Maternal Vaginal Fluids Play A Major Role In The Colonization of The Neonatal Intestinal Microbiota

Chen Tang

Xiamen University

Xueqin Zhang

Xiamen University

Jingxian Xie

Xiamen University

Lingyu Zeng

Third Institute of Oceanography

Yuntian Xin

Key Laboratory of Urban Environment and Health

Jie Zhang

Xiamen University

Yi Lin

Xiamen University

Yunshan Xiao

Xiamen University

Quanfeng Wu

Xiamen University

Heqing Shen (✉ hqshen@xmu.edu.cn)

Xiamen University

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Abstract

Background: Caesarean delivery (CD) is associated with newborns' health risks due to the blocking of microbiome transfer. To understand the vertical bacterial seeding and reduce CD disadvantages, microbiome transmissions via anal and genital routes were investigated, and the efficiency of vaginal fluid swabbing treatment was evaluated using 16s rDNA sequencing-based techniques.

Results: Pregnant women were recruited in the Women and Children's Hospital, School of Medicine, Xiamen University from June 1st to August 15th, 2017. Maternal faeces (n = 26), maternal vaginal fluids (n = 26), and neonatal transitional stools (n = 26) were collected, while the participants underwent natural delivery (ND) (n = 6), CD (n = 4) and CD with vaginal fluid swabbing (CS) on their newborns (n = 16). 26 mothers with the median age 26.50 (25.00-27.25) years showed no substantial clinical differences. The newborns' gut microbiota altered among ND, CD and CS, and clustered into two groups (PERMANOVA $P < 0.01$) of swabbing and no-swabbing exposure. Gut colonization of ND babies majorly originated from maternal vaginal microbes (PERMANOVA $P = 0.08$), no vertical transmission was observed via anal route in any group. The vaginal transfer partially occurred by swabbing, in which the phyla Firmicutes and Proteobacteria and the genera *Lactobacillus*, *Bacteroides*, and *Escherichia-Shigella* in newborns were similar to their mothers. The recovered taxa predicted KEGG functions of biosynthesis, metabolism, and DNA replication and repair with benefits of low risks of digestive, cardiovascular, and immune diseases.

Conclusions: The newborn's gut microbiota is mainly shaped by maternal vaginal microbiota, and aberrant colonization initiated by CD is partly mitigated by the swabbing treatment.

Background

Caesarean delivery (CD) is a common obstetric surgical procedure entailing the incision of a woman's abdomen/uterus to deliver their offspring(s) [1] with the intent to increase the chances of successful childbirth and to protect the life security of both the mother and the newborn [2, 3]. Over the past two to three decades, global CD surgery rates have been growing steadily but rapidly for women of all ages, races, and medical conditions. According to the World Health Organization (WHO), an estimated 21.1% of births occurred by CD in 2015, which was almost twice that in 2000 (12.1%), and the rates were even higher in certain developed countries and regions [4–6]. Similar patterns could also be observed in China, in which 28.8% and 34.9% of babies were delivered by CD in 2008 and 2014, respectively [7]. However, prior publications have demonstrated that caesarean birth was associated with adverse short- or long-term effects on newborns, including the dysplasia of the immune system, infections, allergies, and inflammatory disorders [8–10]. The conventional view concerning this correlation was that CD newborns were subjected to different hormonal, physical (mechanical forces), bacterial, and therapeutic conditions [11]. Among these conditions, differences in microbial transmission induced by delivery mode were thought to be the determining factor [8, 12]. Vaginally delivered newborns are first exposed to a wide array of microbes during labour via direct contact with the birth canal, whereas CD newborns are initially contacted with microbes from the delivery room and the mothers' skin [13]. In addition, prophylactic

antibiotics administered before or during caesarean surgery may also result in the failure of newborns to acquire the normal microbial inoculum [14]. All these differences inevitably change the composition and abundance of the neonatal gut microbiota, which was hypothesized to result in poorer health outcomes [15]. A better understanding of the theoretical underpinnings of the initial gut colonization of newborns and further actions are required to break this cycle. As mentioned above, exploring an effective intervention measure that is simple, convenient, and cost effective to compensate for the differences in newborn intestinal colonization of CD births could be a prospective application.

In the present research, transitional stool of newborns was used as the outcome bio-sample to assess alterations in the newborns' intestinal microbiome after treatment. Here, transitional stool is defined as the faeces excreted by newborns between 36 h and 72 h after birth, which represents the transition state of meconium and may vary quickly via early-life microbial colonization in the surrounding environment. In addition, because meconium is generated beginning in the 13th week of gestation and is mainly composed of amniotic fluid, shed epithelial cells, intestinal secretions, and gut microbes (e.g., *Enterococcus* and *Staphylococcus* species) [16, 17], previous studies have reported that transitional stool was able to reflect both colonization in utero and early-life development of the intestinal microbiome after birth [18]. However, only a few studies have aimed to explore the microbial community in transitional faeces. In this study, we aimed to understand maternal-newborn vertical microbiota transmission and assess feasible swabbing of CD newborns with maternal vaginal fluids to reduce the potential risks associated with microbiome colonization in CD newborns. By analyzing the pros and cons of the treatment through a population-based intervention study in Xiamen, PR China, we assess its effectiveness and performance in altering the microbes in newborns' transitional stools.

