Longitudinal Analysis of Oral Microbiome Changes at Early Stages in Full Term and Preterm Newborns

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Longitudinal Analysis of Oral Microbiome Changes at Early Stages in Full Term and Preterm Newborns

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마지막으로, 언제나 변함없이 저를 믿어 주시고 아낌없는 사랑과 응원을 보내주신 부모님 (아버지 이석길님, 어머니 최명희님) 그리고 하나뿐인 동생 이상백님께 진심으로 감사의 말을 전합니다. 지난 세월 부모님의 헌신과 지지가 없었다면 오늘의 제가 있을 수 없었음을 고백합니다. 부모님께서 가르쳐 주신 사랑과 가치를 기억하며 앞으로도 바르게 살아가겠습니다.

부족한 제 능력보다 넘치게 받은 사랑과 가르침을 항상 기억하며, 이를 베풀고 겸손히 살아가겠습니다. 감사합니다.

2024년 12월

이태양 드림

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ABSTRACT

Longitudinal Analysis of Oral Microbiome Changes at Early Stages in Full term and Preterm Newborns

The neonatal period is critical for establishing the oral microbiome, which has significant implications for long-term health outcomes. Preterm newborns are particularly vulnerable to delayed microbial stabilization and dysbiosis, increasing their susceptibility to infections. However, the development of the oral microbiome in preterm newborns has not been fully investigated. This study aimed to compare the temporal dynamics of the oral microbiome in full term and preterm newborns and identify the key clinical and environmental factors influencing microbial development.

The study included 23 full term and 75 preterm newborns. Oral swab samples were collected on days 0 and 2 for full term newborns and on days 0, 7, and 28 for preterm newborns. The microbial composition and diversity were assessed using 16S rRNA gene sequencing. Key factors, such as gestational age, birthweight, delivery mode, feeding type, and antibiotic use, were evaluated for their impact on the oral microbiome.

Preterm newborns exhibited delayed microbial stabilization compared to full term newborns, with a prolonged predominance of *Proteobacteria* and late colonization by *Firmicutes* and *Actinobacteria*. Full term newborns rapidly transitioned to a balanced microbiota dominated by *Firmicutes*, with *Streptococcus pneumoniae* emerging as the dominant pathogen by day 2. In contrast, preterm newborns showed persistent dominance of *Escherichia coli* and *Ralstonia pickettii* on day 0, followed by *Serratia marcescens*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* on days 7 and 28. Breastmilk feeding enhanced microbial diversity and stability, whereas cesarean delivery was associated with delayed stabilization and increased dysbiosis. Functional analysis identified virulence-related genes, such as *PspA*, *nanA*, *hla*, and *scpA*, as contributors to pathogenicity in preterm newborns.

This study suggested that gestational age, birthweight, delivery mode, feeding type, and antibiotic use may influence the development of the newborn oral microbiome. The delayed microbial transitions and heightened infection susceptibility in preterm newborns emphasize the need for targeted interventions. Longitudinal studies are warranted to explore the long-term health implications of early oral microbiome development.

Key words: Newborn, Full term, Preterm, Oral microbiome, Microbial diversity, Neonatal health, Microbiota stabilization, Longitudinal study

1. Introduction

The World Health Organization (WHO) defines a baby born before full term (before 37 weeks of gestation) as a preterm newborn. The incidence of preterm births has been steadily rising worldwide. A recent WHO report stated that approximately 9.9% of births are globally preterm, whereas in the East Asia region, 6.8% of babies are born before 37 weeks of pregnancy (Ohuma et al., 2023). The increase in preterm births is attributed to various factors, including preterm labor, premature rupture of membranes, maternal age, genetic predisposition, and infections (Stephansson, Dickman, Johansson, & Cnattingius, 2001). Although advancements in neonatal care have significantly improved the survival rates of preterm newborns, the incidence of various complications has also increased (Raju et al., 2017). Consequently, the importance of comprehensive medical and dental interventions for preterm newborns has been increasingly emphasized (Stoll et al., 2010).

In the early stages of life, the microbiome, defined as the collection of microorganisms and their genomes residing in and on the human body, begins to establish itself (Dominguez-Bello et al., 2010). In particular, neonatal health is severely impacted during the first few weeks of life, when the immune system and various body systems develop rapidly (Lawn et al., 2014). The neonatal period is a critical window for microbiome development, with early microbial colonization influencing immune system maturation, metabolic function, and long-term health outcomes (Kim et al., 2024). Therefore, understanding the oral microbiome composition and its changes in preterm newborns is of great importance because the early composition and development of the oral microbiome may have a significant impact on health outcomes in the first weeks and months of life, especially in preterm newborns (Pammi et al., 2017). Key factors, such as the early postnatal environment, antibiotic use, and nutritional intake methods, can influence the oral microbiome in preterm newborns (Kim et al., 2024).

An imbalance in the microbiome of preterm newborns can increase vulnerability to infections and long-term health complications (Pammi & Weisman, 2015). One of the critical aspects of preterm newborn health is their microbiome, which forms an ecosystem composed of various microorganisms, such as bacteria, viruses, fungi, and archaea, primarily concentrated in areas such as the skin, mouth, intestines, and genitals (Cho & Blaser, 2012). An imbalance in the oral microbiome can be related to not only oral diseases such as periodontitis and stomatitis but also systemic health problems such as respiratory

infections and systemic inflammatory responses (Fardini, Chung, Dumm, Joshi, & Han, 2010). Therefore, the oral microbiome plays a key role in oral health and in systemic health.

