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RESEARCH

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Effect of β-lactam antibiotics on the gut microbiota of term neonates

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Abstract

β-Lactam antibiotics are a class of antibiotics commonly used to treat bacterial infections. However, the effects of β-lactam antibiotics on term neonatal intestinal flora have not been fully elucidated. Hospitalized full-term newborns receiving β-lactam antibiotics formed the antibiotic group ($n=67$), while those without antibiotic treatment comprised the non-antibiotic group ($n=47$). A healthy group included healthy full-term newborns ($n=16$). Stool samples were collected for 16 S rDNA sequencing to analyze gut microbiota variations. Further investigation was carried out within the β-lactam antibiotic group, exploring the effects of antibiotic use on the newborns' gut microbiota in relation to the duration and type of antibiotic administration, delivery method, and feeding practices. The antibiotic group exhibited significant difference of microbial community composition compared to the other groups. Genera like *Klebsiella, Enterococcus, Streptococcus, Alistipes*, and *Aeromonas* were enriched, while *Escherichia-Shigella, Clostridium sensu stricto 1, Bifidobacterium*, and *Parabacteroides* were reduced. *Klebsiella* negatively correlated with *Escherichia-Shigella*, positively with *Enterobacter*, while *Escherichia-Shigella* negatively correlated with *Enterococcus* and *Streptococcus*. Regardless of neonatal age, β-lactam antibiotics induced an elevated abundance of *Klebsiella* and *Enterococcus*. The impact on gut microbiota varied with the duration and type of antibiotic (cefotaxime or ampicillin/sulbactam). Compared to vaginal delivery, cesarean delivery after β-lactam treatment heightened the abundance of *Klebsiella, Enterobacteriaceae_Unclassified, Lactobacillales_Unclassified*, and *Pectobacterium*. Feeding patterns minimally influenced β-lactam-induced alterations. In conclusion, β-lactam antibiotic treatment for neonatal pneumonia and sepsis markedly disrupted intestinal microbiota, favoring *Klebsiella, Enterococcus, Streptococcus, Alistipes*, and *Aeromonas*. The impact of β-lactam varied by duration, type, and delivery method, emphasizing heightened disruptions post-cesarean delivery.

Keywords Gut microbiota, β-lactam antibiotics, 16s rDNA sequencing, Newborn, Cesarean delivery

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Background

Infections continue to be a leading cause of neonatal mortality, with neonatal pneumonia and sepsis representing two prevalent and globally significant health concerns [1–3]. Antibiotics, particularly β-lactam antibiotics, have transformed medicine, saving millions of lives since their first use to treat a bacterial infection [4, 5]. However, while these antibiotics are critical for the treatment of bacterial infections in newborns, they can also strongly disturb the commensal microbiota, select for resistant pathogens and facilitate subsequent infections [4, 6].

Intestinal microbes play a crucial role in maintaining human health by regulating and maintaining the intestinal barrier function, preventing the invasion of harmful microorganisms, aiding in nutrient synthesis and digestion, and promoting the development of the host's innate and adaptive immune systems [7]. The developing infant gut microbiota, especially in neonates, is highly dynamic and prone to disruption by external factors, including delivery mode, feeding patterns, infections and perinatal antibiotics [8–10]. Disturbance of the crucial developmental window predisposes the host to diseases such as neonatal pneumonia, neonatal sepsis, neonatal enteritis and allergies, asthma, cardiovascular disease, obesity, inflammatory bowel disease, irritable bowel syndrome, and neurodevelopmental disorders in later life [10, 11].

Neonatal antibiotic exposure is one of the most important factors for the colonization and development of intestinal microflora. The neonatal gut microbiota is influenced by the timing, the duration, and the type of antibiotic exposure [10]. A study showed that one-week empirical antibiotic therapy related to lower intestinal bacterial diversity and an enrichment of harmful bacteria such as *Streptococcus* and *Pseudomonas* in preterm infants [12]. Moreover, in full-term infants, antibiotic administration during the first hours of life reduced the level of *Bifidobacterium* in the days immediately after birth and subsequently increased the levels of *Enterobacteriaceae* [13]. In addition, β-lactam antibiotic treatment for less than seven days significantly affected fecal microbes and metabolites in the late preterm infants, including a reduction in the diversity of the gut microbiota overall and some beneficial bacteria such as *Bacteroides* [5]. The data indicated that while β-lactam antibiotics effectively eliminate pathogenic microorganisms, they may also disrupt the growth and development of beneficial bacteria in the gut microbiome. However, it is currently unclear how β-lactam antibiotic use impacts the early period of term neonates. The primary objective of this study is to investigate the impact of β-lactam antibiotics on the gut microbiota of term neonates. Specifically, we aim to compare the intestinal microbiota composition and diversity among neonates treated with β-lactam antibiotics, those who did not receive antibiotic treatment, and healthy controls. Furthermore, we will explore how the duration and type of antibiotic administration, as well as delivery and feeding methods, influence these effects. By establishing a clearer understanding of these impacts, we aim to provide a theoretical framework for guiding the regulation of β-lactam antibiotic usage in the neonatal period.

Methods

Study designs and subjects

The newborns who were hospitalized in the neonatology department of the Children's Hospital, Zhejiang University School of Medicine, between February 2019 and April 2019, were eligible for the study. Based on the clinical use of beta-lactam antibiotics, the newborns were divided into two groups: the beta-lactam antibiotic group ($n=67$) and the non-antibiotic group ($n=48$). The primary infections in the antibiotic-treated children included neonatal pneumonia and sepsis. Within the beta-lactam antibiotic group, newborns were further categorized into cefotaxime (Keflong) group ($n=31$) and ampicillin/sulbactam group ($n=36$), with both antibiotics administered intravenously at a dose of 50 mg/kg every12 hours. Additionally, based on the duration of antibiotic use, the β-lactam antibiotic group was subdivided into 3 days ($n=29$), 5 days ($n=26$) and 7 days ($n=12$) groups. The non-antibiotic group consisted of newborns hospitalized for non-infectious diseases, such as neonatal hyperbilirubinemia, who did not receive antibiotics. Healthy term newborns living at home were also enrolled in the healthy group ($n=16$) with voluntary participation from their families. The study protocol was approved by the Ethics Committee of the Children's Hospital, Zhejiang University School of Medicine (Ethics Approval Number: 2018-IRB-103).

Inclusion criteria and exclusion criteria

The inclusion criteria were as follows: (1) hospitalized newborns; (2) gestational age between 37 and <42 weeks; (3) birth weight between 2500 g and <4000 g; (4) mothers with healthy pregnancies and no history of probiotic use; (5) for the β-lactam antibiotic group, newborns who were confirmed to have infections and were treated with β-lactam antibiotics for a duration of 3 to 7 days; for the non-antibiotic group, newborns who were hospitalized for non-infectious diseases and did not receive antibiotics; for the healthy group, healthy term newborns living at home were enrolled with voluntary participation from their families. The exclusion criteria were as follows: (1) infants who were older than 28 days; (2) newborns with congenital gastrointestinal abnormalities; (3) infants who had taken probiotics within 2 weeks prior to admission; (4) infants who had a history of antibiotic use within 2

weeks prior to admission; (5) neonatal enteritis; (6) high-risk newborns; (7) significant organ dysfunction.

Fecal sample collection

Fecal samples were collected from newborns who met the inclusion criteria. For the β-lactam antibiotic group, fecal samples were collected on days 3, 5 and 7 after the initiation of β-lactam antibiotics. In the non-antibiotic group, fecal samples were collected during admission. Fresh feces were collected from the neonatal diapers or the residual feces after routine bowel movements using disinfected cotton swabs, with each sample weighing approximately 2 g. The samples were then placed into sterile containers and stored in a -20 °C refrigerator. Within an hour of collection, the samples were transferred to a laboratory refrigerator at -80 °C for subsequent analysis. In addition, in the healthy group, fecal samples were collected by parents into sterile containers and immediately frozen in a -20 °C refrigerator. These samples were then transported to the laboratory using dry ice by investigators and stored at -80 °C for subsequent experimentation.

