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Impacts of ceftriaxone exposure during pregnancy on maternal gut and placental microbiota and its influence on maternal and offspring immunity in mice

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Abstract: This study aimed to investigate the association between microbiota found in the maternal gut and placenta, and whether ceftriaxone exposure during pregnancy could alter these microbiota, and consequently affect the immunity of the mothers and their offspring. The microbiota in the feces and placenta of the dams were comprehensively analyzed using16S rRNA sequencing. Furthermore, viable bacteria in the placentas and blood of pups were also isolated by plate cultivation then taxonomically identified in detail by clone sequencing. Serum cytokines collected from dams and pups were quantitatively profiled using Luminex. The spleen organ index of dams was significantly lower and the offspring serum interleukin-6 levels were significantly higher in ceftriaxone-treated mice compared with the control group. The maternal fecal microbiota community was drastically altered in ceftriaxone-treated mice with significantly decreased diversity, depletion of *Bacteroidetes* and the blooming of *Tenericutes*. However, the placenta microbiota was dominated by *Proteobacteria* especially characteristically by *Ralstonia*, which was distinct from the maternal gut microbiota, regardless of whether ceftriaxone treatment or not. Viable bacteria have been found in placenta and blood cultures. These results indicated that ceftriaxone exposure in pregnancy could dramatically alter maternal intestinal microbiota, which affected the immunity of the mothers and their offspring at least partly, characteristically by enhanced pro-inflammatory responses. This study also indicated that the placenta might harbor its own microbes and the microbes were distinct from maternal gut microbiota, which may not be affected by oral administration of ceftriaxone during pregnancy.
Key words: ceftriaxone, immunity, microbiota, placenta, pregnancy

Introduction

Emerging studies have suggested that early-life gut microbiota communities play an important role in the immunity of their host animals [1, 2]. The colonization by gut microbes in early life has long been assumed to begin only after delivery, with the fetus and uterus considered to be sterile. However, although controversial, the "sterile uterus" hypothesis has been challenged by recent studies that have shown low abundance micro-

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biota in placentas, amniotic fluid, and fetal membranes [3–7]. One recent study demonstrated that the most of the vaginal delivery infant's gut microbiota comes from the mother's gut, not from vagina [8]. Other studies also indicated that the maternal gut microbiota during pregnancy potentially determines the development of allergic and autoimmune phenotypes in offspring, and the placental microbiota is emerging as a source for antigenic determinants during this process [9, 10]. It is possible that the placental microbiota has a previously unconsidered role in early life immune development.

Events during pregnancy and lactation, such as diet and medical treatment, may influence offspring immunity by way of modifying the gut microbiota [11, 12]. Among such events, antibiotic use was considered to be one of the strongest modifiers of microbiota due to their lethal effects on bacteria [13]. In recent years, antibiotics have been frequently prescribed to women during pregnancy to prevent or treat infections. Studies based on populations in Germany and the Netherlands found that about 20% of pregnant women had received antibiotics [14, 15]. Ceftriaxone is a β-lactam antibiotic, a third-generation cephalosporin, and is a broad-spectrum antibacterial targeting against most Gram-positive and negative bacteria. It has been widely used in clinical practice, even in pregnant women. However, antibiotics usage has been found to possess many adverse effects to humans, including bacterial resistance, blooming of opportunistic pathogens and its destruction of host-microbiota interactions. Meta-analysis from observational studies showed that maternal exposure to antibiotics is associated with eczema by one-year age and may have a prolonged effect on eczema after 1-year age [16]. Rodent and human studies have found that antibiotic treatment during pregnancy can lead to disruption of the gut microbiota, with long lasting immunological, metabolic and cognitive consequences to offspring [17–20]. Our previous study has found that ceftriaxone treatment during nursing can cause significant dysbiosis of the gut microbiota, and dysfunction of immunity in neonatal mice, and increasing of the susceptibility of IgE-mediated allergy, in their own characteristic ways [21]. However, additional studies are necessary to investigate whether the administration of ceftriaxone during pregnancy could affect placental microbiome, for which could consequently alter the immunity of the newborns.

The present study aimed to investigate the association between microbiota found in the maternal gut and placenta, and whether ceftriaxone exposure during pregnancy could alter these microbiota, and consequently affect the immunity of the mothers and their offspring. Pregnant BALB/c mice were gavaged with ceftriaxone from gestation day 13 to delivery. Spleen, placental, and fecal samples were collected from the dams, and blood was collected both from dams and their pups. The fecal and placental microbiota of dams were investigated using 16S rRNA sequencing, live placental bacteria were isolated using plate cultivation, and serum cytokine levels in both dams and pups were measured by multiple cytokine measurement using the Luminex System.

Materials and Methods

Animals

Twenty-four pregnant BALB/c mice, at gestation day 13, were purchased from the Institute of Laboratory Animals at the Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital (Sichuan, China). They were kept in individually ventilated plastic cages at an ambient temperature of 23 ± 1°C, and a humidity of 50% to 70%, under a 12 h light/dark cycle, with unrestricted access to water and food in Experimental Animal Center of West China School of Public health, Sichuan University. All experimental procedures were performed in accordance with the Guidelines for Animal Experiments at West China School of Public Health, Sichuan University (Sichuan, China).The animal experiment facility and animals used for the present study were officially approved by the Experimental Animal Management Committee of Sichuan Government (Approved number: SYXK2013-011). The experimental protocols were approved by the West China School of Public Health Medical Ethics Committee of Sichuan University (Sichuan, China)

Antibiotic treatment and cesarean section

Pregnant mice were randomly divided into two groups: a control group (n=13) and a ceftriaxone group (n=11). Mice in the ceftriaxone group were orally gavaged with 0.2 ml of 150 mg/ml ceftriaxone for once a day (Aladdin Shanghai Biochemical Technology, Shanghai, China) dissolved in saline; the mice in control group were gavaged with the same volume of saline. Gavage was discontinued after delivery.

On gestation day 21, mice underwent sterile cesarean section (C-section). The detailed process of sterile C-section was as followed: before experiment, all the items (including the plates) used in the experiment were sterilized at 121°C for 20 min and pasted the sterilization indicators on the items. First, the sterilized trypticase soya agar (TSA, Land Bridge Technology, Beijing, China) plates were aerobic cultured at 37°C overnight to do the sterile verification. Then, all the sterilized items were placed on the ultra-clean bench and were irradi-

ated with ultraviolet for 1 h. Sacrificed pregnant mice were soaked in 75% ethanol for 1 min immediately after sacrificed. We carefully cut open the abdomen and uterus of mice to avoid poking into the intestines as much as we can and obtain offspring and placentas sterilely. After caesarean-section, the spleens of dams and pups were weighed. The spleen organ index was calculated through the weight of spleen divided by their respective body weight after delivery.

