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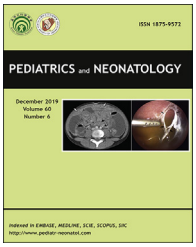

Original Article

Impact of maternal intrapartum antibiotics on the initial oral microbiome of neonates

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Neonatal oral microbiome;
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16S rRNA sequencing

Objectives: Prior studies have proposed that maternal intrapartum antibiotic exposure shapes the gut microbiota and, subsequently the child's health. However, the effect of maternal intrapartum antibiotic exposure and its influence on the development of the neonatal oral microbiota in early infancy has not yet been reported. The aim of this study was to compare the initial oral microbiota immediately after birth of healthy infants with and without intrapartum antibiotic exposure.
Methods: Twenty-two newborns of the BaoAn Maternal and Child Care Hospital (Shenzhen, China) were recruited for this study, 11 born to mothers without intrapartum antibiotic exposure (NT group) and 11 to mothers with intrapartum antibiotic prophylaxis with cefamezin (AT group). Oral microbiome profiles were determined by 16S rRNA sequencing based on the V3–V4 hyper-variable regions.
Results: Phylum *Firmicutes* was most frequently detected in subjects both groups and a higher frequency was observed in the NT group than the AT group. Phyla *Actinobacteria*, *Bacteroidetes* and *Proteobacteria* were more abundant after intrapartum antibiotics exposure. Genus *Lactobacillus* belonging to *Firmicutes* was predominant in the neonates not exposed to antibiotics, while significantly higher percentages of genera *Klebsiella*, *Roseburia*, *Propionibacterium*, *Faecalibacterium*, *Escherichia/Shigella*, *Corynebacterium*, *Bifidobacterium*, and *Bacteroides* were noted in AT infants than NT infants. Further function analysis demonstrated that lipopolysaccharide biosynthesis and amino acid–related metabolic function was enriched in the AT group, and carbohydrate metabolism pathways were more abundant in the NT group.
Conclusions: These findings revealed distinctions in both taxa and metabolic function of oral microbiota between antibiotics-treated and unexposed groups, which indicated that maternal intrapartum antibiotic treatment is a key regulator of the initial neonatal oral microbiome.

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1. Introductions

Interaction of variable oral microorganisms helps the human body against casual invasion. Imbalance of oral microbial flora contributes to oral disease such as dental caries, periodontitis,[1] oral mucosal diseases,[2] and systemic diseases, such as gastrointestinal and nervous systemic diseases.[3–6] Oral microbiota plays an important role in the human microbial community and human health,[7] and identification of pioneer colonizers is essential to elucidate the early stages of microbiota development. The first microbial colonizers of the oral cavity in the first day of life stimulate changes in the oral cavity that favor the growth of subsequent species.[8] These pioneer bacteria usually bind to mucosal epithelium (*e.g, Streptococcus*) where they produce some metabolites to causes a selective pressure for certain bacteria in the oral cavity.[9]

The acquisition of the first oral microbial colonizers was affected by multiple maternal and infant factors,[10–12] such as mode of delivery,[13] amniotic fluid quality and the type of rupture of the membranes, which may result in differences in oral microbiota development. Among these factors, the mother is the primary source of oral bacteria for newborns[14] because there occurs a host–microbial interaction *in utero,* and the fetus commonly encounters bacteria that inhabit the maternal vaginal tract after rupture of fetal membranes. However, maternal intrapartum antibiotic was often administrated for the prophylaxis of vaginal *Group B Streptococcus* (GBS) clinically. Maternal antibiotics exposure has been recognized to affect infant's intestinal microbial habitat,[15] and to disrupt the microbiota transmission from mother to infant, which has been associated with many diseases later in life.[16,17]

Additionally, maternal antibiotics exposure might segregate the neonatal oral microbiota profile into two distinct types.[14] However, to what extent the influence presented and the initial community structure existed in the oral microbiome of newborns remains unclear. The oral cavity is a diverse ecosystem colonized by numerous microorganisms immediately following delivery.[14] Thus the objective of this study was to estimate, to what extent the administration of intrapartum antibiotic contributed to the oral microflora of newborns immediately after birth.