Previous studies have explored various aspects of neonatal microbiome development, focusing on how gestational age, delivery mode, and feeding practices influence microbial colonization. Research has shown that preterm newborns exhibit delayed and altered microbiota development compared to full term newborns, with lower microbial diversity and a predominance of potentially pathogenic taxa (Arrieta, Stiemsma, Amenyogbe, Brown, & Finlay, 2014; Chernikova et al., 2018). Studies on the gut microbiota of preterm newborns have linked dysbiosis to adverse outcomes such as necrotizing enterocolitis (NEC) and sepsis (Pammi et al., 2017). Similarly, the role of the delivery mode has been extensively studied, with vaginally delivered newborns acquiring maternal vaginal microbiota and infants delivered by cesarean section retaining a colony of microbes present on their mothers' skin (Dominguez-Bello et al., 2010). Although much of the neonatal microbiome research has focused on the gut, studies on the oral microbiome remain limited. Previous studies have indicated that the oral cavity is an important microbial reservoir that can influence respiratory and gastrointestinal health (Aas, Paster, Stokes, & Dewhirst, 2005; Bhandary et al., 2024; Selway et al., 2023). However, the temporal diversity changes of the oral microbiota and the related factors have not been studied, especially in preterm newborns. Although the impact of antibiotics on the gut microbiota is well-documented (Elvers et al., 2020), their effects on the oral microbiome in newborns have received little attention.

Despite the growing interest in newborn microbiome research, the dynamics of oral microbial communities during the first month of life remain underexplored, particularly in preterm newborns. Moreover, no comprehensive studies have systematically compared the oral microbiome development in full term and preterm newborns while considering the influence of clinical factors, such as gestational age, feeding practices, and antibiotic exposure. These reasons highlight the need for a detailed investigation of the oral microbiome of preterm and full term newborns.

This study investigated the temporal dynamics of oral microbiota in full term and preterm newborns, focusing on the effects of gestational age, birthweight, feeding practices, delivery mode, and antibiotic use. In addition, this study aimed to identify the factors influencing microbiome development and assess their impact on early health outcomes.

2. Materials and Methods

2.1. Study population

This study was approved by the Institutional Review Board of Yonsei University Dental Hospital (IRB No. 2-2021-0091) and the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine (IRB No. 4-2021-0419). The study was conducted in accordance with institutional guidelines.

The study included 23 full term and 75 preterm newborns admitted to the neonatal ward or the neonatal intensive care unit (NICU) of Severance Hospital. Inclusion criteria were as follows: healthy neonates born at Severance Hospital at a gestational age of 37 weeks and preterm newborns admitted to the NICU or neonatal ward due to a gestational age of 32 weeks. Exclusion criteria included neonates with congenital anomalies or other disabilities diagnosed during the preterm newborn screening test and high-risk neonates with a low probability of surviving beyond 1 month.

All study participants consented to the collection of oral samples and medical records from the children and mothers (Table 1).

Table 1. Demographic distribution of full term and preterm newborns

		Full term (n = 23)	Preterm (n = 75)
Sex	Male	10	43
	Female	13	32
Gestational age	≥37	23	
	<28		21
	<32		40
	<37		14
Birthweight	Normal	23	1
	<1,000 g		26
	<1,500 g		39
	<2,500 g		9
Fetal polymorphism	Singletons	19	49
	Twins	4	26
Advanced maternal age	>35	14	33
	≤35	9	42
Pregnancy method	Natural	15	42
	Artificial	8	33
Delivery mode	NSVD	4	2
	C/S	19	73
Oral feeding*	No	0	63
	Yes	23	12
Feeding type	NPO	1	8
	Breastmilk	1	0
	Formula	18	61
	Breastmilk + Formula	3	6
Oral medication	No	19	30
	Yes	4	45
Oral antibiotics	No	11	8
	Yes	12	67

Abbreviations: NPO, nothing per oral; NSVD, normal spontaneous vaginal delivery; C/S, cesarean section.

*Since it is not possible to determine immediately after birth whether oral feeding is provided, its type, or whether medication and antibiotics are administered, data for full term newborns were collected based on their status 2 days after birth, whereas data for preterm newborns were collected based on their status 7 days after birth.

2.2. Oral sample collection

Oral swab samples were collected from newborns in collaboration with the Division of Neonatology at Severance Children's Hospital. Specimens from full term newborns were collected on the day of birth [day 0 (D0)] and within 48 h [day 2 (D2)]. For preterm newborns, specimens were collected on D0, D7, and D28 after birth. For full term newborns, samples were collected on D0 and D2 because they are typically discharged from the hospital within 48 h. Given that deciduous teeth do not begin to erupt until approximately 6 months of age, swabs were taken from the oral mucosa (Figure 1A). Specifically, the posterior region of the tongue, buccal mucosa, alveolar ridge, and palate were gently swabbed using a sterile cotton swab. The swabs were placed into a swab collection kit (OMNIgene·ORAL OMR-110, DNA Genotek, Inc., Ontario, Canada; Figure 1B). The collection timeline highlighted the differences in sampling schedules between full term and preterm newborns, with additional sampling in preterm newborns at later stages to capture developmental events (Figure 1C).

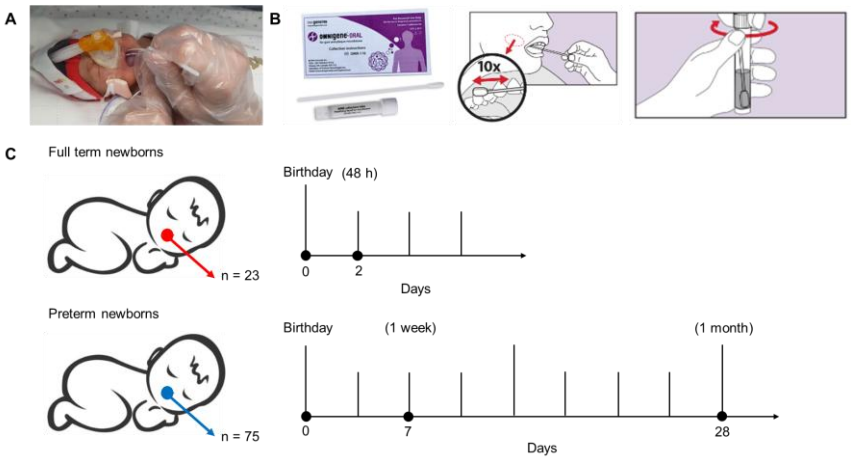

Figure 1. Sampling timeline and methods for oral tissue collection in full Term and preterm newborns. (A) Procedure for collecting oral mucosa swabs from newborns. (B) Instructions to use the OMR-110 kit. The collected swabs were stored in the kit for preservation. Instructions adapted from the manufacturer's guide (DNA Genotek Inc., Ontario, Canada). (C) Time points for collecting oral swabs from full term and preterm newborns. Filled circles (●) indicate the sampling time points. Full term: Samples were collected on D0 and D2; Preterm: Samples were collected on D0, D7, and D28

2.3. Variables

Oral microbiome diversity in full term and preterm newborns was evaluated in relation to several perinatal and neonatal factors. These factors included gestational age (<28, <32, <37, and ≥37 weeks), birthweight (<1,000, <1,500, <2,500, and ≥2,500 g), feeding type (breastmilk, formula, breastmilk + formula, or nothing per oral [NPO]), fetal polymorphism (singletons vs. twins), advanced maternal age (<35 vs. ≥35 years), pregnancy method (artificial vs. natural), and mode of delivery (cesarean section[C/S] vs. normal spontaneous vaginal delivery [NSVD]).