Bacterial cultivation and identification

Sterile whole blood samples were collected by beheading the offspring delivered by cesarean section. The placentas of dams were also collected in a sterile manner during cesarean section. Three pups and the placentas from each dam were used for cultivating bacteria. Three duplicate plates were made for each sample. Resected placental tissue samples (100 mg) were cut into small pieces, homogenized in 600 μl of phosphate-buffered saline (PBS) with the grinding rod of an electric grinder, and 100 μl aliquots were plated out on TSA plates. The whole blood samples (100 μl) were mixed with 500 μl PBS, mixed by vortex, and 100 μl aliquots were plated out on TSA plates.

Three types of the controls were designed for this study including the positive, blank and PBS control, respectively. As the positive control, the disposable sterile L-shaped rod was used to wipe on the abdomen of the mouse, then coated it on the TSA plates to check the contamination from the tested mice skin. As blank control: opened the TSA plates to expose TSA medium to the air during the whole experiment to check contamination from experimental environment, especially inside of bench. PBS control: 100 μl of the tested PBS were coated on the TSA plates to check contamination from the PBS (Hyclone) used to dilution placentas lysates and blood samples. All the plates were cultured for aerobic or anaerobic, each of them had two parallel repetitions, respectively.

The TSA plates were incubated under aerobic or anaerobic conditions at 37°C for 3 to 5 days. Representative colonies for each different colony type grown were examined by Gram-stain and microscopy. Bacterial DNA was obtained by boiling the bacterial suspension for 5min, then template DNA was amplified with the universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Amplified samples were cloned and sequenced (Sangon Biotech Co., Ltd., Shanghai, China) after verification using 2.0% agarose gel electrophoresis. The GenBank 16S rRNA (bacteria and archaea) database was searched using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the closest relatives of the partial 16S rDNA sequences. The 16S rDNA base sequences of identified bacterial strains in plate cultivation were listed in Supplementary data.

Serum cytokine detection

Whole blood samples were collected from dams and pups after delivery. Sera samples were isolated and frozen at −80°C. Serum tumor necrosis factor (TNF)-α, IL-12p70, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-17A and interferon (IFN)-γ levels were determined using a Luminex assay (R&D Systems Inc., Minneapolis, MN, USA). Assays were performed according to the manufacturer's instructions and read using a Luminex 200^{TM} multiplexing instrument (Merck Millipore, Burlington, MA, USA). All the procedures were performed by a professional experimental technician who was blind to our study design.

Bacterial DNA extraction and 16S rRNA amplification

Fresh stool pellets and the placentas of dams were collected and frozen at −80°C. Total DNA was extracted using the TIANamp Stool DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) and E.Z.N.A. Tissue DNA Kit (Omega Biotek, Norcross, GA, USA), in strict accordance with the manufacturer's instructions. The 5' ends of the primers were tagged with unique sample-specific identifiers (barcodes) and sequenced with the universal bacterial primers V3-338F (5'-ACTCCTACGGGAGGCAGCAG-3') and V4-806R (5'-GGACTACHVGGGTWTCTAAT-3'). Polymerase chain reaction (PCR) amplification and PCR product purification were performed as previously described [22].

16S rRNA sequencing and diversity analysis

Briefly, as previously described [22], purified amplicons were pooled and sequenced on an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. All raw sequences were screened using QIIME 1.9.1 for quality filtering. Operational taxonomic units (OTUs) were clustered with a cut-off of 97% similarity using the *de novo* UCLUST algorithm against the Greengenes database 13.8. Sequence alignment was conducted using the PyNAST 1.2.2 software, and a phylogenetic tree was built using FastTree 2.1.90 to study the phylogenetic relationships of different OTUs. The phylogenetic tree and modified relative abundance tables generated were used to calculate microbial alpha diversity (Chao 1, Shannon and Simpson index) with QIIME script. Weighted UniFrac or Bray-Curtis distance metrics analysis was performed using OTUs for each sample, and principal coordinates analysis (PCoA) was conducted according to the matrix

of distance in Phyloseq 1.20.057. Variations in microbial abundance at different taxonomic ranks were determined using Metastats performed by the EDDA R-package 1.10.0. Sequence Read Archive (SRA) accession number for the 16S rRNA sequencing of murine fecal and placenta microbiota reported in this paper are SRP155014 (https://www.ncbi.nlm.nih.gov/sra/SRP155014) and PRJNA491812 (https://www.ncbi.nlm.nih.gov/sra/PRJNA491812), respectively.

Linear discriminant analysis (LDA) effect size (LEfSe) analysis

To identify biomarkers which differentiated abundance in the different treatments, the linear discriminant analysis (LDA) effect size (LEfSe) algorithm was used [23]. LEfSe couples a robust test for measuring statistical significance (Kruskal–Wallis test) with a quantitative test for biological consistency (Wilcoxon-rank sum test). Any differentially abundant and biologically relevant features are ranked by effect size after undergoing LDA. An effect size threshold of more than 2 or 3 (on a log10 scale) was used for all biomarkers discussed in this study.

Inferred metagenomics by PICRUSt

The functionalities of the different metagenomes, grouped by different treatments, were predicted using the PICRUSt software (v1.1.2) (http://picrust.github.io) [24]. This software allows the prediction of functional pathways from the 16S rRNA reads. The resulting OTU table was obtained as described above, and then used for microbial community metagenome prediction with PICRUSt. PICRUSt was used to derive relative Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway abundance. Supervised analysis was done using LEfSe to elicit the microbial functional pathways that were differentially expressed in the different treatments.

Statistical analysis

R 3.4.1was used to analyze the sequence data and to visualize the color plots showed in this study. The specific analysis methods of sequence data have been introduced clearly above. Other data and plots including cytokines, body weight, spleen organ index and alpha index were statistically analyzed and graphed with Graphpad Prism 7.0. The Student's *t* test or Mann-Whitney U test were applied with Graphpad Prism 7.0, and a probability (*P*) value <0.05 was considered to be statistically significant. All statistical tests were two-tailed.

Results

Body weight, spleen organ index, and serum cytokines of dams and pups

For dams, no significant differences in body weight, cytokines (including TNF-α, IL-12p70, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-17A, and IFN-γ) were found between the control and ceftriaxone groups. The spleen organ index was significantly lower in ceftriaxone-treated dams compared with the control group (Table 1). For pups, differences in body weight, spleen index, and TNF-α, IL-12p70, IL-1β, IL-2, IL-4, IL-10, IL-17A, and IFN-γ between the two groups were also not significant. However, serum IL-6 levels in pups whose dams were treated with ceftriaxone before delivery were found to be significantly higher compared with pups in the control group (Table 2).