2. Methods

2.1. Subjects and samples

The prospective randomized pilot study enrolled 22 full-term newborns, delivered vaginally. The study was conducted at the Shenzhen Baoan Maternal and Child Health Hospital in October 2016. Mothers were aged between 20 and 37 years (mean 27.77 years). To be eligible for enrollment, infants must have to be born with gestational age >37 weeks, birth weight >2500 g, and without any significant congenital anomaly, chromosomal abnormality or metabolic diseases. Premature neonates were excluded, and only normal births were included. Perinatal and infant demographics, feeding type, body weight and antibiotics during delivery were checked against medical records prospectively. Infants in the AT group were supplied with cefazolin. The Ethical Committee on Clinical Research of the Hospital approved all study protocols and signed informed consents were obtained from parents or legal guardians of all enrolled subjects. Oral samples were taken as soon as the newborns were delivered and before feeding using sterile swabs by carefully swabbing the oral mucosa. Swabs were stored in 1000 μL of cell lysis solution immediately after collection and then immediately placed in a −80 °C freezer until DNA extraction.

2.2. Sequencing and sequence processing

DNA was extracted using commercially available kit (Qiagen) according to the manufacturer's instructions. Extracted DNA samples were then purified by electrophoresis on a 0.7% agarose-gel, followed by phenol-chloroform extraction[18] and eluted in a final volume of 100 μL. DNA concentration and quality were determined by Qubit (Invitrogen) and verified with agarose gel electrophoresis. 5′CCTACGGGNGGCWGCAG3′ forward primer and 5′GACTACHVGGGTATCTAATCC3′ reverse primers were used to amplify the 16s rRNA V3–V4 variable region. Equimolar amounts of cleansed PCR products were pooled and processed for sequencing. DNA sequencing was performed on an Illumina MiSeq instrument with barcoding using sequence kit version 3.0 to achieve the desired pair-end sequence reads. Custom perl and python scripts as well as Mothur pipeline were used to process the sequencing files.

The quality of the raw sequence data was initially evaluated with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and then demultiplexing was performed to remove the PhiX sequences. Individual sample sequence was assigned based on dual-index barcodes, allowing for 1 mismatch by using custom Perl scripts. The selected high quality sequences were further processed using Mothur.[19] Paired end reads of sufficient length were first merged into full-length sequence (tag), removing either tags with high amount of ambiguous bases and homo-polymers, or tags out of the expected range. Then sequences were aligned to SILVA 119[20] 16S rRNA gene sequences, selected according to the correct aligned region and consistent with the same alignment coordinates. For each sample, the frequency of the unique sequence was identified and a pre-clustering algorithm was utilized for further denoising. The resulting sequences were then screened and the chimeric sequences

were discarded based on prediction by UCHIME using the reference database mode.[21] Substantial taxonomic ranks were assigned to each sequence using Ribosomal Database Project (RDP) Naive Bayesian Classifier[22] trained on the RDP 16S rRNA gene training set (version 10). Then an 80% pseudo-bootstrap confidence score was required as a cut-off[22] to achieve a balance between accuracy and the number of reads retained. Those sequences that either were not classified to the level of kingdom or that were classified as *Archaea*, *Eukaryota*, chloroplasts, or mitochondria were culled. Finally, sequences were split into groups corresponding to their taxonomy at the level of order and then assigned to operational taxonomic units (OTUs) at 97% similarity level.

2.3. Statistical analysis

Characteristics were analyzed using R. Continuous variables were reported as means ± standard deviations, and categorical data were presented as ratios or percentages. Unpaired *t*-tests were used to study differences in continuous variables and $\chi 2$ tests were used to analyze categorical variables. Difference with *P* value of less than 0.05 was considered statistically significant.

The LDA Effect Size (LEfSe: Linear Discriminant Analysis Effect Size) algorithm[23] was used to identify taxa with differentiating relative abundance. The threshold for the logarithmic LDA score was set at 3.0 for biomarker discovery.

The STAMP program[24] was used in this study to generate an extended error bar plot to show the properties which differed significantly between AT and NT group with filter parameters (q value < 0.05).

