Full title: Vaginal microbiota of adolescents and their mothers: A preliminary study of vertical transmission and persistence

Short title: Mother-daughter vaginal microbiota

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Abstract

Background: The composition of the human vaginal microbiota is related to many aspects of health from infection susceptibility to preterm birth. Factors that influence human vaginal microbiota composition, including its source, are not well understood.

Objective: The goal of this study was to determine if vaginal microbiota transmission from mother to daughter at birth influences the human vaginal microbiota composition in adolescence.

Study Design: Weekly vaginal swab samples from 13 adolescents and their mothers were collected for up to 4 weeks. After DNA was isolated from the swabs, the V4 region of the bacterial 16S rRNA genes were amplified, sequenced and analyzed. We calculated distances between the bacterial communities in different samples to investigate the relationship between the vaginal microbiota of the mother/daughter pairs and the daughter's birth mode. We also cultivated *Lactobacillus crispatus* from the mother and daughter of 1 pair. To investigate the possibility of direct transmission and persistence of one member of the vaginal microbiota, we isolated DNA from the *L. crispatus* isolates and compared their genomes with each other and other publicly available *L. crispatus* genome sequences.

Results: The vaginal microbiotas of mother/daughter pairs were more similar to each other if the daughter was born by vaginal delivery rather than by C-section. Additionally, genome sequences from an important member of the vaginal microbiota, *L. crispatus*, isolated from one mother/daughter pair in which the daughter was born by vaginal delivery, were highly similar.

Conclusion: Both community-level analysis and isolate genome sequence analysis are

consistent with birth-mode dependent transmission and persistence of at least some members of the vaginal microbiota.

Introduction

The vaginal microbiota plays an important role in human health. The community structure of the vaginal microbiota is linked to HIV susceptibility and preterm birth (1-3). The composition of the vaginal microbiota is distinct from other body sites and contains types of bacteria that seem specific to the vagina (4). For example, the vaginal microbiota is often dominated by specific types of *Lactobacillus,* most commonly *L. crispatus* and *L. iners* (5, 6). Vaginal *Lactobacillus* sp. are thought to maintain dominance and inhibit colonization of other microbes through lactic acid production (7, 8).

Despite strong evidence that the vaginal microbiota can have significant impacts on a woman's reproductive tract health, the factors that influence the composition of the vaginal microbiota are not well understood. It is not known how this vagina-specific community is maintained from generation to generation. One possibility is that at least some members of the vaginal microbiota are transmitted from mother to daughter at birth and maintained in daughters through adolescence.

In healthy babies, the first large, direct exposure to microbes occurs at birth. Birth mode has been shown to influence the composition of the newborn microbiota (gut, skin, mouth), likely due to different bacterial exposure in vaginal delivery and C-sections (9-11). However, the effect of birth mode on the composition of the vaginal microbiota has not been investigated. In this study, we compared the vaginal microbiotas of 13

mother/daughter pairs and investigated the effect of birth mode on mother/daughter microbiota similarity. We also compared the genome sequences from *Lactobacillus crispatus* isolates from one mother/daughter pair. We hypothesized that the vaginal microbiota of mothers and daughters would be more similar if the daughter was born by vaginal delivery than by C-section.

Materials and Methods

Subject recruitment and sample collection

Mother/daughter pairs were recruited from the Pediatric and Adolescent Gynecology Clinic at the University of Michigan Health System in 2014 and 2015. Exclusions were pregnancy and age of less than 15 years. Written, informed consent was obtained and participants completed a baseline survey on their demographics and pertinent gynecologic and medical history. Vaginal samples were self-collected using a dual-headed swab (Starplex Scientific, S09D) at baseline and then weekly for 4 weeks. The baseline swab was obtained in the clinic, with immediate storage on ice and transfer to -80°C within a few hours. The subsequent swabs were returned via mail at ambient temperature. After the fifth swab was received and a completion incentive was mailed to the subject, the link between samples and subject names was destroyed, irreversibly de-identifying all samples. The study was approved by the University of Michigan IRB (HUM00086661).