Table 1. Body weight, spleen organ index, and serum cytokines of dams

Group	Body weight (g)		Spleen organ index (mg/g)	Serum cytokines (pg/ml)								
	Before delivery	After delivery		TNF-α	IL-12p70	IL-1β	IL-2	IL-4	IL-6	IL-10	IL-17A	IFN-γ
Control	37.31 ± 4.40	27.04 ± 2.33	3.61 ± 0.61	0.20 ± 0.11	1.84 ± 0.87	17.76 ± 23.59	0.13 ± 0.16	6.41 ± 2.29	0.95 ± 1.30	N.D.	N.D.	0.41 ± 0.37
Ceftriaxone	35.12 ± 5.07	25.19 ± 3.01	2.82 ± 0.31***	0.15 ± 0.06	1.74 ± 1.28	5.26 ± 6.74	N.D.	7.00 ± 1.34	0.39 ± 0.27	N.D.	N.D.	0.32 ± 0.13

Data were showed as mean ± SD. For control group, n=13; for ceftriaxone group, n=11. For cell cytokines, n=7 per group. ***: *P*=0.0008, comparing with control. N.D.: not detected.

Table 2. Body weight, spleen organ index, and serum cytokines of pups

Group	Body weight (g)	spleen organ index (mg/g)	Serum cytokines (pg/ml)								
			TNF-α	IL-12p70	IL-1β	IL-2	IL-4	IL-6	IL-10	IL-17A	IFN-γ
Control	1.09 ± 0.30	2.16 ± 1.38	0.42 ± 0.12	5.13 ± 1.60	1.82 ± 1.04	0.01#	9.51 ± 3.12	2.56 ± 1.91	8.38 ± 11.36	N.D.	1.76 ± 1.85
Ceftriaxone	1.10 ± 0.31	2.26 ± 1.32	0.43 ± 0.12	6.30 ± 1.81	N.D.	0.01#	9.23 ± 2.86	9.26 ± 7.98*	8.55#	N.D.	2.38 ± 1.58

Data were showed as mean ± SD. Pups obtained from one dam were pulled up and treated as n=1 to avoid maternal effects. For body weight and spleen organ index, control group, n=13; for ceftriaxone group, n=11. For cell cytokines, control group, n=10; for ceftriaxone group, n=12. *: *P*=0.0248, comparing with control. The "#" represents that there's a missing SD because only one sample had been detected. N.D.: not detected.

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Maternal fecal microbial diversity and composition

The fecal microbiota were found to be significantly altered following orally administered ceftriaxone. The alpha diversity indices of maternal fecal microbiota, including Chao 1, Shannon and Simpson indices, were significantly lower in ceftriaxone-treated mice compared with those of the control group (Fig. 1A).

The composition of the maternal fecal microbiota was also dramatically changed by exposure to orally administered ceftriaxone. At the phylum level, the fecal microbiota of the mice in the control group predominately consisted of *Bacteroidetes, Firmicutes* and *Proteobacteria.* However, *Tenericutes* was found to be significantly higher in ceftriaxone-treated dams, and was the most common phylum, since *Bacteroidetes* and *Proteobacteria* were significantly lower in the ceftriaxone-treated dams (Fig. 1B, Supplementary Fig. 1). No significant differences were found in the relative abundance of *Firmicutes* between the two groups. At the genus level, *Mycoplasma* and *Staphylococcus* were significantly higher in the ceftriaxone-treated dams, while *Bacteroides, Odoribacter, Ruminococcus, Lactobacillus, Prevotella, Oscillospira,* AF12, *Desulfovibrio,* [Prevotella], and *Helicobater* were all significantly lower in the treatment group (Fig. 1C, Supplementary Fig. 2).

According to the LEfSe analysis and the cladogram, bacteria from *Bacteroidetes* almost represented and featured the maternal fecal microbial community of mice in control group, however, *Mycoplasma* from *Tenericutes* almost featured microbiota community of ceftriaxone-treated mice during pregnancy (Figs. 2A and B). Weighted Unifrac distance is a beta diversity index, which considers not only the evolutionary information but also the abundance of OTUs between each sample to compare the differences in microbial communities. Here we used the weighted Unifrac distance for the PCoA analysis because of the significantly altered maternal fecal microbial abundance and diversity caused by ceftriaxone. And two obvious clusters were observed in the plot, 85.05% of the variability on axis PC1 with a larger coordinate values, which showed significant alterations in the microbiota community due to ceftriaxone treatment during pregnancy (Fig. 2C).

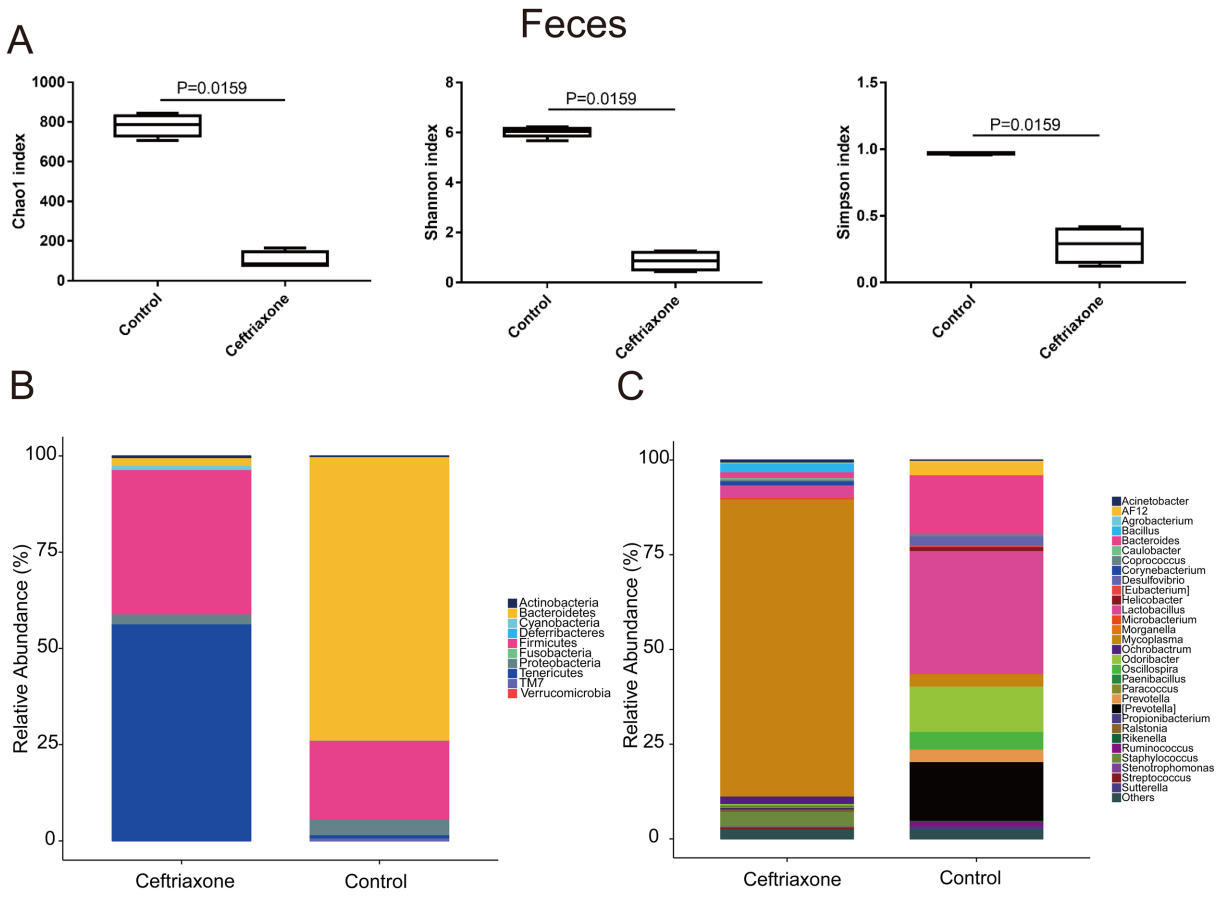

Fig. 1. Diversity and community structure of maternal fecal microbiota. (A) alpha-diversity (Chao 1, Shannon and Simpson indexes) of fecal microbiota in two groups. Mann-Whitney U test was used for statistical analysis. (B) Fecal microbiota composition at the phylum level. (C) Fecal microbiota composition at the genus level. n=4–5/group.