Medications for preterm newborns were evaluated based on whether they were administered orally or not. The medications assessed included ubacillin, claforan, meropenem, oneflu, penbrex, and ceftazidime.

2.4. DNA extraction, PCR amplification, and sequencing

Total DNA was extracted using the FastDNA Spin kit (MP Biomedicals, Solon, OH, USA) in accordance with the manufacturer's instructions.

Polymerase Chain Reaction (PCR) amplification was performed using primers targeting the V3–V4 regions of the 16S rRNA gene with extracted DNA. Amplification was carried out under the following conditions: initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final elongation at 72°C for 5 min. Secondary amplification for attaching the Illumina NexTera barcode was performed. The condition of secondary amplification was equal to the former, except that the amplification cycle was set to 8.

The PCR product was confirmed using 1% agarose gel electrophoresis and visualized under a Gel Doc system (Bio-Rad, Hercules, CA, USA). The amplified products were purified by magnetic bead-based cleanup. The proper concentrations of the purified products were pooled together, and short fragments (nontarget products) were removed with the ProNex® Size-Selective Purification System (Promega, Southampton, England).

The quality of the products was determined by the PicoGreen assay (Molecular Probes, Invitrogen, USA). Mixed amplicons were pooled, and sequencing was performed at CJ Bioscience, Inc. (Seoul, Korea), with the Illumina MiSeq Sequencing system (Illumina, USA) according to the manufacturer's instructions.

2.5. Data analysis pipeline

raw reads started with a quality check and the filtering of low-quality (<Q25) reads by Trimmomatic version 0.32 (Bolger, Lohse, & Usadel, 2014). After the quality-control pass, filtering and clustering were performed through the EzBioCloud database. The secondary analysis, which included diversity calculation and biomarker discovery, was conducted by in-house programs of CJ Bioscience. The α-diversity indices [ACE (Chao & Lee, 1992), Chao (Hamady, Lozupone, & Knight, 2010), Jackknife (Burnham & Overton, 1979), Shannon (Magurran, 2003), NP Shannon (Chao & Shen, 2003), Simpson (Magurran, 2003) and phylogenetic diversity (Faith, 1992)], rarefaction curves (Heck, van Belle, & Simberloff, 1975), and rank abundance curves (Whittacker, 1965) were estimated. To visualize the sample differences, β-diversity distances were calculated by several algorithms [Jensen-Shannon (Lin, 1991), Bray-Curtis (Beals, 1984), generalized UniFrac (Chen et al., 2012), and Fast UniFrac (Hamady, Lozupone, & Knight, 2010)]. With functional profiles predicted by PICRUSt (Ye & Doak, 2009) and MinPath (Langille et al., 2013) algorithms, taxonomic biomarkers and functional biomarkers were discovered by statistical comparison algorithms [linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011) and Kruskal-Wallis H test (Kruskal & Wallis, 1952)]. All the above-mentioned analyses were performed in EzBioCloud16S-based MTP, which is CJ Bioscience's bioinformatics cloud platform.

2.6. Data sequence processing and ASV table construction

Raw paired end reads from the Illumina sequencer were processed using the dada2 R package version 1.32.0 (Callahan et al., 2016). In brief, the reads were trimmed and filtered using the filterAndTrim function. Reads were filtered to remove those with >4 (forward) and 6 (reverse) expected errors based on the quality scores and truncated after 280 (forward) and 260 (reverse) bp. The dada2 algorithm was applied to infer the exact amplicon sequence variants (ASVs) from the process. Taxonomy was assigned to the ASVs using a naive Bayesian classifier method against the Expanded Human Oral Microbiome Database (eHOMD version 15.1) with a minimum bootstrap confidence of 50. An ASV table was constructed by recording the number of times each ASV was observed in each sample.

2.7. Microbiome analysis with phyloseq

The ASV table, sample data, and taxonomy table were imported into the R environment as a phyloseq object using the phyloseq R package (version 1.48.0; McMurdie & Holmes, 2013). ASVs with no phylum assigned were filtered out and agglomerated at the species level. α- and β-Diversity metrics were calculated using the estimate_richness and ordinate functions of the phyloseq package, respectively.

LEfSe was performed using the microbiomeMarker R package (version 1.10.0; Cao et al., 2022). Heatmaps were generated using the ComplexHeatmap package (version 2.20.0; Gu, 2022), and all other plots were created with the ggplot2 package (version 3.5.1).

2.8. Statistical analysis

Multivariate dispersion analysis and pairwise permutation-based analysis of variance for β-diversity were conducted using the vegan R package (version 2.6-8). A pairwise *t*-test for samples showed statistically significant differences. The minimum significance level for all tests was set at 5% ($p = 0.05$). Significant differences are represented as *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, and ****$p < 0.001$. “ns” indicates nonsignificant differences ($p > 0.05$).

All analyses and visualizations were performed in the R environment (version 4.4.1) and RStudio.

3. Results

3.1. Comparative analysis of oral microbiome diversity in full term and preterm newborns

3.1.1. Temporal changes in the α-diversity of the oral microbiome

The oral microbiome communities of full term and preterm newborns were analyzed using boxplots to represent alpha-diversity (Figure 2A). In full term newborns, microbiome counts decreased over time from D0 to D2. Similarly, in preterm newborns, microbiome counts decreased over time from D0, D7, and D28.