DNA isolation and 16S rRNA gene sequencing

One of the swab heads from each sample was clipped directly into the bead plate of a PowerMag Microbiome RNA/DNA Isolation Kit (Mo Bio Laboratories, Inc.). DNA isolation was performed according the manufacturer's instructions using an epMotion

5075 liquid handling system. The V4 region of the bacterial 16S rRNA gene was amplified from 1 or 7µl DNA and sequenced with a MiSeq (Illumina, San Diego, CA) using the 500 cycle MiSeq Reagent Kit, v. 2 (Illumina, catalog No. MS-102–2003) by the University of Michigan Microbial Systems Molecular Biology Laboratory as described previously (12).

Bacterial community analysis

The 16S rRNA gene sequences were processed using mothur v.1.36.1 and v.1.39.5 following the mothur MiSeq SOP (13, 14). Details of the processing steps are available in mother.daughter_mothur.batch (https://github.com/cbassis/MotherDaughter_Vaginal_Microbiota.study). After sequence processing and alignment to the SILVA reference alignment (Release 102) (15), sequences were binned into operational taxonomic units (OTUs) based on 97% sequence similarity using the average neighbor method (16, 17). Samples with fewer than 1000 sequences were excluded from the analysis. OTUs were classified to the genus level within mothur using a modified version of the Ribosomal Database Project (RDP) training set (version 9) (18, 19). To further classify the *Lactobacillus* OTUs, representative sequences were analyzed using standard nucleotide BLAST for highly similar sequences (megablast) on the National Center for Biotechnology Information (NCBI) BLAST web page (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (20). To compare bacterial communities between pairs, within pairs and within subjects, we calculated θ_{YC} distances (a metric that takes relative abundances of both shared and non-shared OTUs into account) (21). A Kruskal-Wallis test with a Dunn's posttest or a Wilcoxon (Mann-Whitney) test were used to determine if differences in θ_{YC} distances were

105 statistically significant. R Studio (Version 1.1.456) with R (Version 3.5.1) was used for
106 the statistical tests and plotting the heat map, box and whisker plots, and the ordination
107 using the code available:
108 https://github.com/cbassis/MotherDaughter_Vaginal_Microbiota.study/tree/master/R_co
109 de. Adobe Illustrator (CS6) was used for labeling and formatting all figures.

110 **_Lactobacillus crispatus_ isolation**

111 For pair I, the second swab head from the freshly collected baseline vaginal
112 sample was swabbed onto an MRS agar plate and incubated in an anaerobic chamber
113 (Coy Laboratory Products) at 37°C. Individual isolates were identified via Sanger
114 sequencing of the near-full length 16S rRNA gene.

115 **DNA isolation and genome sequencing**

116 Three *Lactobacillus crispatus* isolates from pair I, 2 from the mother and 1 from
117 the daughter, were grown overnight in 1 ml liquid MRS in an anaerobic chamber (Coy
118 Laboratory Products) at 37°C. Genomic DNA was isolated from the liquid cultures using
119 the PowerMicrobiome™ RNA Isolation Kit (Mo Bio Laboratories, Inc.) without the DNase
120 treatment. Genome sequencing was performed by the Microbial Systems Molecular
121 Biology Laboratory at the University of Michigan using an Illumina Nextera™ sequencing
122 kit and a MiSeq (Illumina, San Diego, CA).

123 **Genome sequence analysis**

124 Phylogenetic relationships between *L. crispatus* isolates from mother/daughter pair I
125 and all *L. crispatus* strains with genome sequences available as fastq files from NCBI
126 on December 27th, 2018 were determined based on recombination-filtered single
127 nucleotide polymorphisms (SNPs). Quality of reads was assessed with FastQC v0.11.3

(22), and Trimmomatic 0.36 (23) was used for trimming adapter sequences and low-quality bases. Variants were identified by (i) mapping filtered reads to reference genome sequence *L. crispatus* ST1 (SAMEA2272191) using the Burrows-Wheeler short-read aligner (bwa-0.7.17) (24, 25), (ii) discarding polymerase chain reaction duplicates with Picard (picard-tools-2.5.0) (26), and (iii) calling variants with SAMtools (samtools-1.2) and bcftools (27). Variants were filtered from raw results using GATK 's (GenomeAnalysisTK-3.3-0) VariantFiltration (QUAL, >100; MQ, >50; >=10 reads supporting variant; and FQ, <0.025) (28). In addition, a custom python script was used to filter out single-nucleotide variants that were (i) <5 base pairs (bp) in proximity to indels, (ii) fell under Phage and Repeat region of the reference genome (identified using Phaster (29) and Nucmer (MUMmer3.23) (30)), (iii) not present in the core genome, or (iv) in a recombinant region identified by Gubbins 2.3.1 (31). A maximum likelihood tree was constructed in RAxML 8.2.8 (32) using a general-time reversible model of sequence evolution. Bootstrap analysis was performed with the number of bootstrap replicates determined using the bootstrap convergence test and the autoMRE convergence criteria (-N autoMRE). Bootstrap support values were overlaid on the best scoring tree identified during rapid bootstrap analysis (-f a). The final maximum likelihood tree was plotted and pairwise SNP distances were calculated in R Studio (Version 1.1.463) with R (Version 3.5.3): https://github.com/cbassis/MotherDaughter_Vaginal_Microbiota.study/blob/master/R_code/Mother_Daughter_Figure_3_Genome_Tree_and_Genome_Analysis.Rmd.