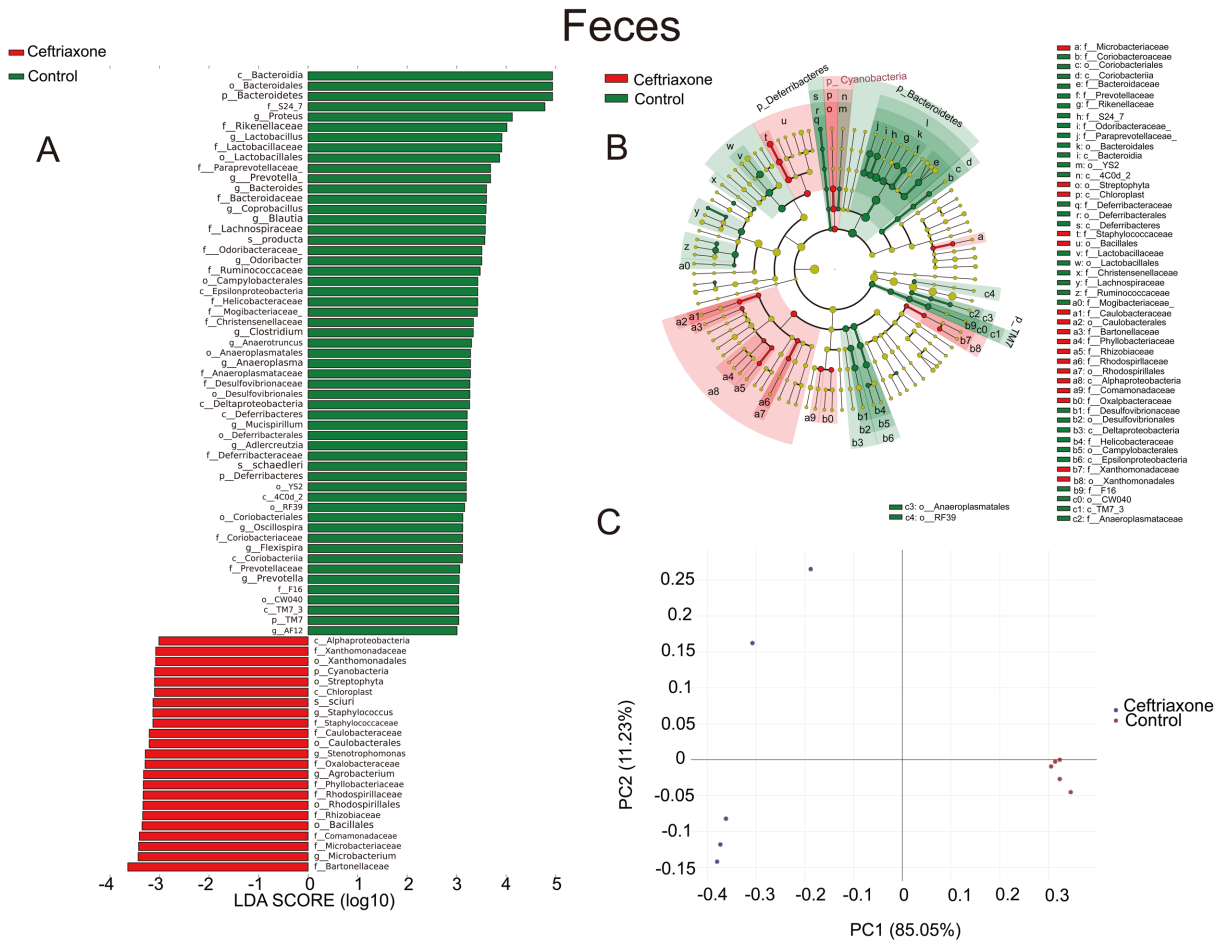

Fig. 2. Lefse and PCoA analysis of maternal fecal microbiota alterations. (A-B) Bacteria taxa from feces were identified as differentially abundant between control and ceftriaxone group as analyzed by Lefse and projected as a cladogram. Taxa with Log LDA score >3 were showed and considered as statistically significant in each group. (C) PCoA analysis based on weighted Unifrac distance. Individual samples of maternal feces are shown as single points. n=4–5/group.

Placental microbial diversity and composition

Plate cultivation and high-throughput sequencing were both employed to observe alterations in maternal placental microbiota. The placental microbiota communities were characterized by low abundance, low richness and low diversity, especially compared with fecal microbiota populations. No significant differences were found in the three alpha-diversities between the two groups of maternal placental microbiota. However, all the three alpha indices of placental microbiota in ceftriaxone treated dams seemed to be lower than that in control, which implied a possible decreased tendency of alpha index in placental microbiota following ceftriaxone treatment (Fig. 3A).

Interestingly, the placenta microbiota community was totally different from the microbes detected in maternal feces. At the phylum level, *Proteobacteria* was the most predominant organism in both groups (Fig. 3B). However, differences in the relative abundance of all detected phyla between the two groups were not found to be significant. At the genus level, *Ralstonia* and *Sphingomonas* were the two predominant organisms in both groups, with a similar abundance (Fig. 3C). The relative abundance of *Acinetobacter* and *Dechloromonas* in the placentas of control mice was significantly higher than in the placentas of mice treated with ceftriaxone (Supplementary Fig. 3).