A pairwise t-test for samples showed statistically significant differences ($p < 0.05$) in full term and preterm newborns (Figure 2B): full term D0 and D2, preterm D0, D2, and D28. Significant differences were also observed between preterm D0 and D2 and preterm D0 and D7.

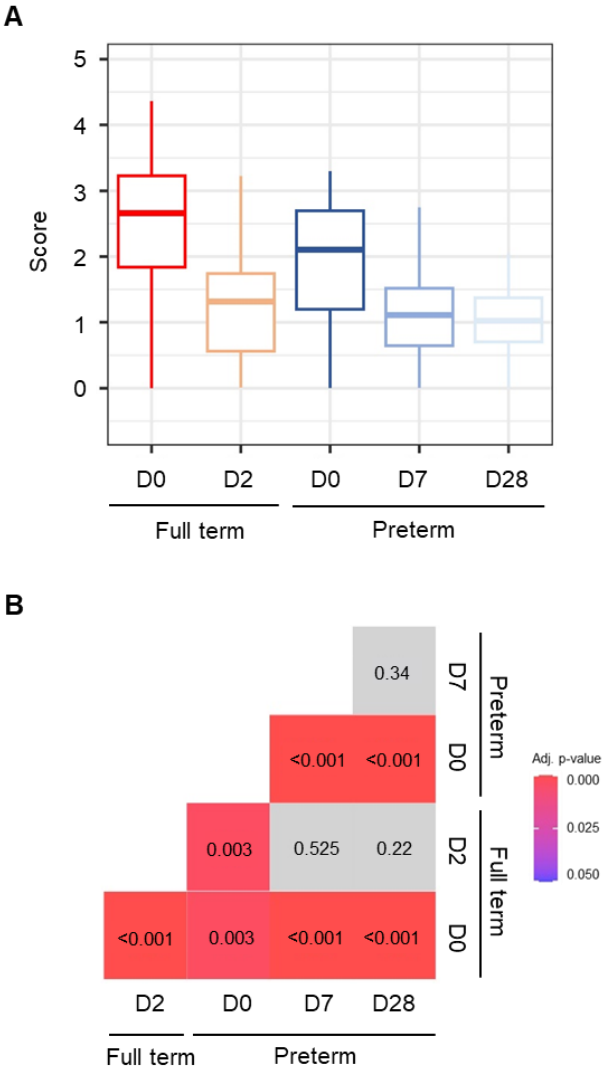

Figure 2. Changes in the α-diversity of the oral microbiome in full term and preterm newborns over time. (A) Box plot showing the α-diversity index of the oral microbiome of full term and preterm newborns. Each color represents a specific group: red for full term D0, orange for full term D2, dark blue for preterm D0, blue for preterm D7, and light blue for preterm D28. The X-axis represents the group category, and the Y-axis represents the diversity index value. (B) Pairwise t-test results for comparison between full term and preterm groups. Statistically significant differences ($p < 0.05$) are indicated by red boxes, and the numbers in the boxes indicate the *p*-value for each comparison

3.1.2. Temporal changes in the β-diversity of the oral microbiome

β-Diversity, represented by principal coordinate analysis (PCoA) plots, revealed distinct group clustering, indicating differences in the microbiome community composition. Each dot represents the oral microbiome of an individual newborn, and the groups are distinguished by color (Figure 3A).

The changes in the microbiome community diversity over time are further illustrated in the PCoA plot (Figure 3B). The oral microbiome diversity increased over time, as indicated by the expanded polygons in the PCoA plot. Full term D2 was wider than D0, preterm D7 was wider than D0, and preterm D28 was wider than D7. The centroids, marked by plus signs, represent the average composition of each group. These results indicated that the oral microbiota gradually diversified from birth to the later stages.

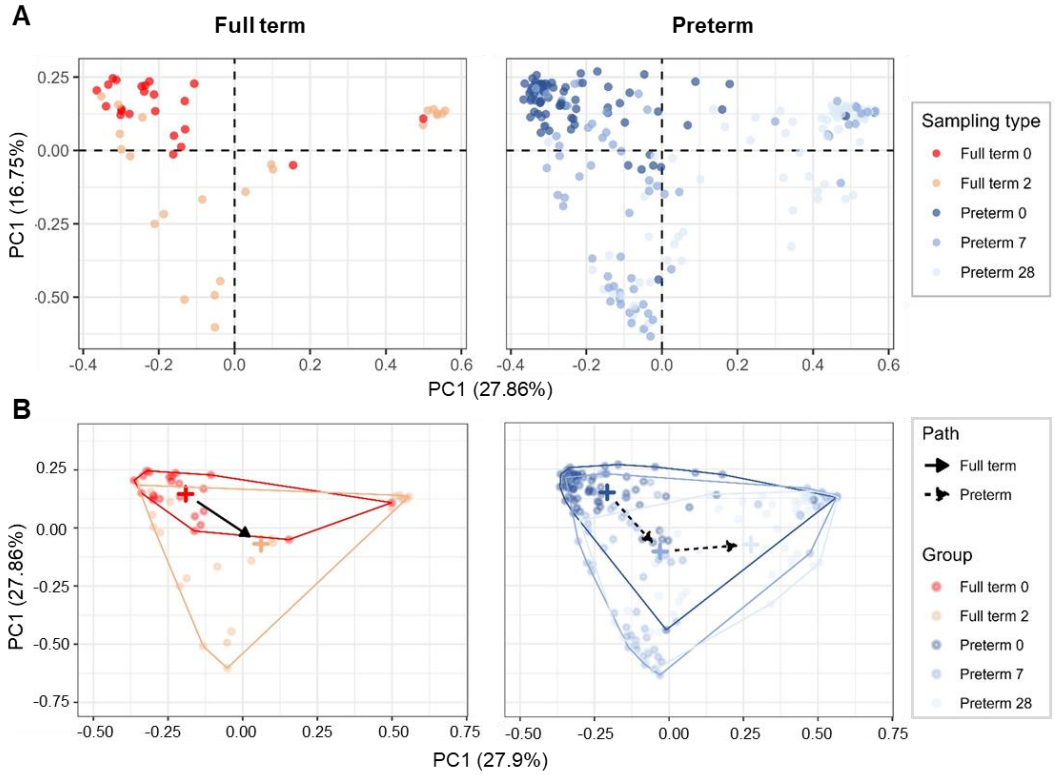

Figure 3. Temporal changes in the β-diversity of the oral microbiome in full term and preterm newborns. (A) PCoA plot showing β-diversity. Each dot corresponds to the microbiome composition of an individual newborn. Red corresponds to full term D0, orange to full term D2, dark blue to preterm D0, blue to preterm D7, and light blue to preterm D28. Clustering indicates distinct microbiome compositions between groups. (B) PCoA plot showing polygons showing microbiome β-diversity over time. The center (plus sign) represents the group mean, tracking changes in diversity over time for full term (solid line) and preterm (dashed line) newborns