Calculation of doubling time estimate for vaginal *L. crispatus in vivo*

We used the number of SNPs between the pair I mother and daughter *L. crispatus* isolates to estimate the doubling time of vaginal *L. crispatus in vivo* if all SNPs in the recombination-filtered core genome were due to mutations acquired since the daughter's birth:

Doubling time=(mutation rate)(daughter's age)(genome length)/(# of mutations)

The mutation rate of *L. crispatus* is unknown, so for this estimate we used the published mutation rate of another *Lactobacillus, L. casei* Zhang, *in vitro,* without antibiotics ($1.0x10^{-9}$ bp/generation) (33). The pair I daughter's age in hours was: 175,200 hours =(20 years)(365 days/year)(24 hour/day). The average length of the recombination-filtered core genome (940,943 bp) was used for genome length. We assumed that the isolates arose from a common ancestor and that all mutations were non-convergent, so the number of mutations acquired would equal the number of SNPs between the mother's isolate and the daughter's isolate divided by 2.

Results

Subject characteristics and sequencing results

A total of 107 self-collected, vaginal swab samples were obtained from 26 subjects (13 mother/daughter pairs) (Table 1). Each subject returned 1-5 weekly samples (median=5 samples/subject, IQR=1). After sequence processing and exclusion of samples with fewer than 1000 sequences, a total of 2,336,437 high quality bacterial 16S rRNA gene sequences from 101 samples were analyzed with an average of 23,133 +/- 10,212 sequences per sample (BioProject PRJNA547595).

Table 1. Subject Characteristics

	Mother (n=13)	Daughter (n=13)
	n (%)	n (%)

Age, mean ± SD, years	47±6	17±2
Race: White (vs. Black, Asian, Hispanic, other)	12 (92)	12 (92)
Birth mode: Vaginal (vs. C-section)	10 (77)	10 (77)
Reproductive stage: Premenarchal	0 (0)	1 (8)
Reproductive stage: Reproductive	8 (62)	12 (92)
Reproductive stage: Postmenopausal	5 (38)	0 (0)

An individual's vaginal microbiota is relatively stable over 4 weeks

During the sampling period, the vaginal microbiota of each subject was relatively stable. The high stability of the vaginal microbiota is apparent from the consistent within subject community composition (Fig 1). For example, high relative abundances of OTU1 (*L. crispatus*) and/or OTU2 (*L. iners*) persisted from week to week in many subjects. Additionally, average θ_{YC} distances were significantly lower within subjects than between subjects (Fig 2A) and samples clustered by subject in a PCoA based on θ_{YC} distances (S1 Fig).

Fig 1. Vaginal bacterial community compositions of mother/daughter pairs. Relative abundances of OTUs in weekly vaginal swab samples from 13 mother/daughter pairs. OTU 1 represents *L. crispatus* and OTU 2 represents *L. iners.* Mother/daughter pairs were ordered by average within pair θ_{YC} distances, with the most similar pair (I) on top and the least similar pair (XIII) on the bottom. OTUs with a minimum of 200 sequences in the dataset overall and present at a relative abundance greater than 2% in at least 1 sample were included in the heat map.