Methods

The recruitment of subjects

This intervention study invited pregnant women who received antenatal care at the Women and Children's Hospital, School of Medicine, Xiamen University, from June 1st, 2017 to August 15th, 2017. The inclusion criteria were briefly listed as follows: 1) gestational age between 37 and 42 weeks; 2) pregnant women who had regular prenatal visits and for whom all clinical data could be obtained; and 3) newborns who were full-term deliveries. Pregnant women with the following complications were excluded: 1) infectious diseases caused by bacteria, viruses, or parasites; 2) inflammatory diseases (e.g., ankylosing spondylitis); 3) metabolic diseases such as diabetes mellitus; 4) pregnancy-associated illnesses including preeclampsia and gestational hypertension; 5) reproductive system disorders (e.g., an ovarian cyst); 6) abnormal pregnancy state (e.g., preterm birth); 7) genetic diseases such as thalassemia; and 8) tumours, including pituitary adenomas and uterine fibroids. The specific inclusion and exclusion criteria are shown in Table S1. The basic information of the pregnant were collected through the last prenatal health checkup and lifestyle questionnaires.

The protocol of our present research was approved by the Medical Ethics Committee of the Women and Children's Hospital, School of Medicine, Xiamen University (KY-2018-020). All procedures were conducted in accordance with the Declaration of Helsinki. All patients were required to provide written informed consent prior to participation. No adverse events were reported for any of the newborns in this study.

Treatment and sample collection

Pregnant women scheduled to have a CD surgery were administrated with prophylactic antibiotics 15 to 60 minutes before skin incision and divided into the two groups according to their willingness to have their newborns swabbed with the vaginal fluids. Before delivery (CD or natural delivery), a 7 × 5 cm four-layered piece of gauze was double-folded and soaked in sterile saline and then inserted into the lower vagina for 30 mins. The gauze was removed as close to the initiation of delivery as possible and kept at room temperature in a sterile collector. The gauze was then divided vertically into two equal parts. One part of the gauze was temporally stored in a 50 ml microcentrifuge tube for subsequent analyses of microbiome, while the other part was used to treat CS newborns (n = 16) as soon as they were born. The principle of 'center-to-periphery' was closely followed during the swabbing procedure, which began on the lips, followed by the face, the thorax, the arms, the legs, and finally the back; the complete process took approximately 30 s for each newborn. Six vaginal-born (n = 6) and four CD-born (n = 4) babies were swabbed with gauze containing sterile saline solution and were used as positive or negative references to assess microbiome transmission. All treatments were performed in the delivery room. Transitional stool was first collected by sterile diapers between 36 h and 72 h after birth, and then 1-2 g of the sample was transferred into a 50 ml microcentrifuge tube. Approximately 1-2 g of fresh maternal faeces was collected into a 50 ml microcentrifuge tube at the last excretion before delivery. All samples were placed into containers of ice and transported to the laboratory within 1 h after collection and stored at -80°C for long-term storage until further processing.

Microbial DNA extraction and 16S rDNA amplification

Microbial DNA in all samples was extracted utilizing the MoBio Powersoil DNA Isolation Kit (QIAGEN, German) according to the manufacturer's instructions. A NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) was used to determine the DNA concentration. The extracted DNA was stored at -20°C until further analysis. The hypervariable V3-V4 regions of the 16S rDNA gene were amplified with the specific primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 806R (5'-GGACTACHVGGGTATCTAAT-3'). PCRs were performed in triplicate in a 50 µL mixture containing 5 µL of KOD buffer (10 ×), 5 µL of dNTPs (2 mM), 3 µL of MgSO₄ (25 mM), 1.5 µL of 341F/806R primer (10 µM), 1 µL of KOD Polymerase, and 100 ng of template DNA. The PCR reagents were purchased from TOYOBO, Japan, and the PCR protocol was carried out for 30 cycles using the following parameters: 94°C pre-denaturation for 2 min, 98°C denaturation for 10 s, 50°C annealing for 30 s, 68°C annealing for 30 s, and hold at 4°C. After amplification, all of the PCR products were pooled, purified, and quantified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA) and the ABI Step-One-Plus Real-Time PCR System (Life Technologies, USA) according to the standard protocols.