LEfSe analysis showed only the characteristic placental bacteria taxa for the control mice, especially *Pseudomonadales*, and no significant bacteria taxa were found in the placentas of ceftriaxone-treated mice compared with the control group (Figs. 3D and E). Bray-Curtis distance is another beta diversity index for comparing the differences of microbial community, which only considers the abundance of OTUs without considering the evolutionary relationship between OTUs from different samples. Here we used the Bray-Curtis distance for the PCoA analysis aimed to find something of significance because that there were no significant changes in the abundance and diversity of placental microbiota

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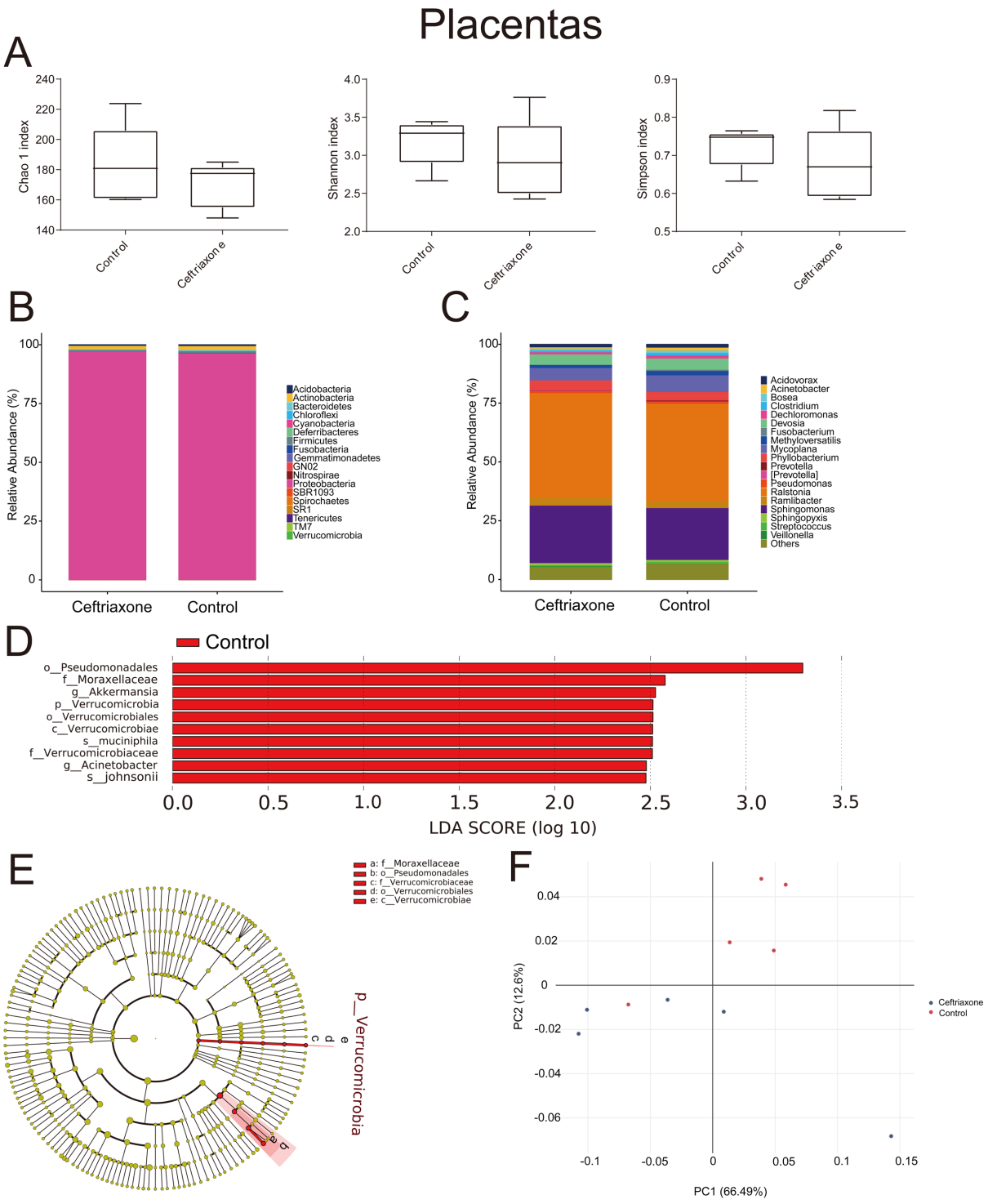

Fig. 3. Maternal placenta microbiota alterations. (A) alpha-diversity (Chao 1, Shannon and Simpson indexes) of placental microbiota in two groups. Mann-Whitney U test was used for statistical analysis. (B) Placenta microbiota composition at the phylum level. (C) Placenta microbiota composition at the genus level. (D-E) Bacteria taxa from placenta were identified as differentially abundant between control and ceftriaxone group as analyzed by Lefse and projected as a cladogram. Taxa with Log LDA score >2 were showed and considered as statistically significant only in control group. (F) PCoA analysis based on weighted Bray-Curtis distance. Individual samples of maternal placentas are shown as single points. n=5/group.

in Figs. 3A–E. However, Fig. 3F showed two clusters separated by PC2, explained with 12.6%, although the coordinate values were relatively small.

Bacteria detected in placenta and blood through cultivation

As we can see in Table 3, more types of viable microbes were found in placental tissue homogenates of

Table 3. Bacteria identification of plate cultivation

Cultures	Groups	Serial number	Bacteria identification		Similarity (%)
			Species name	Phylum	
Placenta	Control	1	*Staphylococcus epidermidis*	*Firmicutes*	98
		2	*Micrococcus yunnanensis*	*Actinobacteria*	95
		3	*Acinetobacter lwoffii*	*Proteobacteria*	92
		4	*Bacillus megaterium*	*Firmicutes*	96
		5	*Staphylococcus nepalensis*	*Firmicutes*	96
	Ceftriaxone	6	*Staphylococcus equorum*	*Firmicutes*	97
		7	*Lactobacillus plantarum*	*Firmicutes*	96
Blood	Control	8	*Micrococcus yunnanensis*	*Actinobacteria*	95
		9	*Exiguobacterium acetylicum*	*Firmicutes*	96
		10	*Pseudomonas koreensis*	*Proteobacteria*	96
		11	*Pseudomonas moraviensis*	*Proteobacteria*	94

The serial number 1–11 represents the targeted bacteria found by cultivation in two groups.

mice in the control group compared with those in the ceftriaxone group. Four viable microbial types were found in placentas in control group, including *Staphylococcus, Acinetobacter*, *Bacillus,* and *Micrococcus*, two types were found in ceftriaxone group, including *Staphylococcus* and *Lactobacillus*. Furthermore, *Staphylococcus* was found in placentas both in control and ceftriaxone group but with different species. However, only the blood of pups in control group have detected viable bacteria, including *Micrococcus, Exiguobacterium* and *Pseudomonas*. No viable bacteria were found in blood of pups in ceftriaxone group. Interestingly, *Micrococcus yunnanensis* was the only microbes found both in placenta and blood in control group.

Prediction of fecal and placental microbial community functions

The prediction of microbial community functions between groups are shown in Fig. 4A. The relative abundance of KEGG pathways at level 2, encoded in the microbiota present in feces and placenta from both groups, showed that amino acid metabolism, carbohydrate metabolism, energy metabolism, membrane transport, and replication and repair were the most predominant microbiota activities. Significant differences were observed between the functional activities of the fecal microbiota from the control and ceftriaxone group. The relative abundance of metabolic and immunological activities encoded in the fecal microbiota of the control group were significantly higher than those of the ceftriaxone group. However, the relative abundance of transport and repair activities encoded in the fecal microbiota of the control group was significantly lower than that of the ceftriaxone group. No significant differences were found in placental microbial functional activities between the two groups (Fig. 4B).