DNA extraction and quality control

Stool samples were subjected to genomic DNA extraction using the QIAamp Fast DNA Mini Kit (QIAGEN, Hilden, Germany). The integrity of the extracted genomic DNA was evaluated by performing 1% agarose gel electrophoresis, while the DNA concentration was determined using the Qubit Picogreen fluorescence quantitative system.

MiSep library construction

To construct the MiSep library, PCR primers containing sequencing connector sequences were used to amplify and enrich the library template. The resulting target fragments were then screened using a magnetic bead method. Finally, single-stranded DNA fragments were generated through denaturation using sodium hydroxide.

Illumina MiSep sequencing

The DNA fragment is attached to one end of the chip, which is complementary to the primer base. The other end is also fixed and randomly paired with another primer to form a bridge. PCR amplification produces DNA clusters, which are then denatured into single strands. A modified DNA polymerase and dNTPs with four fluorescent markers are added, incorporating one base at a time. The surface of the reaction plate is scanned with a laser to read the nucleotide classes polymerized in the first round of reaction. Before the second nucleotide is added, the fluorophore and terminator groups are chemically cleaved to restore the 3' -end viscosity. The fluorescence signals collected in each round are counted, and the sequence of the template DNA fragment is obtained.

Analysis process

After obtaining data from the MiSep sequencing platform, the sequences were optimized through merging and quality control filtering. Operational Taxonomic Unit (OTU) clustering was performed to generate an abundance matrix of samples and OTU. The representative sequences of each OTU were selected based on their highest abundance and annotated against a species classification database to obtain a matrix of sample and species abundance. These matrices were used to perform various analyses, including alpha diversity analysis, beta diversity analysis, species composition analysis, difference significance analysis, and evolutionary analysis. Alpha diversity analysis focused on individual samples, involving the calculating of species diversity indices and the constructing of dilution curves. Beta diversity analysis was used to compare species composition similarities between samples and included cluster analysis (such as Hcluster) and ordinal analysis (such as PCA and NMDS).

Species composition analysis displayed the species composition and corresponding abundance information of samples. Significance difference analysis highlighted the significance differences between different samples or groups, various species, and their corresponding information. Additionally, evolutionary analysis and correlation studies were performed using sequence information and other sample data to conduct in-depth statistical analyses and visualizations.

Statistical analysis

The distribution of the data was assessed using the Shapiro-Wilk normality test, revealing that the intestinal microbiota data did not adhere to a normal distribution. Consequently, the data were presented using the median and interquartile range (IQR) and subjected to non-parametric tests. To compare differences among the three groups, the Kruskal-Wallis test was employed. In cases where the Kruskal-Wallis test yielded a significant result, post-hoc pairwise comparisons were conducted using the Mann-Whitney U test to assess specific group differences. All data were analyzed by IBM Statistical Package for the Social Sciences (SPSS), version 23 (IBM Corporation). $P<0.05$ was considered statistically significant.

Results

General clinical characteristics

The comparison of the general clinical characteristics among the β-lactam antibiotic group, non-antibiotic group and healthy group showed no significant differences in terms of age, gender, or mode of delivery ($P>0.05$). While there were significant differences in

feeding methods among the three groups, no significant difference was observed between the β-lactam antibiotic group and the non-antibiotic group ($P>0.05$) (Table 1). In the non-antibiotic group, one sample was excluded due to the poor quality of DNA extracted from the feces. In subsequent analyses, the number of samples in the non-antibiotic group group was 47.

Comparison of gut microbial diversity among the β-lactam antibiotic group, non-antibiotic group, and healthy group

The rarefaction curves reached a plateau, indicating that the sequencing depth was sufficient (Fig. 1A). The Shannon indexes, representing microbial alpha diversity and microbiota richness, showed no significant difference among the β-lactam antibiotic group, non-antibiotic group, and healthy group (Fig. 1B). Principal Component Analysis (PCA) (Fig. 1C) and Principal Coordinates Analysis (PCoA) (Fig. 1D), which represent microbial beta diversity, revealed distinct clustering patterns among the β-lactam antibiotic group, non-antibiotic group, and healthy group.

Comparative analysis of the bacterial composition of intestinal flora among the β-lactam antibiotic group, non-antibiotic group, and healthy group

Among the three groups, *Actinobacteria, Firmicutes, Proteobacteria*, and *Bacteroidetes* were the predominant phyla (Fig. 2A). There were no significant differences in the proportions of *Firmicutes, Proteobacteria*, and *Bacteroidetes* between the β-lactam antibiotic group and the other two groups (Fig. 2B, D, E). However, *Actinobacteria* were significantly lower in both the β-lactam antibiotic and non-antibiotic groups compared to the healthy group (Fig. 2C). Moreover, significant differences were observed in the phyla *Actinobacteriota, Campylobacterota* and *Cyanobacteria* among the three groups (all $P<0.01$) (Figure S1A). *Cyanobacteria* were more abundant in the β-lactam antibiotic group compared to the healthy group ($P<0.05$), whereas *Campylobacterota* and *Actinobacteriota* were more abundant in the healthy group compared to the β-lactam antibiotic group ($P<0.01$ and $P<0.001$, respectively) (Figure S1B). Compared to the non-antibiotic group, *Cyanobacteria* were more abundant in the β-lactam antibiotic group ($P<0.05$), while *Bacteroidota* and *Deinococcota* were more enriched in the non-antibiotic group (all with $P<0.05$) (Figure S1C).

At the genus level, the top 10 genera in each group were displayed in Fig. 3. *Klebsiella* was most abundance in the β-lactam antibiotic group, followed by the non-antibiotic group and the healthy group. Genera such as *Enterococcus, Streptococcus, Alistipes* and *Aeromonas* were more enriched in the β-lactam antibiotic group compared to the other groups, whereas *Escherichia-Shigella, Clostridium sensu stricto 1, Bifidobacterium* and *Parabacteroides* were less abundant (Fig. 4A and B).

Correlation analysis of the significant top ten genera

Linear regression correlation analysis was conducted on the 10 most significant genera. As shown in Fig. 5, the relative abundance of *Klebsiella* negatively correlated with *Escherichia-Shigella* ($R^2=0.1641$, $P<0.0001$) and positively correlated with *Enterobacter* ($R^2=0.04477$, $P=0.0084$). In addition, *Escherichia-Shigella* negatively correlated with *Enterococcus* ($R^2=0.02655$, $P=0.0435$) and *Streptococcus* ($R^2=0.02892$, $P=0.0350$). *Streptococcus* abundance was positively correlated with *Enterococcus* ($R^2=0.04412$, $P=0.0089$) and *Aeromonas* ($R^2=0.04344$, $P=0.0095$).