16S rDNA sequencing and data processing

The microbiome profiles were analysed by 16S rRNA gene amplicon sequencing with the Illumina MiSeq 250PE platform (Illumina, San Diego, CA, USA). USEARCH software (version 8.1.1861) was applied to turn paired-end sequencing reads into merged, denoised, chimera-free, inferred sample sequences, and the sequence processing steps are described below in detail. Raw sequencing reads were filtered using FASTP software (version 0.18.0) to remove adapters and low-quality reads that would affect the subsequent assembly and analysis to obtain clean high-quality paired-end reads. Paired-end reads were merged as raw tags using FLASH (version 1.2.11), with a minimum overlap of 10 bp and mismatch error rates of 20% [32], and further merged as raw amplicon sequence variants (ASVs) with a minimum overlap of 10 bp. Noisy sequences of raw tags were filtered under specific filtering conditions to obtain clean tags. The high-quality clean tags were clustered into OTUs of $\geq 97\%$ similarity using the UPARSE (version 9.2.64) pipeline. All chimeric tags were removed using the UCHIME algorithm, and effective tags were finally obtained for the next analysis step. The tag sequence with the highest abundance was selected as the representative sequence within each cluster. The representative OTU sequences were classified into organisms by a naive Bayesian model using the RDP classifier (version 2.2) [33] based on the SILVA database (version 132) [34]. The overall sequencing and data processing protocols are shown in Figure S2.

Statistical and bioinformatic analyses

Because the number of subjects was less than 50, the Shapiro-Wilk test and Levene's test were performed to assess the normality of distributions and the homogeneity of variance, respectively. One-way analysis of variance (ANOVA) was performed to compare normally distributed continuous variables, while the Kruskal-Wallis H test was conducted to compare unevenly distributed variables. Comparisons of microbiota between any two groups were performed by utilizing Welch's t or the Wilcoxon test, whereas the Tukey HSD test or the Kruskal-Wallis H test was used for the comparison among groups. Statistical analysis of the clinical data was achieved by Statistic Package for Social Science software (SPSS) (SPSS Inc., USA) (version 26.0). $P < 0.05$ was considered to be statistically significant.

Alpha-diversity was assessed by the observed species (Sobs) index, inverse Simpson index, Shannon index, and Pielou evenness index, while beta-diversity was calculated by the Bray-Curtis distance and illustrated by NMDS analysis. Alpha- and beta-diversity, as well as the NMDS of (Un)weighted UniFrac, Jaccard and Bray-Curtis distances, were generated in the R project Vegan package [35] (version 2.5.3) and plotted in the R project ggplot2 package [36] (version 2.2.1). Circular layout representations of species abundance were graphed using circos (version 0.69-3) [37]. A heatmap of species abundance was generated by the pheatmap package (version 1.0.12) in the R project [38]. Between groups, Venn analysis was performed in the R project VennDiagram package [39] (version 1.6.16). A ternary plot of taxa abundance was plotted using the R ggtern package [40] (version 3.1.0). The KEGG pathway analysis of the OTUs was inferred by utilizing PICRUSt2 (version 2.1.4) software [41].

Results

Baseline clinical information of pregnant women

A total of 111 mother-newborn dyads were initially recruited. Due to the lack of integrated bio-samples ($n = 69$), health check-up data ($n = 9$), and self-reported diseases information ($n = 7$), 26 mother-newborn dyads were ultimately included according to our inclusion and exclusion criteria, and they provided 78 samples for the subsequent analyses. Missing values of age ($n = 2$), enrolment age ($n = 1$), haemoglobin ($n = 1$), alanine aminotransferase (ALT) ($n = 1$), aspartate aminotransferase (AST) ($n = 1$), serum total bilirubin (STB) ($n = 1$), and serum conjugated bilirubin (SCB) ($n = 1$) were interpolated using the multiple imputation method. The basic information of the pregnant women is shown in Table 1. The median maternal age was 26.50 (25.00-27.25) years, and the average body mass index (BMI) was 20.82 ± 2.45 . No participants in the present research subjected active or passive smoking, and no participants used antibiotics before delivery. Other demographic and clinical indicators among groups were roughly similar except for spouse age (SA) and AST (ANOVA, $P_{SA}=0.033$; Kruskal-Wallis H test, $P_{AST}=0.022$). Despite a significant difference in AST, other maternal biological factors were well homogenized among the different groups. In combination with the clinical indices, participants in this study were overall of a relatively good health status, and the maternal clinical statuses were comparable.