Relationship of maternal fecal and placental microbiota

When taking feces and placentas from the two groups for PCoA analysis based on weighted Unifrac distance, three clusters were clearly separated, the variability explained by PC1 with 79.48% and PC2 with 16.8%. The placental microbiota from the two groups were mostly clustered in one area but distinct from feces; the fecal microbiota from the two groups were also separated into two clusters due to ceftriaxone treatment (Fig. 5A). A Venn diagram shows the OTUs shared between groups and samples in Fig. 5B. There were 21 OTUs shared by feces and placentas in the control group, and 50 OTUs shared in the ceftriaxone group. There were 112 OTUs shared in feces in both control and ceftriaxone groups, and 217 OTUs in the placenta in both groups. 589 OTUs were detected only in the feces of the control group, while 13 OTUs were detected only in the feces of the ceftriaxone group. 15 OTUs were detected only in the placentas of the control group, with 20 OTUs found only in the placentas of the ceftriaxone group.

Discussion

In the present study, ceftriaxone was administered to pregnant mice to imitate clinical usage of antibiotics in pregnant women. There was no body weight loss, diarrhea, or abortion of ceftriaxone administration in pregnant or neonatal mice. However, the maternal spleen organ index in ceftriaxone-treated mice was significantly lower and no significant differences were found among serum cytokines. Consistently, our previous studies have found that vancomycin and ceftriaxone treatment from neonatal stage to weaning could decrease spleen and thymus organ index of mice [21, 25]. Guo *et al*. reported that long-term ceftriaxone treatment could also decrease the spleen index of adult mice [26]. The

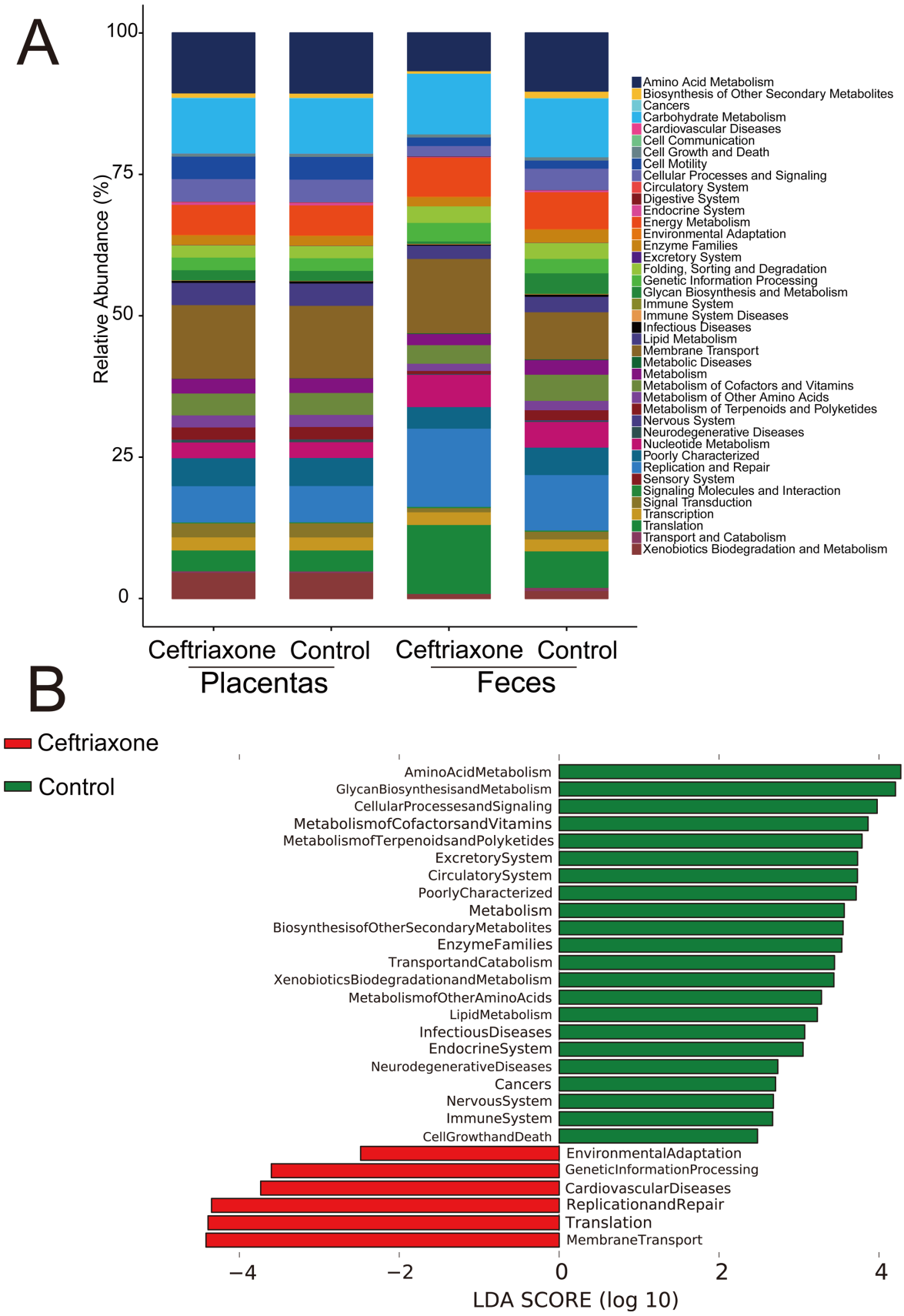

Fig. 4. Microbial metagenomics activity in maternal feces and placenta samples. (A) Relative abundance of KEGG pathways at level 2 encoded in the microbiome present in maternal feces and placenta samples in two groups. (B) LDA scores for differentially abundant PICRUSt predicted microbial genes, pathways and classified functional categories (Log LDA >3) in maternal feces samples in two groups. No significant differences were found in PICRUSt predicted placental microbial functional activities between the two groups.