Fig 2. Average distances between vaginal bacterial communities. A. Average θ_{YC} distances between subjects from different mother/daughter pairs (between pairs), between subjects within a mother/daughter pair (within pair) and between samples from the same subject (within subject). P-values for comparisons that were

significantly different by Dunn's posttest are shown (Kruskal-Wallis p-value= 8.154e-10). B. Average θ_{YC} distances between subjects within a mother/daughter pair for daughters born by vaginal birth and by C-section. Wilcoxon (Mann-Whitney) test p-value is shown. In the box and whiskers plots, the median θ_{YC} distance is indicated by a line, values within the first to the third quartiles are inside the box and the whiskers extend to the smallest and largest values within 1.5x the interquartile range.

Daughters born via vaginal delivery have greater microbiota similarity with their mothers than those born via C-section

To determine if mothers and their daughters had more similar vaginal microbiotas than unrelated subjects, we compared the average θ_{YC} distances between all unrelated subjects (between pairs) and the average θ_{YC} distances between mothers and their own daughters (within pairs) (Fig 2A). There was a trend toward greater similarity (lower θ_{YC} distances) within all mother/daughter pairs than between subjects in different mother/daughter pairs. To determine if birth mode was related to vaginal microbiota similarity within mother/daughter pairs, we compared the average within pair θ_{YC} distances for pairs in which the daughter was born by vaginal delivery and by C-section (Fig 2B). The average within pair θ_{YC} distances were significantly lower for pairs in which the daughter was born by vaginal delivery compared to C-section (Fig 2B). Therefore, the vaginal microbiotas of daughters born by vaginal delivery were significantly more similar to their mothers' than the daughters born by C-section were to their mothers' (Fig 2B).

***Lactobacillus crispatus* isolates from mother/daughter pair I have highly similar genome sequences**

To investigate the possibility of direct transmission and persistence of one member of the vaginal microbiota, we generated draft genome sequences of *Lactobacillus crispatus* strains isolated from the freshly collected second swab head of mother/daughter pair I (NCBI BioProject PRJNA547620). The draft genome sequences of these isolates were compared with publicly available *L. crispatus* genome sequences by constructing a maximum likelihood phylogenetic tree based on a recombination-filtered core genome alignment. Interestingly, the three strains of *L. crispatus* from mother/daughter pair I, UMP1M1, UMP1M2 and UMP1D1, were more similar to each other than to any of the other strains, including others isolated from the female reproductive tract (Fig 3).

Fig 3. Phylogenetic relationships between *L. crispatus* strains. Maximum likelihood tree based on recombination-filtered SNP distances between *L. crispatus* genome sequences of isolates from mother/daughter pair I and other *L. crispatus* strains with publicly available genomes. Tip labels indicate *L. crispatus* strain names and NCBI BioSample identifiers. Bootstrap values were greater than or equal to 0.65.

We also calculated the number of SNPs between our isolates using the recombination-filtered core genome alignment. There were 11 recombination-filtered SNPs between the 2 isolates from the mother (UMP1M1 and UMP1M2) and 25 and 16 recombination-filtered SNPs between the daughter's isolate (UMP1D1) and the 2 isolates from the mother (UMP1M1 and UMP1M2, respectively).

Estimate of *in vivo* doubling time for vaginal *L. crispatus*

To further investigate the plausibility that the *L. crispatus* strain isolated from daughter I descended from a strain transmitted from her mother at birth, we estimated

the doubling time that would allow our isolates to acquire the observed number of SNPs over 20 years. Based on the 25 SNPs between UMP1M1 and UMP1D1, the estimated doubling time for *L. crispatus in vivo* would be 13.2 hours. Based on the 16 SNPs between UMP1M2 and UMP1D1, the estimated doubling time would be 20.6 hours.

Discussion

Our study provides preliminary evidence that the vaginal microbiota may be vertically transmitted from mother to daughter at birth via vaginal delivery and persist into adolescence. Because the daughters in our study were 15-21 years old, both transmission and persistence were required to observe evidence of vertical transmission. The first piece of evidence supporting vertical transmission is that the vaginal microbiotas of mothers and their adolescent daughters were more similar if their daughter was born by vaginal delivery rather than C-section. The second piece of evidence supporting vertical transmission and persistence is that an important member of the vaginal microbiota, *L. crispatus,* isolated from a vaginally-born, 20-year-old daughter and her mother (pair I) had highly similar genome sequences.