3.2. Analysis of clinical and environmental factors influencing the oral microbiome in full term and preterm newborns

3.2.1. Trends in full term and preterm newborns by gestational age, birthweight, and feeding type

Newborns born at <28, <32, and <37 weeks indicated more significant changes in diversity than those born at ≥37 weeks. Full term newborns (≥37 weeks) exhibited a significant difference ($p < 0.005$) between D0 and D2 (Figure 4A). Preterm newborns (<28, <32, and <37 weeks) all indicated statistically significant differences from D0 to D7 and from D0 to D28.

Full term newborns with normal body weight (≥2,500 g) showed a significant difference ($p < 0.005$) over time, whereas low-birth-weight newborns (<1,000 and <1,500 g) showed a more significant decrease in microbial diversity over time (Figure 4B). Significant differences were observed among the body weight categories.

Diversity patterns varied by the feeding method. In full term newborns, a decrease in diversity was observed only in those fed formula (Figure 4C). In contrast, preterm newborns showed significant differences ($p < 0.01$) among D0, D7, and D28 in the breastmilk, formula, and breastmilk + formula groups (Figure 4D). In NPO, a decrease in microbial diversity was observed, but it was not statistically significant.

The gestational age and birthweight categories were compared in terms of microbial community diversity, with significant differences between time points indicated. Changes in the microbiome were observed based on the feeding type in full term and preterm newborns. This figure indicates how various factors influence oral microbiome diversity in full term and preterm newborns, with significant declines observed over time in specific categories.

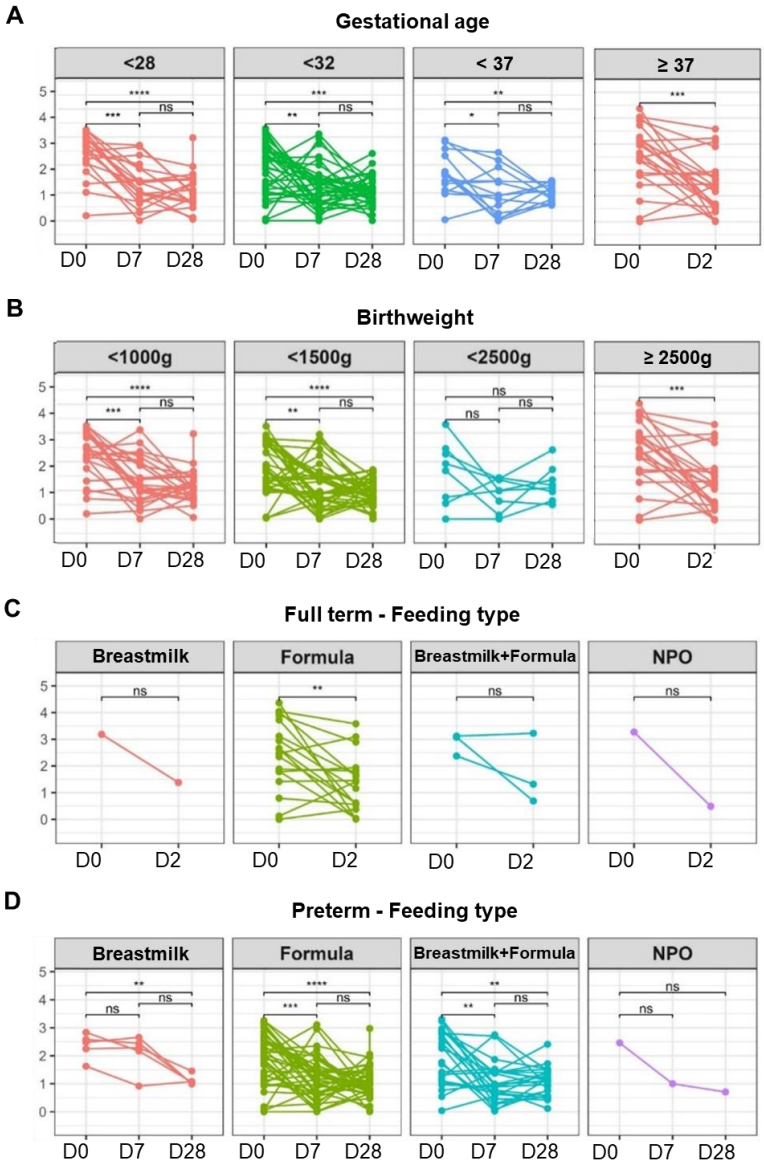

Figure 4. Effects on oral microbiome diversity: gestational age, birthweight, and feeding type. (A and B) Gestational age (<28, <32, <37, and ≥37 weeks) and birthweight (<1,000, <1,500, < 2,500, and ≥2,500 g) were compared in terms of microbial community diversity. (C and D) Feeding methods were categorized into breastmilk, formula, breastmilk + formula feeding, and NPO. Significant differences between time points are indicated by *p < 0.05, **p < 0.01, ***p < 0.001. "ns" indicates nonsignificant differences (p > 0.05)

3.2.2. Differences in the oral microbiome of full term and preterm newborns by fetal polymorphism, maternal age, and pregnancy method

Microbiome diversity in the fetal polymorphism significantly decreased between D0 and later time points in singletons and twins (Figure 5A). Twins did not change from D0 to D2 in full term, but singletons experienced a significant decrease ($p < 0.01$). In preterm newborns, the microbiome diversity significantly decreased from D0 to D7 and from D7 to D28 ($p < 0.001$). However, it was not significant from D7 to D28. Similarly, for twins, there was a significant decrease ($p < 0.05$) from D0 to D7 and a significant decrease ($p < 0.01$) from D0 to D28, but it was not significant from D7 to D28. The magnitude and significance of the changes in the microbiota differed between singletons and twins.