3. Results

3.1. Characteristics of the infants

Clinical characteristics from the 22 infants included in this study are shown in Table 1. All infants were of Han ethnicity and born at term. Intrapartum antibiotic (cefamezin) was administered to the antibiotic group (AT group). Infants had a mean gestational age of 39.6 ± 1.1 weeks, birth weight of 3185.5 ± 373.1 g, with 12 (54.17%) boys. Counterparts in the AT and the control groups (NT group) were compared, 3 outcomes (the gestational age, birth weight, male:female ratio) all showed no significant difference. Also, there were no statistically significant differences between any of the clinical characteristics of mothers in the two groups, except for pregnancy complications. There were 4 mothers with *Group B Streptococcus* infection, 3 with gestational diabetes, 2 with premature rupture of fetal membranes in the AT group, while complications were negligible in the NT group, as shown in Table 1.

Oral samples were randomly collected from 11 infants who were administrated intrapartum antibiotics and 11 infants without antibiotics. Samples were amplified by the universal bacterial 16s rRNA primers, and high-throughput sequencing of the positive PCR products by the Illumina MiSeq platform generated a total of 1,291,757 valid sequences (passing quality control) with an average of 53,823 sequences per sample.

Table 1 Characteristics compared in the antibiotic and control group.

	Antibiotic group (N = 11)	Control group (N = 11)	P-value
Newborn's characteristics			
Gestational age (week)	39.32 ± 1.34	39.91 ± 0.91	0.25
Birth weight (g)	3161 ± 468.7	3210 ± 277.6	0.77
Male/female	7/4	5/6	0.67
Delivered vaginally	11	11	1
Mothers' conditions			
Age (year)	27 ± 3.2	28.5 ± 4.1	0.34
Pregnancy weight gain (g)	12.4 ± 4.7	14.7 ± 3.9	0.23
Perineal tear condition			0.33
Without	1	1	
First-degree	8	10	
Episiotomy	2	0	
Pregnancy complication			0.02
Gestational diabetes	3	1	
Vaginal *Group B Streptococcus*	4	0	
Vaginal Hepatitis B virus	1	2	
Anemia	1	2	
Premature rupture of membranes	2	0	
Contamination of amniotic fluid			0.06
Without (colorless)	6	6	
Level I	4	0	
Level II	1	2	
Level III	0	3	

*$\chi 2$ test was used for categorical variables and *t*-test was used for continuous variables. $P < 0.05$ was considered as significant.

3.2. Antibiotics administrated and non-antibiotics infants showed significant difference in oral microbial diversity

The Shannon and Simpson indices were applied to evaluate microbial diversity. Based on the OTUs distribution, the average value of Shannon was 4.0 ± 1.22 (mean ± SD) and 2.35 ± 1.0 in antibiotics—treated and non-antibiotics—-treated infants, respectively ($p < 0.05$). The Simpson index also reflected significant discrepancy, averaging 0.16 ± 0.23 for antibiotics infants and 0.46 ± 0.25 for non-antibiotics infants ($p < 0.05$) (Fig. 1A). Both the Shannon and the Simpson index showed significant statistical difference between the AT and NT groups.

Principal coordinates analysis (PCoA), based on the relative taxa abundance in the oral microbiome of neonates, showed a significant difference in bacterial composition between the two groups (Fig. 1B). Most subjects were well separated from each other, while AT4 and AT5 clustered similarly to the samples from NT group, and NT3 clustered with AT6. The subjects of the antibiotic group were quite distant from each other based on thetayc dissimilarity.

3.3. Composition analysis of microbiota

The overall microbiota composition of each sample at the phylum and genus levels is shown in Fig. 2. Fig. 2A demonstrates that *Firmicutes* and *Bacteroidetes* were the most abundant in all enrolled subjects, and these accounted for the majority of microflora (>50%). Fig. 2A also shows that phylum *Tenericutes* was rarely detected. There was an approximately 35.8% increase in the proportion of *Firmicutes* (75.4% versus 39.6%), whereas *Proteobacteria* (3.3%