Table 1
Maternal Clinical Information at Baseline

Variables	Overall	VD	CD	CS	<i>P</i> value
	N=26	n=6 (23.08%)	n=4 (15.38%)	n=16 (61.54%)	
Marriage Age (years)	26.50 (25.00-27.25)	26.00 (25.75-31.15)	25.25 ± 1.11	26.16 ± 0.70	0.707
Enrollment Age (years)	30.00 (27.00-32.00)	26.67 ± 1.33	33.25 ± 1.97	30.00 (28.55-32.00)	0.055
Spouse Age (years)	32.08 ± 5.52	26.98 ± 2.51	34.00 (34.00-40.00)	33.00 ± 1.11	0.033
Age of Menarche (years)	14.52 ± 1.68	14.00 ± 0.68	15.75 ± 0.75	14.41 ± 0.42	0.255
SBP (mmHg)	103.04 ± 9.91	105.17 ± 3.44	109.25 ± 5.31	100.69 ± 2.49	0.262
DBP (mmHg)	64.40 ± 7.62	65.33 ± 3.03	68.00 ± 4.97	63.15 ± 1.82	0.512
Height (cm)	158.82 ± 5.14	161.08 ± 2.27	160.50 ± 2.99	157.55 ± 1.18	0.288
Weight (Kg)	53.82 ± 7.91	53.35 ± 1.46	53.50 ± 4.94	54.08 ± 2.24	0.979
BMI (kg/m ²)	20.82 ± 2.45	20.03 ± 0.31	20.18 ± 1.28	21.27 ± 0.70	0.507
Hemoglobin (g/L)	122.60 ± 8.80	125.83 ± 3.04	114.00 (113.00-121.75)	122.98 ± 2.37	0.208
Leukocyte (×10 ⁹ /L)	7.08 (6.50-8.01)	7.86 ± 0.75	5.95 (5.18-7.10)	7.29 ± 0.27	0.155
Platelet (×10 ⁹ /L)	230.52 ± 41.64	232.50 ± 11.68	214.50 ± 4.13	233.78 ± 12.55	0.644
Blood Glucose (mmol/L)	4.82 ± 0.31	4.74 ± 1.60	4.79 ± 0.18	4.86 ± 0.07	0.720

Shapiro-Wilk tests were performed to test the assumptions of normality. Normally distributed variables with even variance were presented as mean ± SD, skewed variables as median (lower quartile to upper quartile), and categorical variables as n (%). Continuous variables were compared using ANOVA or Kruskal-Wallis test depending on distribution.

Abbreviations: VD, Pregnant women underwent vaginal delivery; CD, Pregnant women underwent cesarean delivery; CS, Pregnant women underwent cesarean delivery with vaginal fluid swabbing on their newborns; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; BMI, Body mass index; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; STB, Serum total bilirubin; SCB, Serum conjugated bilirubin; SCr, Serum creatinine; BUN, Blood Urea Nitrogen.

Variables	Overall	VD	CD	CS	<i>P</i> value
	N=26	n=6 (23.08%)	n=4 (15.38%)	n=16 (61.54%)	
ALT (U/L)	13.30 (10.75-18.02)	11.00 ± 1.02	39.52 ± 16.21	15.34 ± 1.33	0.120
AST (U/L)	16.90 (14.00-19.00)	14.18 ± 0.71	22.00 ± 2.86	17.34 ± 1.01	0.022
Albumin (g/L)	42.63 ± 5.78	44.03 ± 1.74	40.60 ± 1.64	41.55 (38.83-44.05)	0.350
STB (µmol/L)	10.82 ± 2.73	10.44 ± 0.97	7.10 (6.63-10.50)	11.64 ± 0.64	0.091
SCB (µmol/L)	3.59 ± 1.54	3.52 ± 0.52	2.91 ± 0.95	3.78 ± 0.40	0.613
SCr (µmol/L)	56.50 ± 11.18	58.05 ± 5.00	50.48 ± 3.80	57.43 ± 2.91	0.518
BUN (mmol/L)	3.07 ± 0.74	3.11 ± 0.24	2.87 ± 0.45	3.10 ± 0.20	0.856
Smoking (n, %)	0 (0)	0 (0)	0 (0)	0 (0)	-
Use of Antibiotic before Delivery (n, %)	0 (0)	0 (0)	0 (0)	0 (0)	-
Use of Prebiotics/ Probiotics (n, %)	0 (0)	0 (0)	0 (0)	0 (0)	-
Shapiro-Wilk tests were performed to test the assumptions of normality. Normally distributed variables with even variance were presented as mean ± SD, skewed variables as median (lower quartile to upper quartile), and categorical variables as n (%). Continuous variables were compared using ANOVA or Kruskal-Wallis test depending on distribution.					
Abbreviations: VD, Pregnant women underwent vaginal delivery; CD, Pregnant women underwent cesarean delivery; CS, Pregnant women underwent cesarean delivery with vaginal fluid swabbing on their newborns; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; BMI, Body mass index; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; STB, Serum total bilirubin; SCB, Serum conjugated bilirubin; SCr, Serum creatinine; BUN, Blood Urea Nitrogen.					

Microbiome overview of the maternal and neonatal samples

The diversity of the microbiota in a given habitat reflects the composition and relative abundance of the community. Approximately 460 bp of PCR products were generated by amplifying the V3-V4 region of the 16S rRNA gene to compare the bacterial diversity among samples. DNA sequencing after quality filtering yielded 8.29 million paired-end reads, which further merged into 7.21 million tags, with a minimum of

64253 tags per sample (average of 92448 ± 8822), and ultimately formed 27801 operational taxonomic units (OTUs).