The influence of β-lactam antibiotics on the gut microbiome in neonates of varied age groups at the genus level

To analyze the impact of β-lactam antibiotics on the gut microbiome in different age groups of neonates, the groups were further divided into four age categories: ≤ 7 days, 8–14 days, 15–21 days, and 22–28 days. As

Table 1 Comparison of general information among the three groups

Category		Non-antibiotic ($n=48$)	β-lactam antibiotic ($n=67$)	Healthy ($n=16$)(40 samples)	χ^2/F	*P* value
Age	≤ 7d	12 (25)	17 (25.4)	10 (25)	0.999	0.310
	8-14d	11 (22.9)	18 (26.8)	10 (25)		
	15-21d	12 (25)	16 (23.9)	10 (25)		
	22-28d	13 (27.1)	16 (23.9)	10 (25)		
	Average age (d)	14.95 ± 8.44	14.19 ± 7.23	13.98 ± 8.40	0.198	0.820
Gender	Boy	19 (39.6)	39 (58.2)	9 (56.3)	4.073	0.131
	Girl	29 (60.4)	28 (41.8)	7 (43.7)		
Feeding methods	Breast	28 (58.4)	28 (41.8)	14 (87.5)	12.314	0.015
	Artificial	10 (20.8)	18 (26.9)	2 (12.5)		
	Mix	10 (20.8)	21 (31.3)	0 (0)	3.135*	0.209*
Delivery method	Cesarean section	18 (37.5)	26 (38.8)	6 (37.5)	0.024	0.988
	Vaginal delivery	30 (62.5)	41 (61.2)	10 (62.5)		

* The variance in feeding methods was compared between the β-lactam antibiotic group and the non-antibiotic group

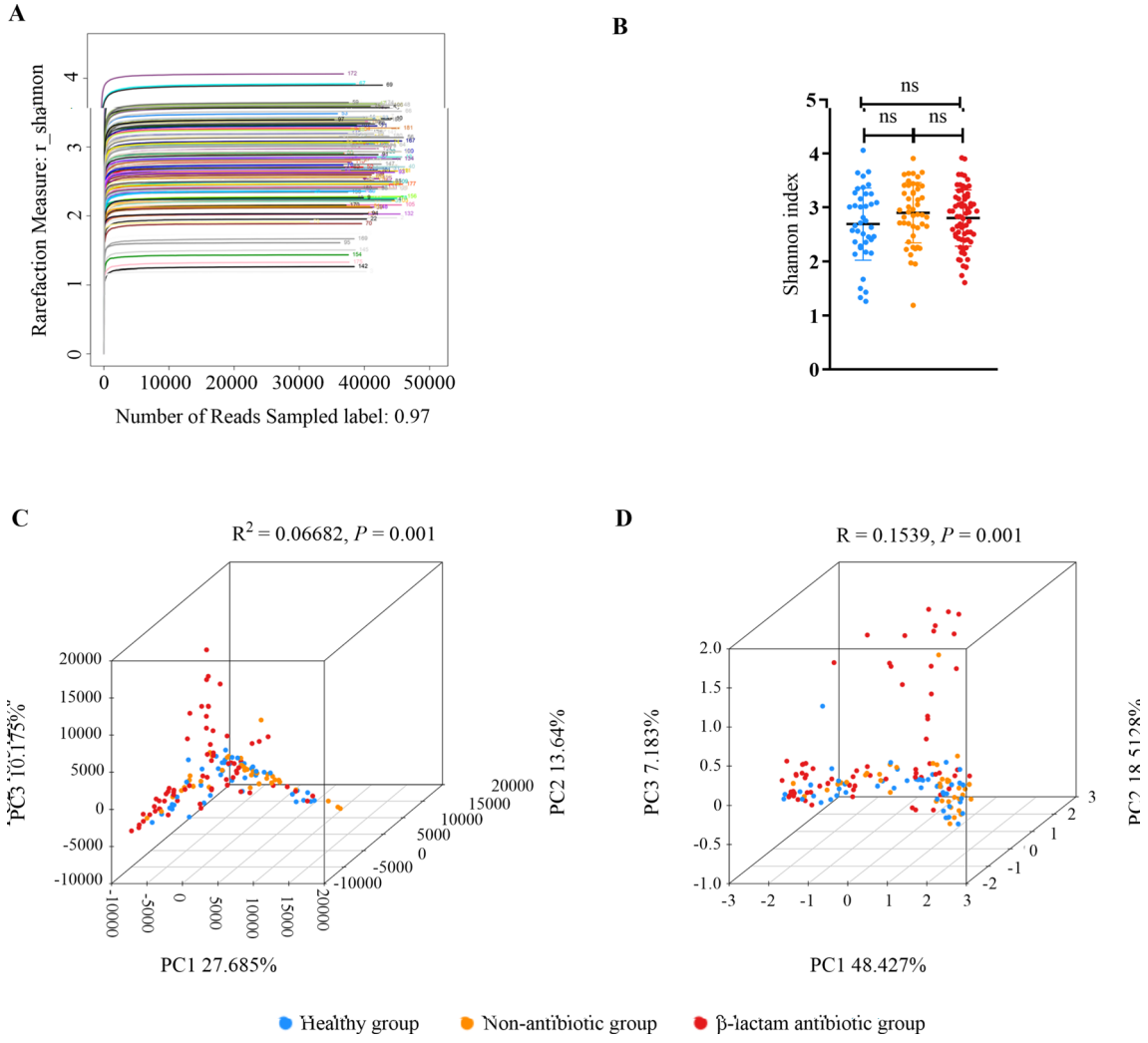

Fig. 1 Diversity of intestinal microbiota in β-lactam antibiotic, non-antibiotic, and healthy groups. **(A)** Rarefaction measure of Shannon diversity index; **(B)** Comparison of α diversity (Shannon index) of intestinal microbiota among β-lactam antibiotic, non-antibiotic, and healthy groups; **(C)** Principal component analysis of the intestinal microbiota among β-lactam antibiotic, non-antibiotic, and healthy groups. R^2 and *P* were calculated using ADONIS; **(D)** Principal coordinates analysis of the intestinal microbiota among β-lactam antibiotic, non-antibiotic, and healthy groups. R and *P* were calculated using ANOSIM.

shown in Fig. 6, the use of β-lactam antibiotics consistently induced the highest abundance of *Klebsiella* and *Enterococcus* across all age groups (Fig. 6A, B). *Streptococcus* abundance was highest in the β-lactam antibiotic group across all age groups, with significant differences observed in the ≤7 days and 8–14 days groups (Fig. 6C). *Alistipes* was most enriched in the β-lactam antibiotic group in the ≤7 days, 8–14 days and 15–21 days groups (Fig. 6D). Similarly, *Aeromonas* was highly enriched in the β-lactam antibiotic group in the ≤7 days, 8–14 days and 15–21 days group, but showed the least enrichment at 22–28 days (Fig. 6E). Conversely, the abundance of *Escherichia-Shigella* was lowest in β-lactam antibiotic group at 15–21 days, with no significant differences observed at other ages (Fig. 6F). *Clostridium sensu stricto 1* was relatively low in the β-lactam antibiotic group, particularly in the ≤7 days and 22–28 days groups (Fig. 6G). The abundance of *Bifidobacterium* significantly increased in the healthy group at 8–14 days, peaking at 22–28 days, while it increased slowly and remained significantly lower in the β-lactam antibiotic group at 22–28 days (Fig. 6I). Additionally, the abundance of *Parabacteroides* was significantly lower in the β-lactam antibiotic group compared to the healthy group at 15–21 days (Fig. 6J).

Impact of the duration of β-lactam antibiotic administration on the composition of intestinal microbiota

The duration of β-lactam antibiotic administration significantly affected the composition of intestinal microbiota. As presented in Fig. 7A, the abundance of *Lactobacillales_Unclassified, Ruminococcaceae_Unclassified, Granulicatella* and *Lawsonella* was highest in the 7 days group.