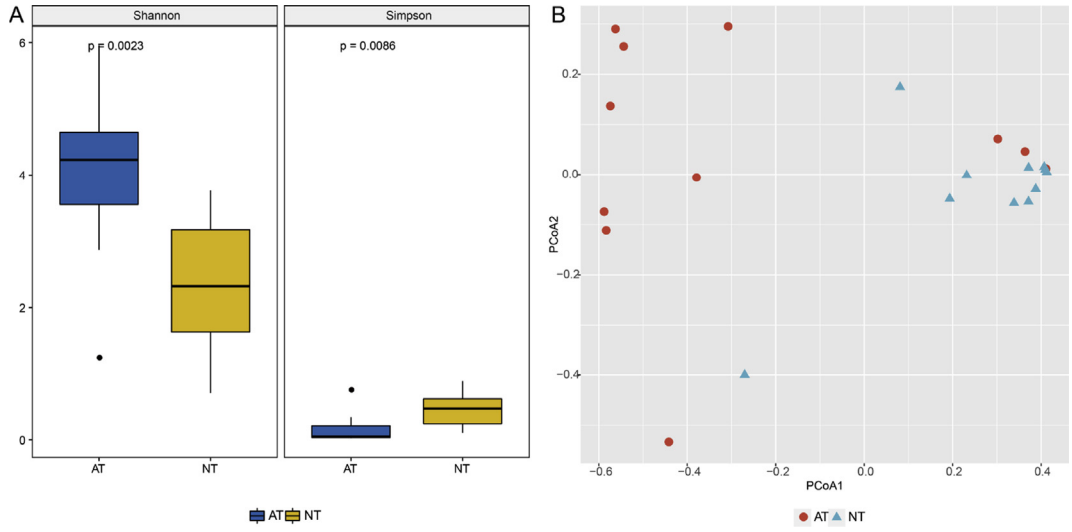

Figure 1 Comparison of the microbiome biodiversity of antibiotics-treated and antibiotics-free newborns. (A) The Shannon index, Simpson index were shown as estimators. (B) PCoA plot based on the abundance.

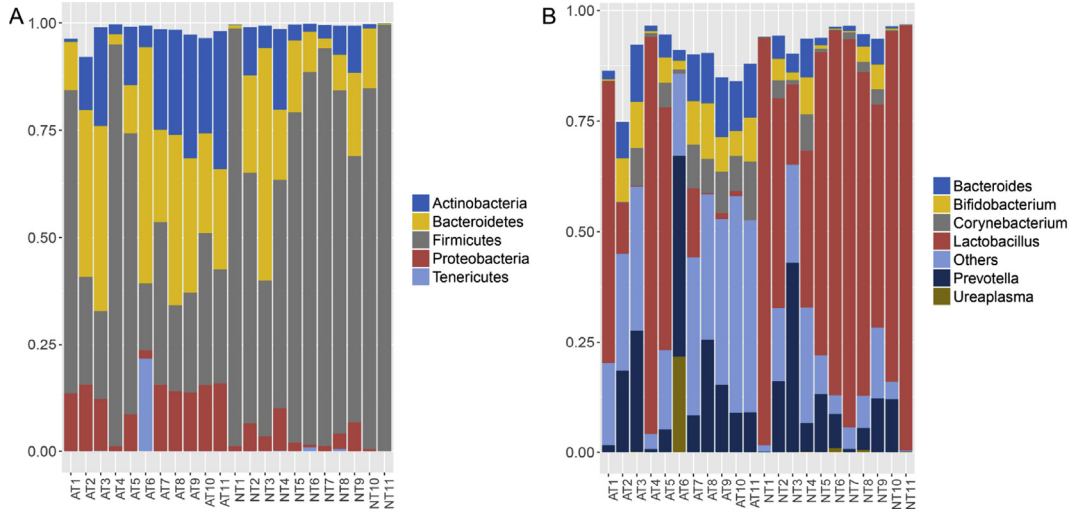

Figure 2 Composition of oral microbiome between AT and NT group. (A) Relative abundance at the phylum level. (B) Relative abundance at the genus level. AT, antibiotics-treated group; NT, antibiotics-free group.

versus 11.6%), *Actinobacteria* (5.7% versus 17.1%) and *Bacteroidetes* (14.9% versus 27.4%) decreased by the same percentage in NT group as compared to AT group.