Rarefaction curves evaluated the OTU richness and represented whether a reasonable sampling size (sequencing depth) was used. As shown in Figure S2, the almost horizontal asymptotic curves indicated that the sequencing depth was sufficient for our research. The values of observed species (Sobs), Pielou evenness, Shannon, and inverse Simpson indices were calculated to thoroughly assess alpha-diversity. The Sobs index indicated the actual detected OTUs, while the Pielou evenness index referred to the species equitability within each group. The Simpson and Shannon indices measured the degree of species asynchrony and stability, and higher values represented higher richness, evenness, or both [19]. As shown in Figure 1A, the highest Sobs value could be observed in the transitional stool samples of naturally delivered newborns (TSN), and the OTUs differed substantially between the transitional stool samples of caesarean-delivered newborns (TSC) and the TSN, which demonstrated that CD surgery reduced the actual number of species of newborn gut microbes compared with vaginal delivery. In contrast, the TSC group presented the highest evenness according to the value of the Pielou evenness index (Figure 1B) and there was a significant difference between the TSC and TSN groups (Wilcoxon test, $P_{Pielou} < 0.01$). The above analyses revealed that CD surgery led to an obvious alteration in bacterial richness and evenness. Good agreement was exhibited in the inverse Simpson index and Shannon index, which suggested that the gut microbial composition of CD newborns was less diverse (Wilcoxon test, $P_{Simpson} < 0.01$, $P_{Shannon} < 0.01$), while a lesser degree of dominant bacteria and a higher distribution homogeneity was observed in CD newborns with swabbing vaginal fluid (CS) (Figure 1C, 1D). The aggregate analyses of alpha-diversity indicated that the neonatal gut microbiota was affected by both the mode of delivery and the swabbing treatment. Beyond that, pregnant women who underwent different labour modes showed no significant differences in intestinal microbiota (Wilcoxon test, $P_{Sobs} = 0.13$, $P_{Pielou} = 0.39$, $P_{Simpson} = 0.27$, $P_{Shannon} = 0.32$), whereas a disparate vaginal fluid microbiome was exhibited according to indices of alpha-diversity (Wilcoxon test, $P_{Pielou} = 0.03$, $P_{Simpson} = 0.04$, $P_{Shannon} = 0.03$), which might result from the different physical conditions and medical administrations during pregnancy.

We further investigated beta-diversity according to the Bray-Curtis distance to compare the microbial community structures among samples, as this method provided a model to describe the overall pattern of community composition based on OTUs. Similar trends as alpha-diversity were obtained (Figure 2). Briefly, the significant differences between the CD and CS newborns (Wilcoxon test, $P = 0.045$), as well as the CD and vaginally delivered newborns (Wilcoxon test, $P < 0.01$), showed that the offspring microbial communities were shaped by maternal vaginal fluids. The gut microbiota of CS and vaginally delivered newborns seemed to show no substantial difference in terms of beta-diversity. From the above analyses, it was concluded that a similar progression towards a natural configuration was observed with swabbing exposure (Wilcoxon test, $P = 0.5$), and a vaginal microbiome-like signature that was absent in CD newborns was exhibited in CS newborns. In parallel, the microbiota of the maternal vaginal fluid (Wilcoxon test, $P = 0.35$) and the stool samples (Wilcoxon test, $P = 0.68$) were not significantly different among the groups.

The visualization of non-metric multidimensional scaling (NMDS) analysis based on the Bray-Curtis distance showed clear ordinations that were able to distinguish the gut microbes of the CD and CS newborns from those of the vaginally delivered newborns (Adonis test, $R^2=0.24$, PERMANOVA $P<0.01$) (Figure 3). Furthermore, the OTU clustering distribution of CS newborns was mainly limited within the elliptical field (95% CI) of vaginally delivered newborns, suggesting that the microbial composition of transitional stool samples of caesarean-delivered newborns swabbed with maternal vaginal fluid (TSS) tended to be similar to that of TSN samples. In addition, the NMDS plot also reflected that the maternal vaginal microbiota (Adonis test, $R^2=0.13$, PERMANOVA $P=0.08$), rather than the maternal intestinal microbiota (Adonis test, $R^2=0.23$, PERMANOVA $P<0.01$), was the major source of neonatal gut colonization among vaginally delivered babies. However, the gut microbiota of CD newborns was inherited neither from the vaginal microbes nor from the intestinal microbes of their mothers. Similar conclusions could also be derived from the distance heatmap in Figure S3. Combined with the above results, swabbing treatment increased the richness of caesarean-delivered newborns' intestinal microbiota and made it more similar to that of the vaginally delivered newborns.