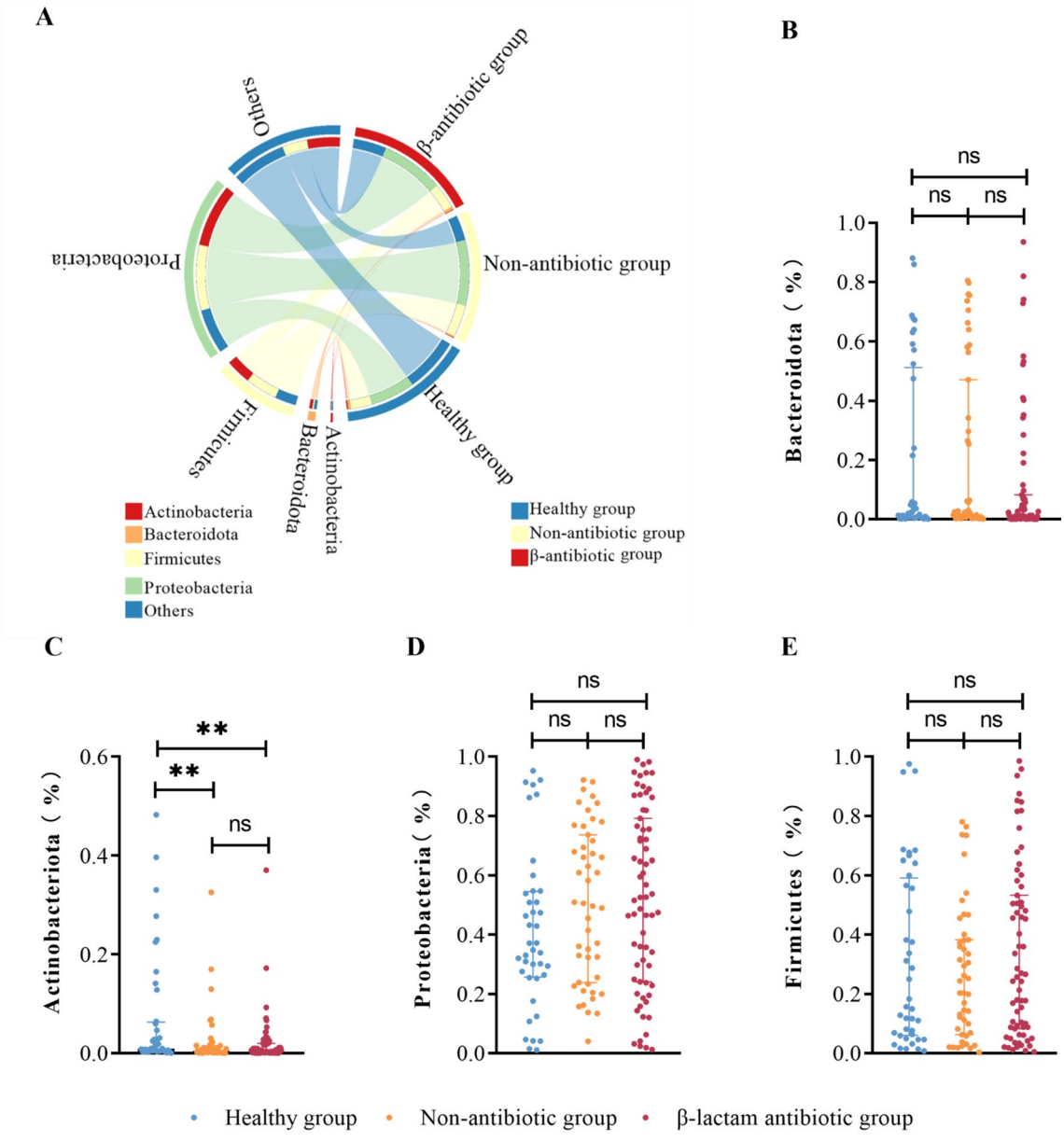

Fig. 2 Comparison of intestinal microbiota among β-lactam antibiotic, non-antibiotic, and healthy groups at the phylum level. **(A)** Composition of intestinal microbiota in phylum level; **(B ~ E)** comparison of intestinal microbiota in phylum level, including *Bacteroidota, Actinobacteriota, Proteobacteria* and *Firmicutes*, respectively. ns: non-significance; **, $P < 0.01$

In contrast, the abundance of *Serratia, Clostridiaceae_Unclassified, Lachnospiraceae_uncultured*, was highest in the 3 days group. Additionally, genera such as *Collinsella, UBA1819, Paenibacillus, [Ruminococcus] torques group, Amycolatopsis* and *Chloroplast_norank* were most enriched in the 5 days group.

The impact of different β-lactam antibiotic types on the gut microbiota

Different types of β-Lactam antibiotics exhibited no significant impact on the α-diversity and β-diversity of the gut microbiota, as shown by the Chao1 index and Shannon index for α-diversity (both $P > 0.05$), as well as PCA and PCOA for β-diversity (both $P > 0.05$). Similarly, there were no differences between the two groups at the phylum level ($P > 0.05$). However, compared to cefotaxime group, genera such as *Enterobacter, Citrobacter, Lachnospiraceae_Unclassified*, and *Staphylococcales_Unclassified* were more enriched in the ampicillin/sulbactam group ($P < 0.05$ and $P < 0.01$) (Fig. 7B).

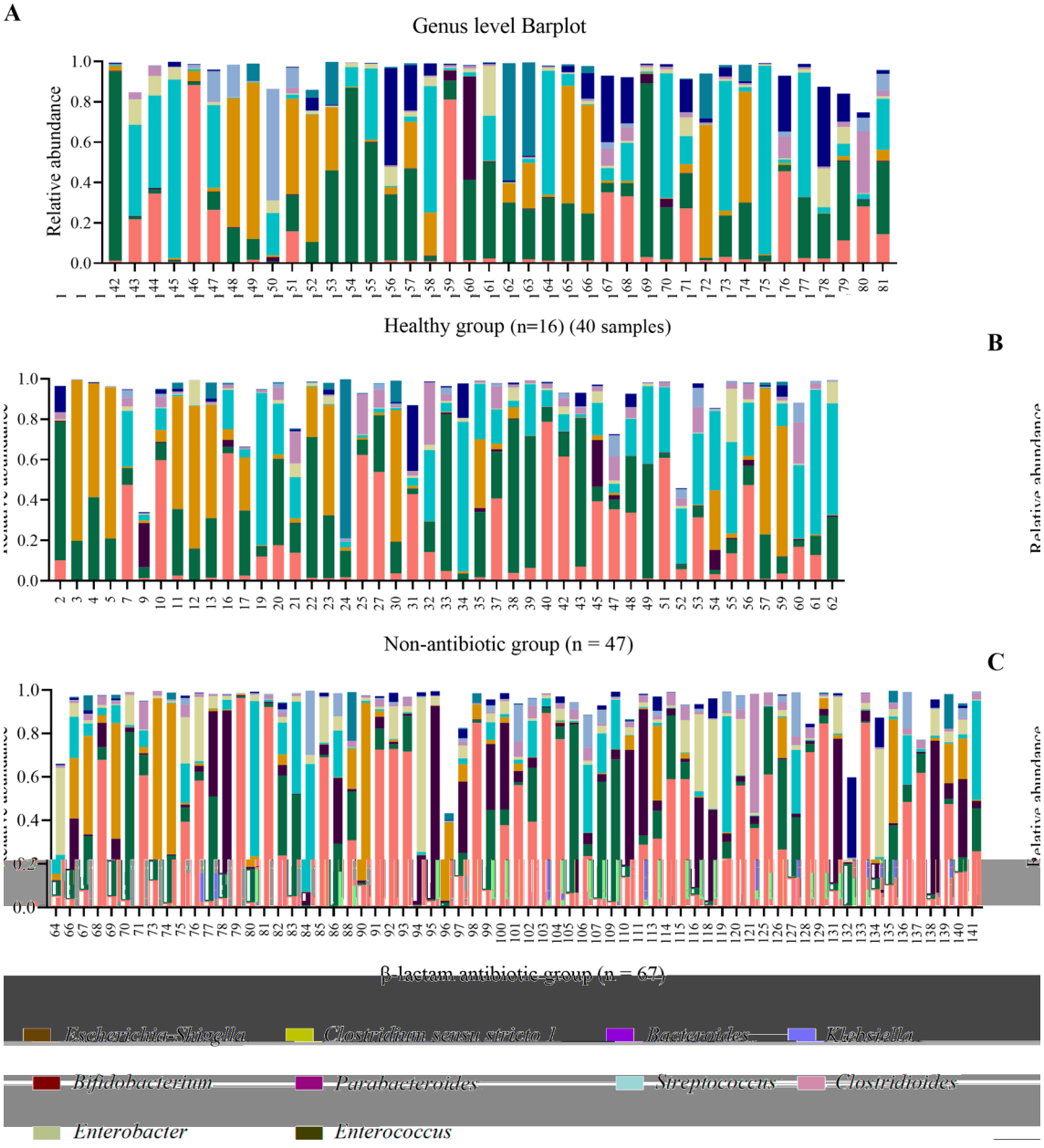

Fig. 3 Top ten genera composition of individual samples among β-lactam antibiotic, non-antibiotic, and healthy groups. **(A)** Top ten genera barplot for individual samples of the healthy group. **(B)** Top ten genera barplot for individual samples of the non-antibiotic group. **(C)** Top ten genera barplot for individual samples of the β-lactam antibiotic group. The healthy group consisted of 16 infants, and a total of 40 fecal samples were collected