At the genus level (Fig. 2B), *Bacteroides*, *Bifidobacterium*, *Corynebacterium*, *Lactobacillus*, *Prevotella* and *Ureaplasma* were the main genera of all the neonates. The proportion of *Lactobacillus* accounted averagely for more than 66.5% microbiota in the NT group, which was higher than in the AT group (21.7%). The proportion of *Bacteroides* (8.2% versus 2.8%), *Bifidobacterium* (6.8% versus 2.3%) and *Corynebacterium* (5.8% versus 2.0%) increased in the AT group compared to the NT group.

3.4. Enriched bacterial residents in antibiotics and non-antibiotics infants

A LEfSe analysis and cladogram were performed to investigate differences in the community composition between the two groups (Fig. 3). The phyla *Actinobacteria*, *Bacteroidetes* and *Proteobacteria* were significantly enriched in the AT group, whereas *Firmicutes* was significantly decreased compared to the NT group. There were 5 significantly different classes, with enrichment of *Actinobacteria*, *Bacteroidia*, *Clostridia*, and *Gammaproteobacteria* in the AT group and *Bacilli* in the NT group (Fig. 3A). The microbial composition also included 9 significantly different genera. *Bacteroides*, *Bifidobacterium*, *Corynebacterium*, *Escherichia/Shigella*, *Faecalibacterium*, *Klebsiella*, *Propionibacterium* and *Roseburia* exhibited relatively higher abundance in the AT group. *Lactobacillus* was relatively more abundant in the NT group (Fig. 3A).

3.5. Functions of metabolism in the oral microbiome in AT and NT neonates

To detect difference in microbiome metabolites induced by changes in the microbiome abundance, the sequencing data were annotated with metabolic pathways from the KEGG database using the PICRUST program.[25] According to the STAMP analysis, extended error bar of the metabolic function pathways demonstrated that most differences occurred in amino acid and carbohydrate metabolism pathways (Fig. 4). The oral microbiome of the infants who were administrated antibiotics included more relative amino acid biosynthesis and amino acid degradation than did antibiotics-free infants, while carbohydrate metabolism was more abundant in antibiotics-free infants. Moreover, lipopolysaccharide biosynthesis and biosynthesis of ansamycins were more active in the AT group, and phosphotransferase system was more active in the NT group.

4. Discussion

Oral microorganisms are important determinants of health and disease. This study aimed to verify whether maternal exposure to intrapartum antibiotics could influence the main microbial groups of the newborn oral microbiota at an early stage of microbial establishment. High-throughput sequencing provides sensitive detection and discrimination for a wide spectrum of phylogenies and quantifies the relative abundance of total bacterial components and the structural variations of communities.[26] In our study, 1,291,757 high-quality sequences were produced and 53,823 reads per sample were analyzed in total, suggesting that sequencing was useful in estimation of oral bacterial microbiota at a more in-depth level. As the initial oral colonizers of neonates were related to status of mothers, mode of delivery, feeding type and living environment, neonatal characteristics and most maternal characteristics presented without significant differences between AT and NT groups in this study. However, pregnancy complications occurred more frequently in the AT group, such as GBS infection, gestational diabetes, anemia, and premature rupture of fetal membrane. These were strongly associated with the antibiotic use.

16S rRNA sequencing was used to detect differences in the oral microbiome immediately after birth between intrapartum antibiotic-exposed and antibiotic-free neonates in this study. The early oral microbiota occurring within a few minutes after delivery was largely composed of *Firmicutes*, *Bacteroidetes*, *Actinobacteria*