The variation in microbiota caused by delivery modes and treatment

The gut microbiome of vaginally delivered newborns was enriched for Firmicutes (40.58%), Proteobacteria (26.45%), and Bacteroidetes (19.67%) at the phylum level. However, the highly abundant phyla in CD newborns were Proteobacteria (58.77%), Bacteroidetes (12.68%), Firmicutes (11.51%), and Cyanobacteria (10.95%), while the intestinal microbiota of CS newborns mainly consisted of Firmicutes (30.38%), Bacteroidetes (30.13%), and Proteobacteria (24.52%), as shown in Figure 4A. The bacterial composition and richness of CS newborns tended to be similar to those of vaginally delivered newborns. At the genus level, the microbial community of CS newborns changed clearly and consisted primarily of *Bacteroides* (17.51%) and *Escherichia-Shigella* (10.10%) after treatment (Figure 4B). The circos plots confirmed the above results from another aspect and are displayed in Figure S4. In addition, we used ternary enrichment and indicating taxa to evaluate the specific effects of swabbing exposure. At the phylum level, Firmicutes (Tukey HSD test, $P<0.01$; Welch's t test, $P_{TSS-TSC}=0.02$, $P_{TSN-TSC}<0.01$, $P_{TSN-TSS}=0.36$) and Proteobacteria (Tukey HSD test, $P<0.01$; Welch's t test, $P_{TSS-TSC}=0.01$, $P_{TSN-TSC}=0.03$, $P_{TSN-TSS}=0.98$) were recovered by swabbing (Figure 5A, Figure 5C), while *Lactobacillus* (Tukey HSD test, $P<0.01$; Welch's t test, $P_{TSS-TSC}=0.20$, $P_{TSN-TSC}=0.80$, $P_{TSN-TSS}=0.79$), *Bacteroides* (Tukey HSD test, $P<0.01$; Welch's t test, $P_{TSS-TSC}<0.01$, $P_{TSN-TSC}=0.99$, $P_{TSN-TSS}<0.01$), and *Escherichia-Shigella* (Tukey HSD test, $P<0.01$; Welch's t test, $P_{TSS-TSC}=0.11$, $P_{TSN-TSC}=0.07$, $P_{TSN-TSS}=0.63$) showed similar patterns of restoration (Figure 5B, Figure 5D). The richness of the genera *Akkermansia* (Welch's t test, $P_{TSS-TSC}=0.01$) and *Alistipes* (Welch's t test, $P_{TSS-TSC}<0.01$) showed elevation in the CS newborns compared to CD newborns but there was no significant difference when compared with the vaginally delivered newborns (Figure 5B). It is worth noting that only vaginal fluid swabbing induced more shared taxa between CS and CD newborns, as displayed in Figure S5, and it could be speculated that the swabbing treatment introduced a considerable amount of microbial colonization via 'mother-to-offspring transmission'. Interestingly, such bacterial transmission was not obviously related to maternal faeces or anal transmission.

The Metabolic Active Effects of the Vaginal Transferred Microbiota

Metagenomic functioning predictions using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) software provided further insight into the pathway abundances for investigating the functional implication of the treatment-induced changes. Compared with vaginally delivered newborns, the percentages of genes related to 'metabolism of terpenoids and polyketides' (Welch's t test, $P = 0.02$) and 'glycan biosynthesis and metabolism' (Welch's t test, $P = 0.048$) decreased in the gut environment in CD newborns, as displayed in Figure 6A. The TSS group had a higher number of bacterial genes related to 'glycan biosynthesis and metabolism' and 'biosynthesis of other secondary metabolites', whereas the future risks of 'neurodegenerative diseases' and 'digestive system diseases' were statistically larger in vaginally delivered newborns, as shown in Figure 6B. As a result of exposure to vaginal fluids, the number of bacterial genes related to metabolism, *e.g.*, carbohydrates (Welch's t test, $P = 0.022$), amino acids (Welch's t test, $P = 0.039$), terpenoids and polyketides (Welch's t test, $P = 0.001$), lipids (Welch's t test, $P < 0.001$), glycans (Welch's t test, $P < 0.001$), and nucleotide metabolism (Welch's t test, $P = 0.032$), increased significantly. Beyond this, the predicted functions of 'DNA replication and repair' (Welch's t test, $P = 0.018$) and 'biosynthesis of other secondary metabolites' (Welch's t test, $P = 0.041$) were also enhanced by the treatment (Figure 6C). In summary, swabbing treatment can compensate for bacterial functional alterations in CD newborns, which could have potential benefits in decreasing the risk of digestive, cardiovascular, and immune diseases.

Discussion

To date, very few studies have focused on the microbiota in transitional stool. A pilot study conducted by Mueller et al. showed that the top five phyla in vaginally delivered babies were Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Cyanobacteria, and significantly higher relative abundances of the genera *Bacteroidetes*, *Parabacteroides*, and *Clostridium* were observed in vaginally delivered babies than in CD newborns [18], which was fundamentally aligned with our findings at the phylum level. However, the results were marginally incongruent at the genus level. These conflicting results might reflect the dissimilarity in the collection, storage processing or analytic platforms used in the different studies [20]. Interestingly, the microbiome appeared to show significant discrepancies in the vaginal fluid samples of pregnant women who were subjected to different labour modes. This conclusion was consistent with Marta Selma-Royo et al. [21] and Romero R et al. [22], which described the variations occurred during gestation, mainly in the intestinal and vaginal microbiomes. These studies also indicated that delivery mode significantly affected the maternal microbiota composition at delivery. One of the probable reasons was that medical decisions for different delivery modes depended on maternal health states and might be directly reflected in the vaginal microbiota. Moreover, previous investigations have proposed that the use of antibiotics during the gestation or the periparturient period would also cause those differences [23]. Early studies observed that the abundance of the phylum Bacteroidetes, including *Bacteroides* species, participated in modulating the weight development of newborns [24,25]. Additionally, it has been well documented that an increase in the abundance of the phylum *Proteobacteria* was associated with onset risks of diabetes and obesity [26]. In our present work, the richness of the phyla