The influence of delivery mode and/or feeding mode on the composition of the gut microbiota community

Different delivery mode did not affect the intestinal microbiota community at the phylum leve in the β-lactam antibiotic group ($P>0.05$). However, at the genus level, distinct microbial community structures were observed (Fig. 7C). Genera such as *Klebsiella, Enterobacteriaceae_Unclassified, Lactobacillales_Unclassified*, and *Pectobacterium* were significantly more abundant in the cesarean delivery group compared to in the vaginal delivery group (all $P<0.05$). Different feeding modes also significantly altered the intestinal microbiota community in the β-lactam antibiotic group. At the phylum level, *Cyanobacteria* varied significantly among breast feeding, artificial feeding and mixed feeding (Figure S2). At the genus level, *Methyloversatilis, Ruminococcus, Acidovorax*, and *Staphylococcales_Unclassified* were most enriched in the artificial feeding group compared to the other two groups. Conversely, *Novosphingobium* was most abundant in the breast-feeding group, while *Chloroplast_norank* was most enriched in the mixed feeding group (Fig. 7D).

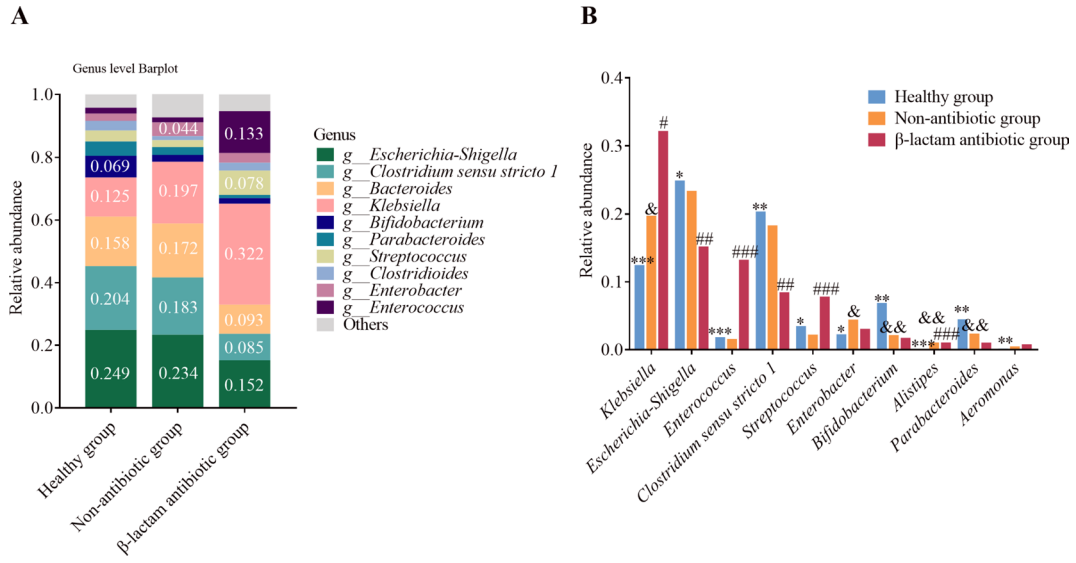

Fig. 4 Comparison of intestinal microbiota community among β-lactam antibiotic, non-antibiotic, and healthy groups at the genus level. **(A)** Top ten genera among β-lactam antibiotic, non-antibiotic, and healthy groups. **(B)** Top ten significant differences of genera among β-lactam antibiotic, non-antibiotic, and healthy groups. *, ** and ***, comparisons between the healthy group and β-lactam antibiotic group ($P<0.05$, $P<0.01$, and $P<0.001$, respectively); & and &&, comparisons between the healthy group and non-antibiotic group ($P<0.05$ and $P<0.01$, respectively); #, ##, and ###, comparisons between the non-antibiotic group and β-lactam antibiotic group ($P<0.05$, $P<0.01$, and $P<0.001$, respectively)

Discussion

β-lactam antibiotics are fundamental in treating most neonatal infections [13]. Among them, cefotaxime and ampicillin/sulbactam are two of the most commonly prescribed antibiotics for neonatal infections [14, 15]. However, antibiotic use is a double-edged sword: while it eliminates harmful pathogens, it also eradicates beneficial bacteria, disrupting the normal development of the gut microbiota. This disruption can lead to the overgrowth of opportunistic pathogens and increase the risk of disease onset [16–18]. The disruptive effect of antibiotics on the gut microbiota is more pronounced in neonates, especially within the first week [19]. Furthermore, the type and duration of antibiotic use have varying impacts on the gut microbiota [19, 20]. This study primarily investigates the effects of β-lactam antibiotics, specifically cefotaxime and ampicillin-sulbactam, on the gut microbiota of neonates. We also explored how these effects varied with neonates' ages and were influenced by different treatment durations, types of antibiotics, delivery modes, and feeding modes.

First, we observed that β-lactam antibiotic administration significantly affected the composition of the gut microbiota. Specifically, the top 5 genera showed significant alterations after β-lactam antibiotic use, with strongly increased proportions of *Klebsiella, Enterococcus* and *Streptococcus* and dramatically decreased *Escherichia-Shigella, Clostridium sensu stricto 1, Bifidobacterium* (Fig. 4). Our findings support an earlier investigation, which found that newborns exposed to antibiotics in their first week of life had higher levels of *Klebsiella* and *Enterococcus* than the control group [19].

Klebsiella and *Enterococcus*, potential pathogens colonizing in neonatal gut, are important organisms for neonatal late onset sepsis [21]. A recent global neonatal sepsis observational cohort study involving 3,195 infants (90.4% neonates aged < 28 days) revealed that of the 17.7% blood culture pathogen positive, *Klebsiella pneumoniae* was the most common pathogen, accounting for 4.1% [22]. Additionally, *Klebsiella* spp are frequently enriched in the gut microbiota of preterm neonates, with overgrowth associated with necrotizing enterocolitis (NEC), nosocomial infections and late-onset sepsis [23]. *Klebsiella*/*Enterococcus*-dominated fecal microbiota is linked to an increased risk of developing NEC in preterm infants [24]. *Klebsiella* spp, through Toll-like receptor-4 activation, induce the recruitment of pro-inflammatory T helper 17 cells, resulting in the release of pro-inflammatory cytokines (IL-17, IL-22), leading to erythrocyte death, mucosal injury, and bacterial translocation to the microvasculature beneath the intestinal epithelium [24, 25]. Therefore, the increased abundance of the *Klebsiella* and *Enterococcus* induced by β-lactam antibiotic administration may predispose individuals to late infections. On the other hand, *Streptococcus*, which also significantly increased after β-lactam antibiotic treatment, is a group of Gram-positive bacteria with the potential to cause severe infections associated with significant morbidity and mortality [12]. It has been reported that late-onset neonatal bloodstream infections can be caused by enteric bacteria, including *Streptococcus*, which commonly

