJ, Knight R et al. Biphasic assembly of the murine intestinal microbiota during early development. *ISME J* 2013; 7: 1112–1115, <https://doi.org/10.1038/ismej.2013.15> PMID: 23535917
19. Zheng W, Tsompana M, Ruscitto A, Sharma A, Genco R, Sun Y, et al. An accurate and efficient experimental approach for characterization of the complex oral microbiota. *Microbiome* 2015; 3: 48, <https://doi.org/10.1186/s40168-015-0110-9> PMID: 26437933
20. Pammi M, O'Brien JL, Ajami NJ, Wong MC, Versalovic J, Petrosino JF. Development of the cutaneous microbiome in the preterm infant: A prospective longitudinal study. *PLoS One* 2017; 12: e0176669, <https://doi.org/10.1371/journal.pone.0176669> PMID: 28448623
21. Chu DM, Ma J, Prince AL, Antony KM, Seferovic MD, Aagaard KM. Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery. *Nat Med* 2017; 23: 314–326, <https://doi.org/10.1038/nm.4272> PMID: 28112736
22. Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, et al. Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics* 2006; 118: 511–521, <https://doi.org/10.1542/peds.2005-2824> PMID: 16882802
23. Kriegel MA, Sefik E, Hill JA, Wu HJ, Benoist C, Mathis D. Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice. *Proc Natl Acad Sci U S A* 2011; 108: 11548–11553, <https://doi.org/10.1073/pnas.1108924108> PMID: 21709219
24. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci U S A* 2010; 107: 11971–11975, <https://doi.org/10.1073/pnas.1002601107> PMID: 20566857
25. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* 2013; 79: 5112–5120, <https://doi.org/10.1128/AEM.01043-13> PMID: 23793624
26. Junicke H, Abbas B, Oentoro J, van Loosdrecht M, Kleerebezem R. Absolute quantification of individual biomass concentrations in a methanogenic coculture. *AMB Express* 2014; 4: 35, <https://doi.org/10.1186/s13568-014-0035-x> PMID: 24949269
27. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 2009; 75: 7537–7541, <https://doi.org/10.1128/AEM.01541-09> PMID: 19801464
28. Zackular J P, Rogers MA, Ruffin MT, Schloss PD. The human gut microbiome as a screening tool for colorectal cancer. *Cancer Prev Res* 2014; 7: 1112–1121, <https://doi.org/10.1158/1940-6207.CAPR-14-0129> PMID: 25104642
29. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010; 7: 335–336, <https://doi.org/10.1038/nmeth.f.303> PMID: 20383131
30. Edgar RC, Haas BJ, Clemente J C, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 2011; 27: 2194–2200, <https://doi.org/10.1093/bioinformatics/btr381> PMID: 21700674

31. Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R. UniFrac: an effective distance metric for microbial community comparison. *ISME J* 2011; 5: 169–172, <https://doi.org/10.1038/ismej.2010.133> PMID: [20827291](https://pubmed.ncbi.nlm.nih.gov/20827291/)
32. Bray JR, Curtis JT. An ordination of the upland forest communities of southern Wisconsin. *Ecol Monogr* 1957; 27: 326–349.
33. Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Austral Ecology* 2001; 26: 32–46, <https://doi.org/10.1111/j.1442-9993.2001.01070.pp.x>
34. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. *Genome Biol* 2011; 12: R60, <https://doi.org/10.1186/gb-2011-12-6-r60> PMID: [21702898](https://pubmed.ncbi.nlm.nih.gov/21702898/)