In older mothers, significant differences in microbiota diversity were observed between the groups (Figure 5B). For full term newborns from mothers <35 years, there was a significant difference ($p < 0.05$), and for mothers aged ≥35 years, there was no significant difference. For preterm newborns from mothers <35 years, there was a highly significant difference ($p < 0.001$) from D0 to D7 and from D0 to D28. However, the difference from D7 to D28 was not significant. For preterm newborns from mothers ≥35 years, there was a significant difference ($p < 0.05$) from D0 to D7 and a high difference of ($p < 0.001$) from D0 to D28.

The diversity of the oral microbiome was analyzed using the pregnancy method (Figure 5C). Preterm newborns had a greater decrease in microbiota diversity than full term newborns, which was statistically significant across time points. For full term newborns, there was a significant difference ($p < 0.05$) between artificial and natural pregnancies. In preterm newborns, there was a significant difference ($p < 0.01$) between artificial and natural pregnancies at D0, D7, and D28. There was no significant difference between D7 and D28.

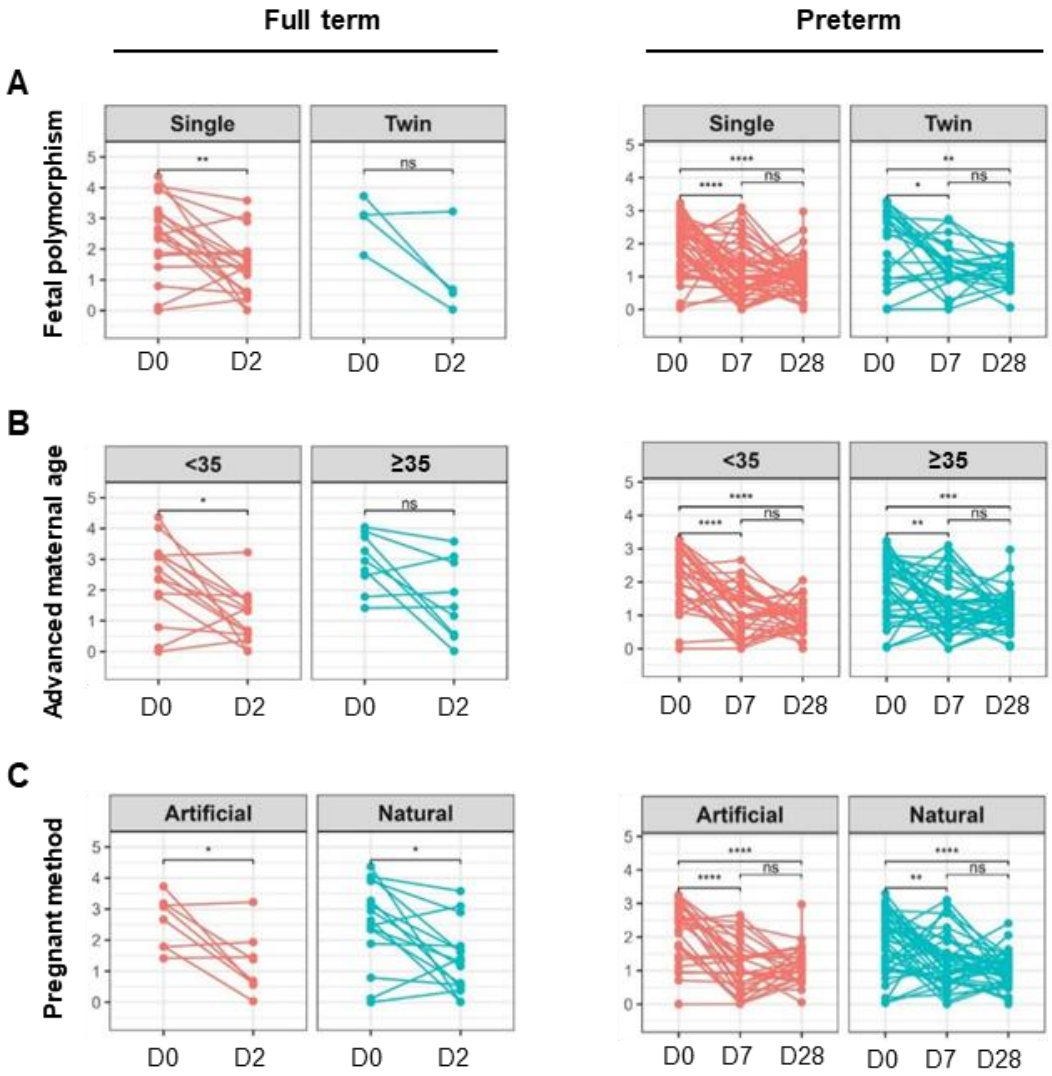

Figure 5. Comparative analysis of α-diversity in full term and preterm newborns: fetal polymorphism, maternal age, and pregnancy method. (A) α-Diversity as a line plot comparing singletons and twins. (B) Comparison of oral microbiome diversity between newborns born to mothers <35 years and mothers ≥35 years. (C) Comparison of the microbiome diversity in newborns conceived by different modes of conception, artificial or natural. Lines represent changes in the microbiome diversity of individual newborns between D0, D7, and D28. Significant differences are indicated by *$p < 0.05$, **$p < 0.01$, ***$p < 0.05$, and ****$p < 0.001$

3.2.3. Oral microbiome patterns in full term and preterm newborns using the delivery method

This study compared the oral microbiome diversity across delivery modes (C/S and NSVD) in full term and preterm newborns (Figure 6).

In full term newborns delivered by C/S, the microbiome diversity decreased significantly from D0 to D2 ($p < 0.01$). For those delivered by NSVD, no significant change was observed between D0 and D2. In C/S-delivered preterm newborns, microbiome diversity decreased significantly from D0 to D7 and from D0 to D28 ($p < 0.0001$). However, no significant difference was observed between D7 and D28. For NSVD-delivered preterm newborns, there were no significant changes in diversity across all time points (D0, D7, and D28; Figure 6A).