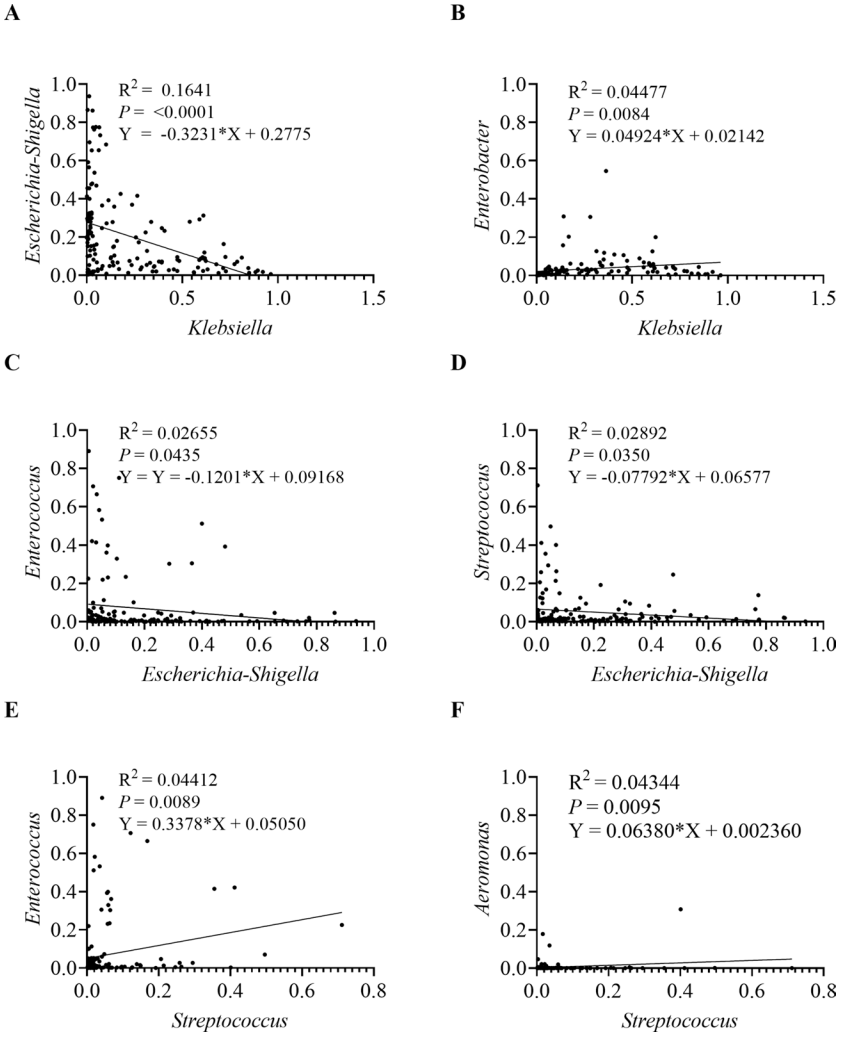

Fig. 5 The relationship between genera shown with scatter diagram. **(A)** Relationship between *Klebsiella* and *Escherichia-Shigella*. **(B)** Relationship between *Klebsiella* and Enterobacter. **(C)** Relationship between *Escherichia-Shigella* and *Enterococcus*. **(D)** Relationship between *Escherichia-Shigella* and *Streptococcus*. **(E)** Relationship between *Streptococcus* and *Enterococcus*. **(F)** Relationship between *Streptococcus* and *Aeromonas*

resides within the mucosal lining of the intestinal tract and can disseminate to various organs, resulting in severe infections [26, 27]. Therefore, the overgrowth of *Streptococcus* in the intestine after β-lactam antibiotic treatment may increase the risk for subsequent infection, such as late-onset sepsis. Our results are largely consistent with previous findings, which showed that empirical antibiotic therapy increased harmful bacteria such as *Streptococcus* and *Pseudomonas* in preterm infants [12]. However, one inconsistency is that in our study, antibiotic use led to an increase in *Streptococcus* abundance, whereas in previous research found a decrease [28]. These variations may be due to differences in the sensitivity of *Streptococcus* to different β-lactam antibiotics or their resistance mechanisms. Specifically, previous studies used amoxicillin/ceftazidime [28] and a combination of penicillin and moxalactam or piperacillin-tazobactam [12], while our study used cefotaxime or ampicillin/sulbactam. Additionally, β-lactam antibiotic administration significantly reduced the abundance of genera such as *Escherichia-Shigella, Clostridium sensu stricto 1*, and *Bifidobacterium*. In term infants, an initially aerobic environment primarily hosts aerobes and facultative anaerobes, such as *Escherichia, enterococci, Enterobacteriaceae, Staphylococcus*, and *Streptococcus species* [29, 30]. As gut luminal oxygen levels rapidly fall due to consumption by these bacteria and secretory immunoglobulin A, strict anaerobes like *Bifidobacterium* and *Clostridium* proliferate [31]. However, antibiotic use significantly decreases the abundance of enteric anaerobic bacteria, including *Bifidobacterium, enterobacteria and clostridia* [19, 32]. Cefotaxime and ampicillin/sulbactam, broad-spectrum β-lactam antibiotics targeting Gram-positive and -negative bacteria, significantly impact gut microbiota composition. *Bifidobacterium* species are particularly sensitive to β-lactam antibiotics, and treatment