Other studies have compared the vaginal microbiotas of mothers and daughters without detecting notable similarity between them (34, 35). There are multiple reasons that high similarity between mothers and daughters was not observed in these studies. First, the effect of birth mode was not analyzed in these previous studies. If many of the daughters in the other studies were born by C-section then high similarity between mothers and daughters would not be expected. With C- section rates of ~30% in the United States (study site for (35)) and ~36% in South Korea (study site for (34)) this is a possibility (36, 37). Additionally, our study focused on adolescent daughters (age 15-21)

while the other studies focused on either younger or older daughters. Since reproductive stage seems to influence the structure of the vaginal microbiota (38), differences in reproductive stage may contribute to differences in vaginal community composition between mothers and daughters. Finally, we used a different method of comparing the vaginal microbiotas of mothers and daughters. We calculated distances between mothers and daughter using θ_{YC}, a metric that accounts for the relative abundances of shared and non-shared OTUs, while the other studies were based on community types (35) and Unifrac (34). Although an overall community similarity was not observed in these studies, specific community members (*Lactobacillus* and *Prevotella*) were identified as most heritable in one study (34).

Based on the number of SNPs observed between the mother and daughter *L. crispatus* isolates and published mutations rates for *L. casei* Zhang (33), we estimated that *L. crispatus* would have an *in vivo* doubling time of 13.2-20.6 hours, depending on the specific isolates compared. The doubling time estimates of 13.2 hours and 20.6 hours for *L. crispatus in vivo* are within the range estimated for other bacteria in their natural environments, including *Escherichia coli* (15 hours) and *Salmonella enterica* (25 hours) (39). Considering the uncertainty in the estimates, transmission of *L. crispatus* from mother to daughter at birth followed by the accumulation of independent mutations during 20 years of persistence in the mother and daughter is a plausible explanation for the observed recombination-filtered SNPs.

The 2 *L. crispatus* isolates from the mother had highly similar genomes, differing by only 11 recombination-filtered SNPs. A previous study also observed high similarity between the genomes of multiple vaginal *L. crispatus* isolates from one individual,

noting that they were indistinguishable (40).

Consistent with a previous study, *L. crispatus* isolates from the human vagina were phylogenetically intermixed with isolates from the human urinary tract, including highly similar vaginal (ERS1867668 (SAMEA104208650)) and bladder (ERS1867667 (SAMEA104208649)) isolates from the same subject (Fig 3) (41).

The health implications of vertical transmission of the vaginal microbiota are unknown and were not addressed in this study. However, because vertical transmission seems to be an important factor in determining the composition of the vaginal microbiota there may be important consequences. Vertical transmission of the vaginal microbiota may be one mechanism for maintaining human microbiota over generations via a consistent and specific seeding of the newborn microbiota. Delivery mode is an important factor in determining the early composition of the gut microbiota (42, 43) and is a risk factor for development of immune-related disorders later in life (44). This suggests an important role for the mother's vaginal microbiota in seeding the infant and setting the stage for development of the gut microbiota. Therefore, maintenance of the vaginal microbiota between generations may be critical for gut microbiota development in each generation.

Additionally, the vaginal microbiota plays an important if not well understood role in reproductive health, with associations between vaginal microbiota composition and infection susceptibility, BV and preterm birth (45-47). Evidence from this study suggests that transmission of microbes from mother to daughter at birth may influence the composition of the daughter's microbiota later in life and may contribute to the maintenance of specific members of the human vaginal microbiota over generations.

This study provides tantalizing evidence of vertical transmission of the vaginal microbiota. However, it was a small study with only 13 mother/daughter pairs (92% white) and 3/13 daughters born by C-section. Most pairs cohabited at least part-time, so the influence of cohabitation on vaginal microbiota similarity could not be addressed. Genomic analysis of isolates was limited to one member of the vaginal microbiota from 1 mother/daughter pair. The mutation rate and growth rate for *L. crispatus* are unknown, so they had to be estimated in our calculations of doubling time. Future studies in larger populations, including more racially diverse subjects, more daughters born by C-section and analysis of more isolate genome sequences or metagenomes are required to validate these findings.

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Supporting Information

S1 Fig. Principal coordinates analysis (PCoA) of vaginal microbiota from 13 mother/daughter pairs. The θ_{YC} distances between 101 vaginal microbiota samples are represented by PCoA. Samples from daughters are represented by triangles and samples from mothers by circles. Each mother/daughter pair is represented by a unique color. Biplot arrows represent the 3 OTUs most correlated with position on the PCoA plot.