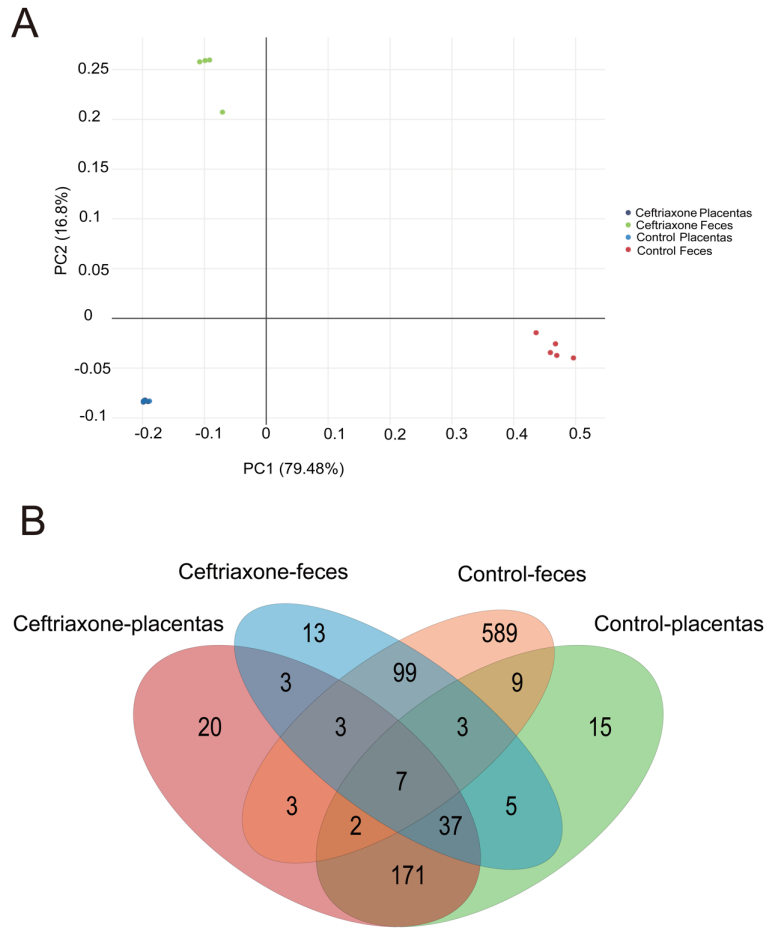

Fig. 5. Possible relationship between feces and placenta microbiota based on OTUs. (A) PCoA analysis of fecal and placenta microbiota based on weighted Unifrac distance. (B) Shared OTUs in four sub-groups visualized as Venn diagram.

spleen is the body's largest immune organ. The spleen organ index (spleen weight divided by body weight after delivery), namely the relative quality of the spleen, which is usually used in toxicology or infected disease as a basic indicator to measure the immunosuppressive effects of the test substances or pathogens, could directly reflect the homeostasis of immune function of the body [27, 28]. Effects of drugs on the spleen organ index can be used as the preliminary indicator for the study on immunopharmacological mechanisms in animals [29]. The significant decreased spleen organ index in treated dams might imply that the ceftriaxone could reduce the body's immune response to pathogens, however, this reduction might be modest because of the non-significant alterations of cytokines. These results indicated that ceftriaxone treatment during pregnancy might affect maternal immunity to a certain degree, although no other significant immunological damage was observed in the present study.

Dysplasia immune system of germ-free neonatal mice and pups born to antibiotics-treated dams indicated the importance of maternal gut microbiota on offspring immune development [30, 31]. In the present study, serum IL-6 levels in the neonatal mice whose mother was treated with ceftriaxone before delivery was significantly higher than those mice whose mother in control group. These results had good agreement with the findings of Fuglsang *et al.* in which perinatal broad-spectrum antibiotics treatment decreased the proportions of bone marrow granulocyte-macrophage progenitor cells, mature granulocytes, spleen granulocytes and B cells in newborn mice. Furthermore, these damages induced by perinatal antibiotics administration in offspring immunity could not be restored with decreased splenic T helper cells and cytotoxic T cells in the later life [32]. On other hand, Nyangahu *et al.* reported that oral administration of vancomycin during pregnancy and nursing not only altered the maternal immunity, as demonstrated by significantly higher levels of both total IgG and IgM in breastmilk, but also altered the immunity of pups, as demonstrated with the significant increased lymphocyte numbers and the elevated numbers of both

$CD4^+$ T cells and B cells, most notable Follicular B cells [19]. In the present study, the significant changes of IL-6 only found in the offspring not in the mothers, these results demonstrate again that antibiotic exposure, such as ceftriaxone, during pregnancy could alter the development of the immunity of offspring even in case that they show limited and modest changes impacts on mothers' immunity. The different immune response between mother and offspring to antibiotic exposure might result from the antibiotic-spectrum, the physiological/healthy condition, and critical window of intervention before or after birth. However, these findings suggest that offspring might be much more sensitive to antibiotic exposure during pregnancy.

Many studies have well demonstrated that IL-6, but not any other cytokines, could alter fetal brain development by maternal immune activation [33, 34]. In addition, Yang *et al*. found that IL-6 promoted follicular helper T (TFH) cell development in immunized adult mice but impaired the vaccine response of neonatal mice due to the higher IL-6 receptor (IL-6R) expression on Foxp3-expressing regulatory TFH (TFR) cells than TFH cells in immunized neonatal mice [35]. These studies indicated that IL-6 played crucial roles in fetal immune and brain development. In the present study, the results that the altered IL-6 express only happened in offspring might because of the offspring's immature immune system such as higher IL-6R expression on TFR cells in offspring compared to their mothers. These characteristics of neonatal mice might make them more susceptible to maternal changes than their mothers and the particularity of IL-6 on fetal immune development make itself more easily affected by external factors, such as gut microbiota and their metabolites.

Pregnancy is a special period for gut microbiota. Even a healthy pregnancy also induces dramatic alterations in the maternal gut microbiota during the course of gestation, with a reduced diversity and an overall increase in *Proteobacteria* and *Actinobacteria* [36]. Antibiotic usage during pregnancy undoubtedly affects the microbiome and later health of the mother and of the fetus, including the maternal vaginal microbial community, early life gut microbiota colonization of offspring, childhood obesity and asthma [37]. In the present study, the alpha-diversities and the relative abundance of *Bacteroidetes* and *Proteobacteria* in maternal fecal samples were significantly lower in mice treated with ceftriaxone during pregnancy, while *Tenericutes* was the most common bacteria in this group. *Lactobacillus* and other genera from *Bacteroidetes* almost disappeared, while *Mycoplasma* from *Tenericutes* became the most predominant genus in the mice treated with ceftriaxone. As others reported before, these results demonstrated that antibiotic treatment during pregnancy destroyed maternal fecal microbiota. However, the altered fecal microbial communities in our study were entirely different from other studies. Fuglsang *et al.* reported that *Cyanobacteria* became the dominate phylum, but not *Tenericutes*, in the fecal microbiota of pregnant mice treated with a mix of ampicillin trihydrate, gentamicin sulfate and metronidazole for 7 days before delivery [32]. Nyangahu *et al.* reported that *Firmicutes* became the most dominate phylum in the maternal fecal microbiota treated with vancomycin 5 days prior to delivery [19]. Despite the inconsistency of microbial community alterations responded to different antibiotic treatment, the decreased diversity was found consistent within these studies. These results demonstrated that the corresponding responses in maternal fecal microbial community changes following different antibiotic administration during pregnancy were special and characteristically related to their antimicrobial spectrums. This reminded us to pay special attention to pregnancy microbiota changes due to various antibiotics.