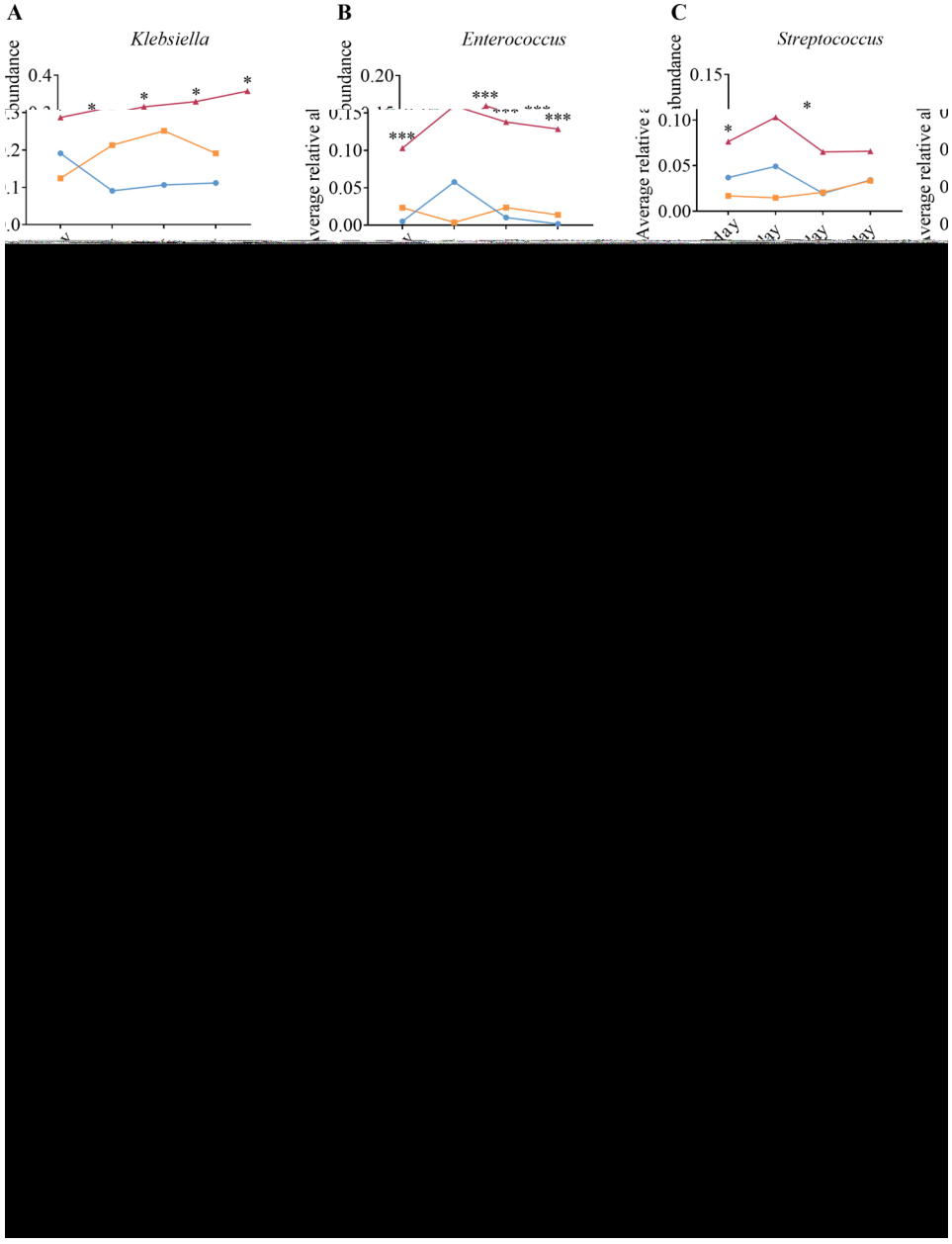

Fig. 6 Comparison of intestinal microbiota community among β-lactam antibiotic, non-antibiotic, and healthy groups at the genus level at different neonatal ages. Comparison the abundance of *Klebsiella* **(A)**, *Enterococcus* **(B)**, *Streptococcus* **(C)**, *Alistipes* **(D)**, *Aeromonas* **(E)**, *Escherichia-Shigella* **(F)**, *Clostridium sensu stricto 1* **(G)**, *Enterobacter* **(H)**, *Bifidobacterium* **(I)** and *Parabacteroides* **(J)** among β-lactam antibiotic, non-antibiotic, and healthy groups. *, **, and ***, comparisons among β-lactam antibiotic, non-antibiotic, and healthy groups, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively

with amoxicillin can greatly influence their composition in infant intestinal microbiota [4, 33]. Moreover, intravenous antibiotic combinations, such as penicillin+gentamicin, co-amoxiclav+gentamicin or amoxicillin+cefotaxime, significantly decreased the abundance of *Bifidobacterium*, and suggesting that antibiotic treatment may directly eliminate these genera [19]. Therefore, the deceased proportion of *Bifidobacterium* after cefotaxime or ampicillin/sulbactam treatment in the present study may be due to their direct elimination. However, the specific species affected by these treatments remain unknown and warrant further investigation. We also found that the overgrowth of *Klebsiella, Enterococcus* and *Streptococcus* may inhibit the growth of *Escherichia-Shigella*, as their relative abundances were negatively correlated (Fig. 5A, C, D). Conversely, certain bacteria exhibit synergistic growth effects, such as *Klebsiella* with *Enterobacter, Enterococcus* with *Streptococcus*, and *Aeromonas* with *Streptococcus*, which show positive correlations in abundance (Fig. 5B, E, F). Bacteria can inhibit

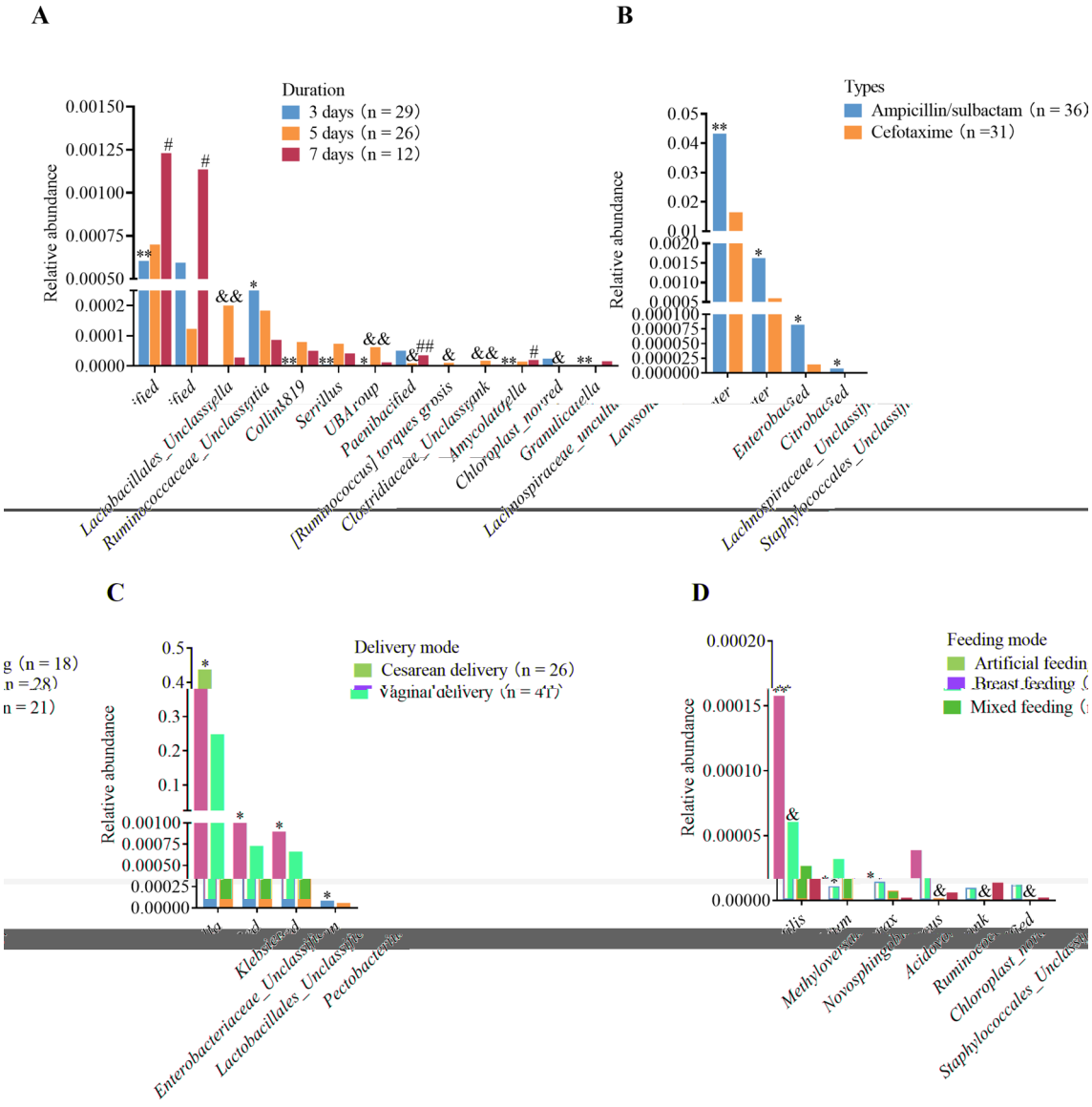

Fig. 7 Comparison of the intestinal bacterial community of the β-lactam antibiotic group in terms of duration, types, delivery mode and feeding mode. Comparison of the intestinal bacterial community of the β-lactam antibiotic group in terms of duration **(A)**. types **(B)**, delivery mode **(C)** and feeding mode **(D)**

each other's growth through mechanisms such as spatial and nutritional competition [34]. For example, *Klebsiella pneumoniae* can produce bacteriocins with antimicrobial effects against closely related species [35]. Furthermore, *Klebsiella* can use the type VI secretion system to secrete and inject, killing surrounding bacteria and aiding in colonization [36]. On the other hand, the positive correlation between microbiota could result from competition for shared resources and nutrients in similar ecological niches or the possibility of a mutualistic relationship that promotes their growth [37]. Certainly, further research is necessary to clarify the potential mechanisms of interactions between microbial communities.