Bacteroidetes and *Proteobacteria* increased, which might exert helpful impacts on the proper development of babies. At the genus level, the relatively abundant distribution of *Akkermansia* was considered to be beneficial due to the production of a membrane protein that improves metabolism in obese and diabetic mice [27]. A later randomized controlled trial of supplementation with *Akkermansia muciniphila* in overweight and obese human volunteers further verified the salutary effects on metabolic functions [28]. Similar effects could also be observed for *Prevotella* [29]. However, the induction of pernicious bacteria in our study, such as *Escherichia-Shigella* and *Alistipes*, might increase potential health risks. Taken together, these findings indicate that the finer targeting of the vaginal fluids before exposure would determine the extent to which this intervention method could be popularized.

The microbiome of transitional faeces reflects the colonization of microorganisms in utero as well as under the influence of external environmental factors shortly after birth [18]. In this research, the gut bacterial composition of vaginally born babies was similar to that of the maternal vaginal fluid, but similar results could not be observed in CD newborns without treatment, while vertical transmission might be achieved by swabbing treatment among CS newborns. This phenomenon has aroused our concern. It is widely accepted that the effects of the natural delivery process are comprehensive, including physical, chemical, and biological effects, which generally last for one to two hours, covering the whole period of the second stage of labour [11]. During childbirth, newborns may inhale (swallow) the vaginal contents in their mother's birth canal [30]. The stomachs of newborns are pH neutral for several hours post birth as a result of swallowing the amniotic fluid in utero, thereby enabling the survival of ingested bacteria [31]. However, the exposure application in our work deliberately avoided contact with the CS newborns' oral cavity, which might eliminate microbial colonization via the oral transmission route. Hence, overall 'mother-to-offspring' microbial transmission may be inefficient due to incomplete biological effects and the absence of chemical and physical effects. On the other hand, antibiotic therapy is a common preventive measure to avoid intra- or post-surgical infections. Nevertheless, residual concentrations of antibiotics in maternal blood might act on the newborn through circulation and last for a period of time post birth, which also means that the microorganisms in the vaginal secretions may not fully colonize the newborn because of the influence of the residual antibiotics.

The above conclusions hinted at the feasibility of swabbing treatment, which may partly reverse the adverse health effects caused by CD, although the specific implementation details still need to be refined, and a larger longitudinal study with a longer follow-up is warranted in the next stage of evaluation.

Conclusion

Through the intervention project conducted in the Chinese population, we first verified the microbial alterations induced by different delivery modes, including noticeable changes in alpha- and beta-diversity, the structure of gastrointestinal bacterial communities, and metabolic functions. Second, the issue of the source of gut microbiota for newborns born via different delivery modes was partly resolved according to NMDS analysis. Maternal vaginal microbes were the main source of neonatal gut colonization among vaginally delivered babies. The intestinal microbiome of CD newborns was derived neither from the

maternal vaginal microbiota nor from the maternal gut microbiota, while ‘mother-to-offspring transmission’ might partially exist in CS newborns. Then, through ternary plots and Venn diagrams, we concluded that maternal vaginal fluids were the main source of neonatal gut microbiota, while swabbing exposure partly provided ‘mother-to-offspring’ transmission to CS newborns. Finally, we analysed the taxa distribution and constructed circos diagrams and distance heatmaps to determine to what extent the exposure restored the aberrant colonization of intestinal microbes. The fractions of Firmicutes and Proteobacteria were recovered by the treatment, while some genera (*e.g.*, *Lactobacillus*) showed a similar trend of restoration.

Abbreviations

ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; ASVs, amplicon sequence variants; BMI, body mass index; Sobs, observed species; CD, caesarean delivery; CS, CD newborns with swabbing vaginal fluid; NMDS, non-metric multidimensional scaling; OUT, operational taxonomic units; PICRUST2, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; SA, spouse age; SCB, serum conjugated bilirubin; STB, serum total bilirubin; TSC, the transitional stool samples of caesarean-delivered newborns; TSN, the transitional stool samples of naturally delivered newborns; WHO, World Health Organization.

Declarations

Ethics approval and consent to participate

The protocol of this study had been approved by the Medical Ethics Committee of the Women and Children’s Hospital, School of Medicine, Xiamen University (KY-2018-020). All patients provided written informed consent before any study procedure. All procedures were conducted in accordance with the Declaration of Helsinki. No adverse events were reported for any of the newborns in this study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are not publicly available due to subjects’ concerns about their personal privacy but are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CT and X-Q Z contributed to design the study, research data and wrote the manuscript. J-X X contributed to data interpretation and the discussion of the results. L-Y Z and Y-T X contributed to methodology. JZ and YL contributed to design the study and critically reviewed the manuscript. Y-S X and Q-F W contributed to subject recruitment, sample collection, and perform clinical examination. H-Q S conceptualized and designed the protocol and critically reviewed the manuscript.