A PCoA plot of β-diversity between the two groups shows a distinct clustering pattern by mode of delivery and gestational age (Figure 6B). C/S neonates showed more pronounced changes in diversity over time, whereas NSVD neonates maintained a more stable microbiota composition.

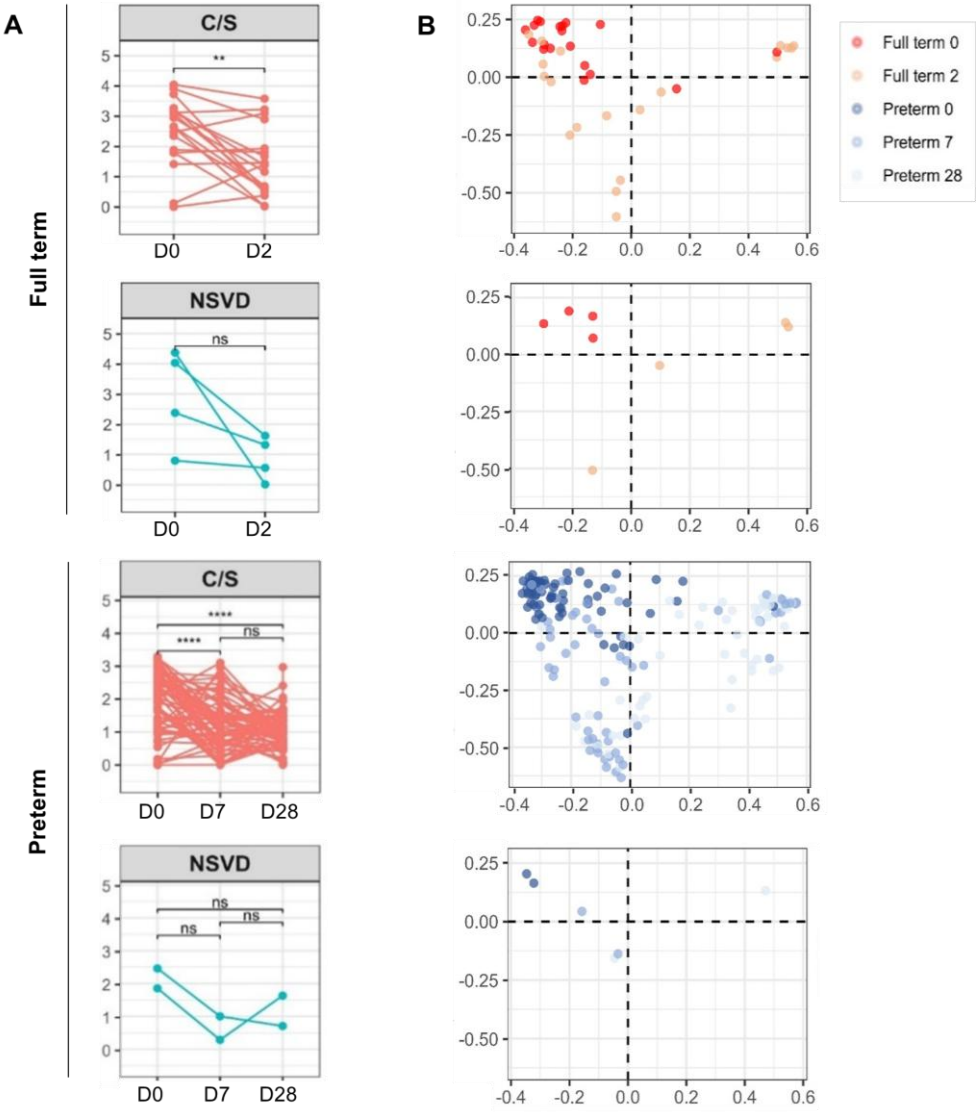

Figure 6. Comparison of oral microbiome diversity by delivery mode in full term and preterm newborns. (A) Linear α-diversity of changes in the microbiome of full term and preterm newborns born by C/S or NSVD. C/S is represented by the red line, and NSVD is represented by the blue line. (B) β-Diversity PCoA plot showing differences in the oral microbiome composition at each time point in full term and preterm newborns. Each dot represents an individual newborn, and the colors correspond to the mode of delivery and time point

3.3. Comparative analysis of preterm newborns under oral drug administration conditions

The analysis of oral microbiota in newborns revealed distinct patterns of microbial diversity changes across three time points (D0, D7, and D28) depending on the drug administered and whether treatment was applied.

Ubacillin showed no significant changes in microbial diversity when the drug was not administered, but a strong reduction in microbial diversity was observed when the drug was administered, particularly from D0 to D7 and D0 to D28 ($p < 0.001$; Figure 7A). Claforan exhibited significant reductions in microbial diversity in both groups, but the reduction was stronger and more sustained in the administered group ($p < 0.001$; Figure 7B). In contrast, meropenem caused significant changes in microbial diversity when the drug was not administered, particularly from D0 to D7 and D0 to D28 ($p < 0.001$), but no significant changes were observed in the administered group, suggesting a lack of effect when administered (Figure 7C). Oneflu significantly reduced microbial diversity in both groups from D0 to D7 and D0 to D28, although the effects diminished over time ($p < 0.05$; Figure 7D). Penbrex caused significant reductions in diversity in the group without treatment from D0 to D7 and D0 to D28 ($p < 0.001$), whereas in the administered group, the reduction was only significant from D0 to D28 ($p < 0.01$; Figure 7E). Lastly, ceftazidime caused significant reductions in microbial variability in both groups from D0 to D7 and D0 to D28 ($p < 0.001$), with no significant changes from D7 to D28, indicating a consistent effect regardless of whether the drug was administered (Figure 7F).

These results highlighted the varying impacts of different drugs on oral microbial diversity in newborns over time.