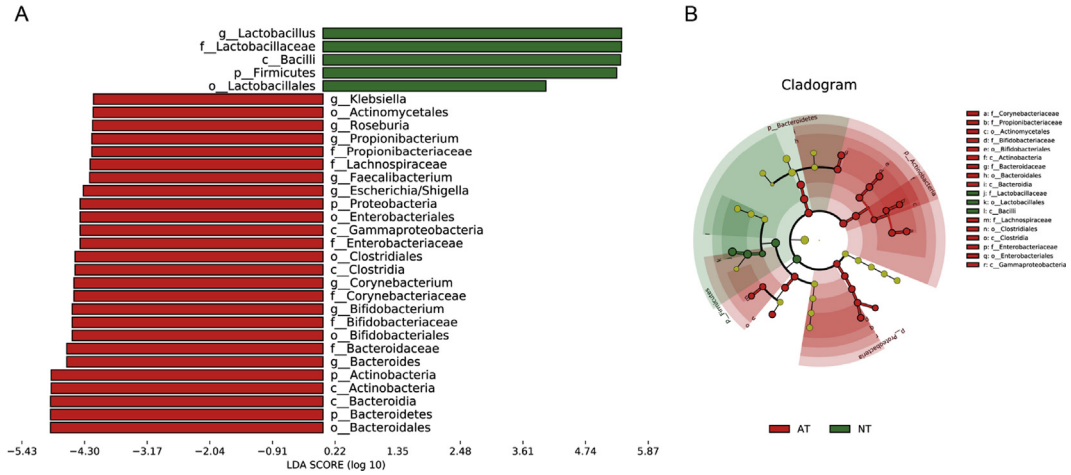

Figure 3 LEfSe analysis of oral microbiota in antibiotics-treated versus antibiotics-free neonates. (A) Cladogram indicating differences at different levels. (B) Each successive circle represents a phylogenetic level.

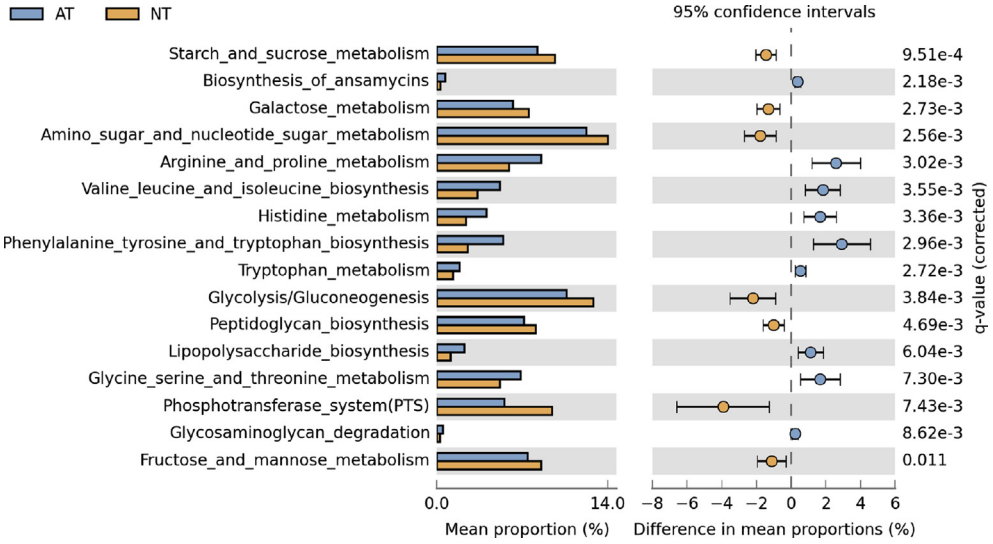

Figure 4 Functional properties that differ significantly between antibiotics-treated and antibiotics-free neonate oral microbiome.

and *Proteobacteria* at the phylum level in this study. Our results demonstrated that the intrapartum antibiotic—exposed neonates were associated with a significant shift in the oral microbiota, a significantly reduced percentage of *Firmicutes* and an increased proportion of the phyla *Actinobacteria*, *Bacteroidetes* and *Proteobacteria*. That *Proteobacteria* increased significantly in the AT group might indicate the dysbiosis of oral microbiota.[27] Furthermore, the dominant genus in the control neonatal oral was *Lactobacillus*, whereas this genus was significantly lower in the AT group, which is consistent with a previous study which showed that maternal intrapartum antibiotics decreased vertical transmission of *Lactobacillus* to neonates during birth.[28] Cefamezin is effective against gram-positive bacteria while *Lactobacillus* is a gram-positive genus.