Tenericutes was a group of bacteria with a relative abundance of less than 1% among normal gut microbiota. *Mycoplasma*, belonging to *Tenericutes*, are usually the smallest organisms which may be potent pathogens of host animals. *Mycoplasma pneumoniae* in particular is known as an etiological agent of pneumonia. Studies have suggested that *Mycoplasma* may be involved in pregnancy complications, including spontaneous abortions and preterm births [38–40]. They also suggested that maternally distinct gut microbiota caused by ceftriaxone treatment during pregnancy might be related to some adverse events such as infections. The disruption of intestinal tissue barrier functions could manifest as damaged tight junctions, with enhanced intestinal permeability possibly being the underlying mechanism of gut microbiota-associated pregnancy infections. These results again reminded us to pay attention to the clinical use of antibiotics, especially in pregnant women and infants.

There have been several studies that indicated the long lasting immunological and metabolic effects of an abnormal early-life microbiota community on hosts [41, 42]. Two recent studies have suggested that the maternal gut microbes provide the largest contribution of colonizing microorganisms which are subsequently transferred to the infant microbiome [8, 43]. In the present study, *Proteobacteria* characterized with more *Ralstonia* became the predominant bacteria in the tested placenta with the main genera totally different from the microbes found colonizing the intestinal tract. These results have good

agreement with those in human placentas reported by Parnell *et al.* [44]. They found that the microbial communities in placentas from term normal pregnancy exhibited spatially variable profiles, and revealed the presence of *Ralstone insideiosa* using species-specific analysis in the basal plate samples. In a recent animal study, Martines *et al.* [45] using source tracker analysis also found that the placenta as the most commonly identifiable origin for fetal bacterial DNA but also over 75% of the fetal gut genera overlapped with maternal oral and vaginal taxa but not with maternal or newborn fecal taxa. Besides analysis of 16S rRNA sequencing, the traditional cultivation methods were also used to directly detect viable bacteria from the tested placentas in the present study. However, the clone sequencing indicated that the isolates were also taxonomically different from the predominant microbes colonizing the intestinal tract. The identified isolates belonged to genus *Staphylococcus* we found using cultivation was also found in healthy human placentas, umbilical cord blood and meconium [4, 46, 47]. These results demonstrated that microbes, even viable bacteria, might exist in murine placental tissue, findings which are consistent with other studies that focused on human placentas or other body sites. All of these suggest that the microbes might be selected in specific manner to colonize in placenta then build the characteristic microbe communication there with specific symbiotic relationship with host animal. Therefore, the contact of infants with microbes might be initiated before birth. The further study should be focused to evaluate the influences of the microbes in the placentas to infant's health with the isolated bacteria.

In the present study, no significant differences in placental microbial diversity, or community and functional composition, were found between the control and the ceftriaxone-treated mice groups. Few OTUs were shared between microbiota found in maternal feces and placenta, regardless of antibiotic treatment. These results indicate that ceftriaxone treatment during pregnancy and its resulting dramatic changes in gut microbiota did not greatly affect the placental microbial community, at least in the present study. The placenta microbiota was quite stable and distinct from maternal gut microbiota. Other recent studies, based on high-throughput sequencing and/or traditional culture methods, have detected commensal microbes in placenta, umbilical cord, amniotic fluid and breast milk [48, 49]. However, the origin of the microbes found in these extra-intestinal body sites remains a mystery. For instance, microbes in breast milk are believed to be partly transferred there from the gut by immune cells, although this process not completely understood [50, 51]. "The revolutionary hypothesis: 'active migration'" suggests that dendritic cells, macrophages and mononuclear immune cells have involved in this process, through which the gut microbes migrate to the mammary glands via an endogenous cellular route (the bacterial entero-mammary pathway) [51–54]. Since then, the underlying mechanisms that how the gut microbes protect themselves from devouring and killing by immune cells and whether there is a window for microbe migrating to extra intestinal body sites are still unclear.

In the present study, we found viable bacteria, through cultivation, in blood samples from neonatal mice, and these bacteria were identified using clone sequencing. To our best knowledge, this is the first study in which viable bacteria have been successfully cultivated using neonatal blood samples. The placenta is a blood vessel-rich organ and plays a key role in blood exchange between mother and fetus. Studies have previously found bacteria or their products in healthy or diseased human blood [55–57]. Bloodstream could be considered as a possible deliverer during gut bacteria transmission with or without the help of immune cells or dysfunction of intestinal epithelial barrier [58]. Therefore, it is reasonable to speculate that any microbes found in the placenta could have migrated from maternal blood circulation. However, Aagaard and colleagues [3] found that the placenta microbiome was most similar to that of oral cavity and hypothesized that bacteria translocate from the mother's oral cavity into the placenta, contributing to in utero colonization of the fetal gut. Because of the lack of detected microbiota in blood or oral cavity using high-throughput sequencing, we are not sure that the similarity of microbiome between placenta and blood or oral cavity could explain the origin of placenta microbiome. The traceability tracking methods with traditional cultivation, PCR, and high-throughput sequencing are needed to demonstrate the origin of microbiome found in placenta.

Cytokines can modulate fetal well-being and contribute to immune programming [59]. IL-6 is a pro-inflammatory cytokine and known to be involved in many pathological processes. Studies have found that maternal serum and/or plasma IL-6 concentration is linked to pregnancy-induced hypertension and neurodevelopmental disorders in offspring [60, 61]. The significantly higher level of serum IL-6 in offspring from mothers treated with ceftriaxone before birth suggests a correlation between pregnancy antibiotic administration and maternal and/or offspring adverse outcomes. Gut commensal microbes have been demonstrated that their interactions with gut-associated lymphoid tissues and their stimulated effects on the immune cells and molecules have participated in the production and functional mod-

ulation of cytokines [62]. LPS from gram-negative bacteria or other microbial products could bind to receptors (such as TLRs) on immune cells surface to activate the production of IL-6, which is an acute response to inflammation and this phenomenon has also been found in cultured explants from healthy term human placentas [63, 64]. Therefore, it is plausible that a relative high IL-6 concentration in serum might be a response to sudden changes in the commensal microbiota due to ceftriaxone administration. However, these microbiota changes did not influence the mother's serum IL-6 level. In another unpublished study which was similar to the present study, we found that no significant changes in mother's serum IgG, IgM, IgA, IgE, sIgA, LPS and fecal sIgA level between control and ceftriaxone group although fecal microbiota of mothers was significantly damaged by antibiotic too. Therefore, in the present study, we speculated that the altered fecal microbiota by ceftriaxone did not affect the IL-6 level in mothers might because that the serum LPS was not significantly promoted enough either. Further studies should focus on the effects during pregnancy that antibiotic administration-induced fetal cytokine changes may have on the health of offspring and their development after birth, especially with regard to the interaction between the immune system and commensal microbiota.

In conclusion, the oral administration of antibiotics such as ceftriaxone during pregnancy could cause maternal distinct gut microbiota. Although oral administration of ceftriaxone during pregnancy may not obviously influence the placenta microbiota, it could partly alter immunity in offspring. Other profound effects of antibiotics administration during pregnancy on the immunity and microbiota of mothers and offspring later in life should be further studied in the future.

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Conflict of Interests

The authors declare that they have no conflict of interests.

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