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Author details

¹State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, School of Public Health, Xiamen University, Xiamen 361102, Fujian, China. ²Department of Obstetrics, Women and Children's Hospital, School of Medicine, Xiamen University, Xiamen 361000, Fujian, China. ³Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, Fujian, China. ⁴Key Laboratory of Marine Genetic Resources, Third Institute of Oceanography, Ministry of Natural Resources of PR China, Xiamen 361005, Fujian, China.

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Figures

Figure 1

Microbial Alpha Diversity Indices. The ecological diversity of microbiota in transitional stools of newborns (36-72h after birth), virginal fluids (before delivery) and stool of mothers (the last excretion before delivery) was measured by Sobs (A), Pielou evenness index (B), the inverse Simpson index (C), and the Shannon index (D). The P values were conducted by Kruskal-Wallis H test. Statistical significance is displayed as * P<0.05 and ** P<0.01. Abbreviations: Sobs, Observed species; TSC, Transitional stools of caesarean delivered neonates; TSS, Transitional stools of caesarean delivered neonates with the treatment of swabbing maternal vaginal fluid; TSN, Transitional stools of natural delivered neonates; VN, Vaginal fluids of the pregnant women who underwent natural delivery; V, Vaginal fluids of the pregnant women who underwent caesarean delivery; FN, Feces of the women who underwent natural delivery; F, Feces of the pregnant women who underwent caesarean delivery.

Figure 2

The Differences of Beta-diversity Index According to Bray-Curtis distance. The P values were conducted by Kruskal-Wallis H test. Statistical significance is displayed as * $P < 0.05$ and ** $P < 0.01$. Abbreviations: TSC, Transitional stools of caesarean delivered neonates; TSS, Transitional stools of caesarean delivered neonates with the treatment of swabbing maternal vaginal fluid; TSN, Transitional stools of natural delivered neonates; VN, Vaginal fluids of the pregnant women who underwent natural delivery; V, Vaginal fluids of the pregnant women who underwent caesarean delivery; FN, Feces of the women who underwent natural delivery; F, Feces of the pregnant women who underwent caesarean delivery.

Figure 3

NMDS Plot Based on Bray-Curtis Distance. Each dot represents one sample from each group. Statistical significance is displayed as * $P < 0.05$ and ** $P < 0.01$. Abbreviations: TSC, Transitional stools of caesarean delivered neonates; TSS, Transitional stools of caesarean delivered neonates with the treatment of swabbing maternal vaginal fluid; TSN, Transitional stools of natural delivered neonates; VN, Vaginal fluids of the pregnant women who underwent natural delivery; V, Vaginal fluids of the pregnant women who underwent caesarean delivery; FN, Feces of the women who underwent natural delivery; F, Feces of the pregnant women who underwent caesarean delivery.

Figure 4

Taxa Distribution Plots of Samples at Phylum and Genus Level. The relative abundances of microbial communities at Phylum (A) and Genus level (B). Abbreviations: TSC, Transitional stools of caesarean delivered neonates; TSS, Transitional stools of caesarean delivered neonates with the treatment of swabbing maternal vaginal fluid; TSN, Transitional stools of natural delivered neonates; VN, Vaginal fluids of the pregnant women who underwent natural delivery; V, Vaginal fluids of the pregnant women who underwent caesarean delivery; FN, Feces of the women who underwent natural delivery; F, Feces of the pregnant women who underwent caesarean delivery.

Figure 5

The Effect Analyses of Swabbing Exposure. The characteristic taxa were measured by indicator analysis (A, B) and ternary plots (C, D). The P values were conducted by Kruskal-Wallis H test. Statistical significance is displayed as * $P < 0.05$ and ** $P < 0.01$. Abbreviations: TSC, Transitional stools of caesarean delivered neonates; TSS, Transitional stools of caesarean delivered neonates with the treatment of swabbing maternal vaginal fluid; TSN, Transitional stools of natural delivered neonates; VN, Vaginal fluids of the pregnant women who underwent natural delivery; V, Vaginal fluids of the pregnant women who underwent caesarean delivery; FN, Feces of the women who underwent natural delivery; F, Feces of the pregnant women who underwent caesarean delivery.

Figure 6

Functional Prediction Profiles Based on PICRUST2. The diagrams of PICRUST2 functional predictions were performed between the TSC and the TSN group (A), the TSS and the TSN (B), as well as the TSS and the TSC group (C). The P values and 95% CI were conducted by Welch's t test. Abbreviations: TSC, Transitional stools of caesarean delivered neonates; TSS, Transitional stools of caesarean delivered neonates with the treatment of swabbing maternal vaginal fluid; TSN, Transitional stools of natural delivered neonates; VN, Vaginal fluids of the pregnant women who underwent natural delivery; V, Vaginal

fluids of the pregnant women who underwent caesarean delivery; FN, Feces of the women who underwent natural delivery; F, Feces of the pregnant women who underwent caesarean delivery.

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