The composition of neonatal gut microbiota, influenced by antibiotic treatment, is determined by various factors, including the timing, duration, and specific type of antibiotics administered [10]. Our study assessed the effects of β-lactam antibiotic treatment on neonatal intestinal microbiota across specific age ranges: ≤ 7 days, 8–14 days, 15–21 days, and 22–28 days. Remarkably, we observed a significant increase in the abundance of *Klebsiella* and *Enterococcus* following β-lactam antibiotic treatment at all four stages compared to the healthy group. Our findings were consistent with a previous study where 147 infants born at ≥36 weeks of gestational age received intravenous antibiotic treatment (penicillin+gentamicin, co-amoxiclav+gentamicin or

amoxicillin+cefotaxime) in the first week of life, resulting in increased abundance of *Klebsiella* and *Enterococcus* spp and decreased abundance of *Bifidobacterium* spp [19]. During the first days of life, the gut microbiota primarily consists of aerobic/facultative anaerobic bacteria belonging to the phyla *Proteobacteria* (e.g., *Enterococcus spp*) and *Firmicutes* (e.g., *Staphylococcus*, and *Streptococcus*) [31, 38]. The abundance of these facultative bacterial taxa decreases rapidly due to oxygen consumption and intestinal secretory immunoglobulin A, along with the expansion of anaerobic bacteria such as *Bifidobacterium* and *Clostridium* during the first months of life [31]. However, antibiotic use, one the most disruptive factors for neonatal gut microbiota development, strongly interferes with normal gut microbiota development at every neonatal period [39]. Our research provides more detailed insights into the influence of antibiotic usage on neonates of varying age groups, indicating that antibiotic use in neonates at any age can substantially impact their gut microbiota.

Additionally, the duration of antibiotic treatment significantly affects the structure of enteric microbiota. Rooney et al. suggested that 1 week of discontinuation of antibiotic treatment, each additional day of antibiotics was associated with lower richness of obligate anaerobes [20]. Zwittink et al. observed a significant reduction in *Bifidobacterium* levels in preterm infants (35±1 week's gestation) following short (≤3 days) or long (≥5 days) antibiotic treatment, which persisted until the third week after birth (P=0.028). For long antibiotic treatments, this reduction continued until the sixth postnatal week (P=0.009) [40]. These studies, along with our findings, suggest that longer durations of antibiotic use have a greater impact on the gut microbiota. Simultaneously, prolonged antibiotic treatment led to the emergence and overgrowth of antibiotic-resistant microbes [16, 41]. Therefore, it is crucial to minimize the duration of antibiotic use as much as possible while treating neonatal infections.

Moreover, the choice of antibiotics administered to neonates can distinctly affect the composition of their intestinal microbiota. We found that ampicillin/sulbactam significantly increased the richness of *Enterobacter, Citrobacter, Lachnospiraceae_Unclassified*, and *Staphylococcales_Unclassified* compared to cefotaxime, indicating that the impact of each antibiotic on neonatal microbiota is not uniform. This suggests that ampicillin/sulbactam may be more harmful to neonatal microbiota due to the enrichment of opportunistic pathogen [42]. A previous study revealed that broad-spectrum antibiotics for suspected early-onset neonatal sepsis, such as amoxicillin+cefotaxime, had the largest effects on microbial community composition and antimicrobial resistance gene profiles, whereas penicillin+gentamicin exhibited the least effects [19]. Antibiotic treatment, especially with broad-spectrum antibiotics, disrupts the gut microbiota and colonization resistance [43]. The choice of antimicrobials for neonatal infection depends on the most frequent causative microorganisms and is often empirical until culture results and antibiograms are available [44]. Ampicillin and gentamicin are the WHO's first-line regimen for empiric antibiotic combinations, with cefotaxime as the second-line regimen [22]. Adding a β-lactamase inhibitor like sulbactam to β-lactam antibiotic like ampicillin can broaden the spectrum of activity to cover gram-negative extended-spectrum β-lactamase producers [45]. However, we found that ampicillin/sulbactam led to an increase in the richness of *Enterobacter, Citrobacter.* We speculated that its effect may be associated with the increased antibiotic resistance microbiota, but the exact mechanisms need further investigation.

Delivery mode significantly affects the colonization and development of neonatal intestinal microbiota [9, 46, 47]. Compared to vaginally delivered infants, those born by cesarean section showed decreased relative abundance of *Bacteroides* and *Parabacteroides* and enrichment of *Clostridium_sensu_stricto_1, Enterococcus, Klebsiella, Clostridioides*, and *Veillonella* [9]. We further found that, compared to vaginal delivery, β-lactam antibiotic treatment in cesarean-delivered infants further increased the abundance of *Klebsiella, Enterobacteriaceae_Unclassified, Lactobacillales_Unclassified* and *Pectobacterium* (Fig. 7C), indicating that β-lactam antibiotics seem to exacerbate intestinal flora disturbance caused by cesarean section. In addition, feeding mode also significantly affects neonatal intestinal composition [48, 49]. However, it did not alter the overall adverse effects of antibiotic use on intestinal flora, as the abundance of the main genera influenced by β-lactam antibiotic treatment, such as *Klebsiella, Enterococcus* and *Streptococcus*, showed no significant differences among artificial feeding, breast feeding, and mixed feeding groups. Only small proportion of generadiffered (Fig. 7D). These results suggest that regardless of previous feeding patterns, β-lactam antibiotic treatment significantly impacts the compositions of neonatal microbiota. Van Daele et al. indicated that antibiotic exposure in the first week perturbated fecal microbiota of term infants, with the perturbation still notable at one month in formula-fed infants, but only until two weeks in breast-fed infants [50]. The results suggests that breast feeding can help restore dysbiosis. Mechanically, breastmilk is abundant in bioactive components, including human milk oligosaccharides, immune cells, lactoferrin, cytokines, antibodies, and antimicrobial proteins and peptides, which aids restoration by stimulating the growth of bifidobacteria and reducing (potential) pathogens [29, 51]. A recent study showed that breast feeding and antibiotics have opposing effects

on the infant microbiome, and that breast feeding enrichment of *Bifidobacterium longum subsp. infants* is associated with reduced antibiotic-associated asthma risk [52]. Taken together, while breast feeding may not prevent the adverse effects of antibiotic on the gut microbiota, it can help restore gut microbiota after antibiotic treatment.

Notable, the study has several limitations. It is limited to examining the effects of ampicillin/sulbactam and cefotaxime, preventing conclusions about which antibiotic treatment causes the least ecological damage or has the shortest duration of impact on the gut microbiome. Future research should explore a broader range of antibiotics to determine the therapies with minimal ecological impact.

Conclusions

In conclusion, our study demonstrates that β-lactam antibiotics treatment for neonatal pneumonia and sepsis significantly disrupts the diversity and composition of the neonatal intestinal microbiota. This disruption is characterized by an increased abundance of genera such as *Klebsiella, Enterococcus, Streptococcus, Alistipes* and *Aeromonas*, and a deceased abundance of genera such as *Escherichia-Shigella, Clostridium sensu stricto 1, Bifidobacterium* and *Parabacteroides.* The use β-lactam antibiotics at any time in newborns can impact the gut microbiota, with the effects determined by the duration and type of β-lactam antibiotics used. β-lactam antibiotic treatment appears to further exacerbate intestinal flora disturbances caused by cesarean section. Additionally, although breast feeding before β-lactam antibiotic administration does not prevent the dysbiosis induced by these antibiotics, it can help restore gut microbiota after antibiotic treatment.

Abbreviations

IQR	Interquartile Range
PCA	Principal Component Analysis
PCoA	Principal Coordinates Analysis
OTU	Operational Taxonomic Unit
NEC	Necrotizing enterocolitis

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Author contributions

MJ and HG designed and conducted the investigation. HG and ET wrote the original manuscript. HG, YF, GL and XJ collected the samples. MJ, HG, and ET revised the manuscript. HG, ET, YF, XJ, TY, LC, XS, and WZ performed the bioinformatics and statistical data analysis. All authors have read and approved the submitted version.

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Data availability

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA1062023.

Declarations

Ethical approval

The study protocol was approved by the Ethics Committee of the Children's Hospital, Zhejiang University School of Medicine (Ethics Approval Number: 2018-IRB-103). The study has been officially registered with the China Clinical Trials Registry (Registration Number: ChiCTR1900021448, Registration Date: February 21, 2019). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin in compliance with the Declaration of Helsinki.

Competing interests

The authors declare no competing interests.

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