The pioneer bacteria that colonize the neonatal oral contribute to the establishment of host—microbe interactions essential for optimal symbiosis, which is essential for health throughout life. Genus *Lactobacillus* dominated in the NT group, which was shown clinical to ameliorate microbial dysbiosis and produce significant improvement in clinical indicators of disease when allied to periodontal treatment.[29] Moreover, some prior studies provided evidence of certain species of *Lactobacillus* as probiotic for oral health.[30] There was also evidence of some species of *Lactobacillus* being beneficial defense against periodontal disease such as gingivitis and periodontitis.[31] This is likely due to its immune modulation; recent in vitro studies showed that probiotic *Lactobacillus* could abolish CXCL8 attenuation to promote Th1 and Th17 responses.[32,33] However, that abundance decreased significantly in the AT group demonstrated that maternal intrapartum antibiotics exposure was associated with decreased transmission of *Lactobacillus* to the neonate. There was also a slightly lower microbial diversity in antibiotics-free infants, which might be attributed to the relative abundance discrepancy of microbiome between the AT and NT groups, for there were much higher presence of *Lactobacillus* among neonates without maternal intrapartum antibiotics administration.

Bacteroides, *Bifidobacterium*, *Corynebacterium*, *Escherichia/Shigella*, *Faecalibacterium*, *Klebsiella*, *Propionibacterium* and *Roseburia* significantly increased in the AT group. This transition within the oral microbiome was a consequence of maternal antibiotic-treatment, suggesting important roles for these genera in the intrapartum antibiotics exposure. Some, such as *Corynebacterium, Escherichia/Shigella and Klebsiella,* can cause human diseases occasionally opportunistically. Previous studies found that genera *Corynebacterium*, *Propionibacterium and Bifidobacterium* played important roles in the development of dental caries and in denture plaque.[34] Moreover, evidence showed that some species of *Bacteroides* were associated with gingivitis, periodontitis, endodontal infections and odontogenic abscesses.[35] Genus *Propionibacterium* played a critical role in the development of inflammation, and some species are opportunistic oral pathogens.[36] Genera *Faecalibacterium* and *Roseburia* had a cytotoxic effect on gingival cells of human and proved to be pathogenic in the oral environment because of their butyrate production.[37] These initial oral colonizers seem to condition the subsequent colonization, leading to a more complex ecosystem, and greatly affect infant oral health.

The initial oral colonizers could be affected by many factors, such as the mode of delivery, diet, drugs and living environment, and metabolic capabilities were altered accordingly. Thus, it is critical not only to identify the types of bacteria that compose the oral microbiota but also to understand their ability to participate in metabolic functions. This study showed that oral microbial genes in AT group were enriched in the amino acid biosynthesis, amino acid degradation, lipopolysaccharide biosynthesis and biosynthesis of ansamycins. However, they were not active in carbohydrate metabolism enzymes, such as glycoside hydrolase and polysaccharide lyase. Lipopolysaccharide, as

an endotoxin, is the major component of the outer membrane of gram-negative bacteria, and it plays an important role in the pathogenesis of certain bacterial infections as an immunostimulator and immunomodulator.[38,39] Furthermore, lipopolysaccharide can be detoxified by removing the two phosphate groups on lipopolysaccharide carbohydrates,[40] while phosphotransferase system was enriched in the NT group. Thus the enrichment of lipopolysaccharide biosynthesis pathway was harmful to the neonatal health.

Overall, administration of antibiotics to the mother during the intrapartum period was significantly associated with a decreased transmission rate of *Lactobacillus* to neonates, and an increased trend for disease-associated taxa and metabolism functions. Our study also presents the prevalence of oral microbiota immediately after birth. These early oral microbial communities play a major role in the development of the adult oral microbiota and may represent a source of both pathogenic and protective microorganisms in a very early stage of human life. Long-term neonatal health should be followed up in the further study on intrapartum antibiotic exposure.

5. Conclusions

Although this study furthers the understanding of oral microbiome involvement in maternal antibiotic exposure, there are some issues that still need to be resolved: detection of microbial interaction and discrepancy at lower taxonomic levels; large cohort studies to confirm these findings; longitudinal studies to track the oral microbiome and its influences on neonatal development; and experimental studies to confirm the identified contributors. In summary, this work lays the foundation for the neonatal oral microbiome change pattern in intrapartum antibiotic treatment. The alternations in the microbiota composition might be associated with various disease, including metabolic disease and immune-related disease. Therefore, larger studies are warranted to validate these findings, and further studies are needed to investigate the long-term influence of intrapartum antibiotic use on oral microbiome and infant health.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

No conflict of interest exists in the submission of this manuscript, and all authors approved this manuscript for publication. And I would like to declare on behalf of all authors that work described was original research and has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pedneo.2019.03.011.