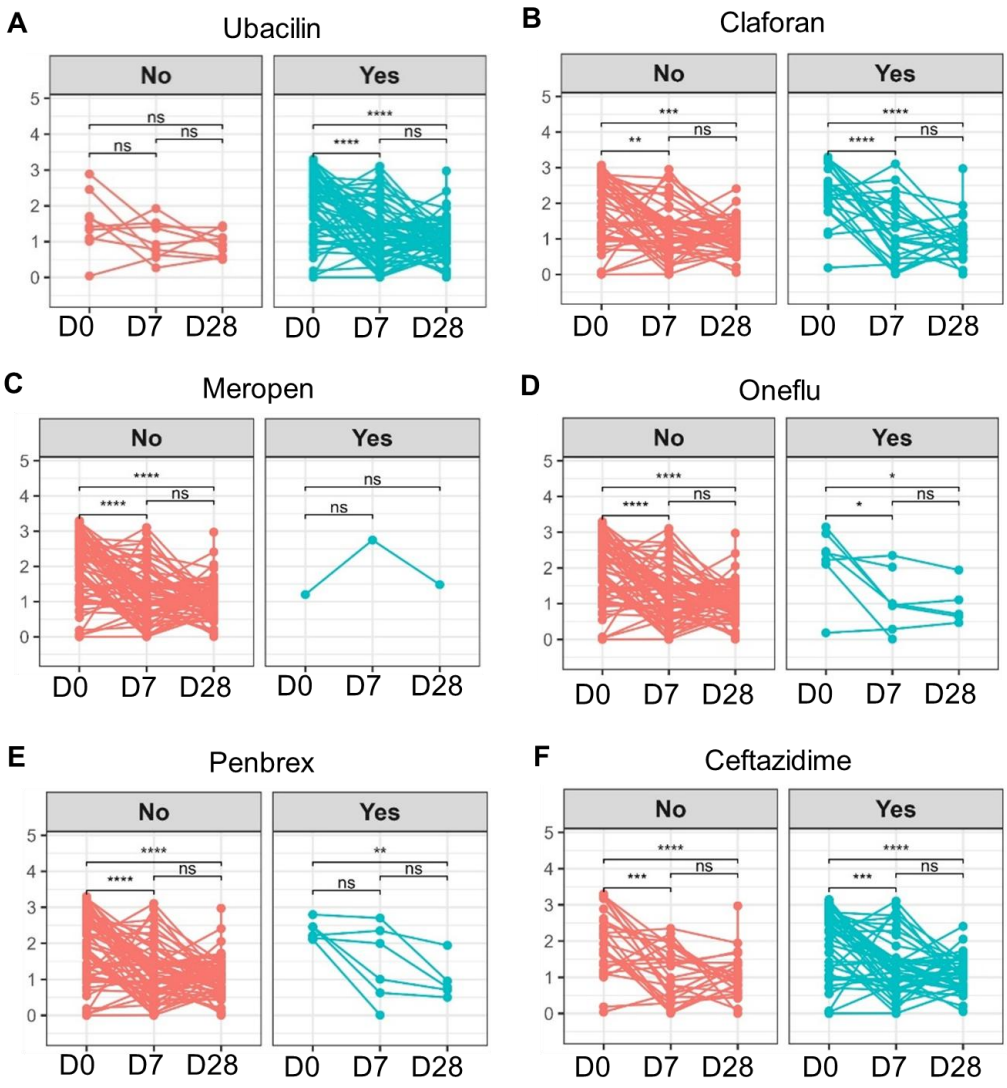

Figure 7. Impact of antibiotics on the oral microbiome of full term and preterm newborns. Each graph represents the microbial diversity for each drug: (A) ubacillin, (B) claforan, (C) meropenem, (D) oneflu, (E) penbrex, and (F) ceftazidime. The red line represents cases where the drug was not administered ("not administered"), and the blue line represents cases where the drug was administered ("administered"). Statistical comparisons across time points within each group and between "administered" and "not administered" groups are indicated as follows: ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, and ns (nonsignificant)

3.4. Oral microbiome phylum level and pie chart trends in full term and preterm newborns

The bar graphs represent the relative abundance of bacterial phylum levels in the oral microbiome of full term and preterm newborns at different time points (D0, D2, D7, and D28; Figure 8A).

The predominant microbial composition in full term newborns is *Firmicutes* (blue), which increases in the microbial community during the first 2 days after birth. In preterm newborns, a significant increase was also observed over time from D0 to D7 and D28. Present in smaller proportions compared to *Firmicutes*, *Actinobacteria* (orange) demonstrated changes in the microbial community, with its abundance increasing over time in full term and preterm newborns. The microbial composition of *Firmicutes* and *Actinobacteria* remained consistent across full term and preterm newborns. A decline in the microbial community was observed over time for the *Bacteroidetes* (apricot) phylum in full term and preterm newborns. This phylum was more noticeable at D0 in both groups. However, its relative abundance decreased significantly as time passed. Its presence became less prominent compared to other phyla. A significant decrease in abundance was observed for *Proteobacteria* (light green) in full term and preterm newborns. By D28, the microbial community in preterm newborns showed a less prominent presence of this phylum. Although full term and preterm newborns initially had similar microbial communities, the diversity of the microbial community in preterm newborns became more dramatic over time.

The pie chart indicated the relative abundance of the most prominent selected bacterial phyla (*Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*) at each time point (Figure 8B).

The pie chart for full term newborns highlighted the dominance of *Firmicutes*, which constituted >50% of the microbiota. In preterm newborns, *Firmicutes* were also the most dominant phylum, with >50% on D7 and D28. *Actinobacteria* increased from 14.1% to 24.8% in full term newborns, from 7.3% to 17.6% in preterm D0, and 34.7% on D28 in preterm newborns, suggesting a colonization pattern. *Bacteroidetes*, which had the smallest relative abundance, decreased in full term newborns from 12.6% initially to 7.3% over time and in preterm newborns from 7.5% to 1.5%, eventually falling to <1%.

In preterm newborns, the abundance decreased from 7.5% to 1.5% and <1% over time. *Proteobacteria* showed an initial abundance of 47.8% full term newborns, which decreased to 15.2% by D28. In preterm newborns, *Proteobacteria* initially constituted 60.9% of the microbial community but decreased to 22.3% and eventually to 8.3% over time.

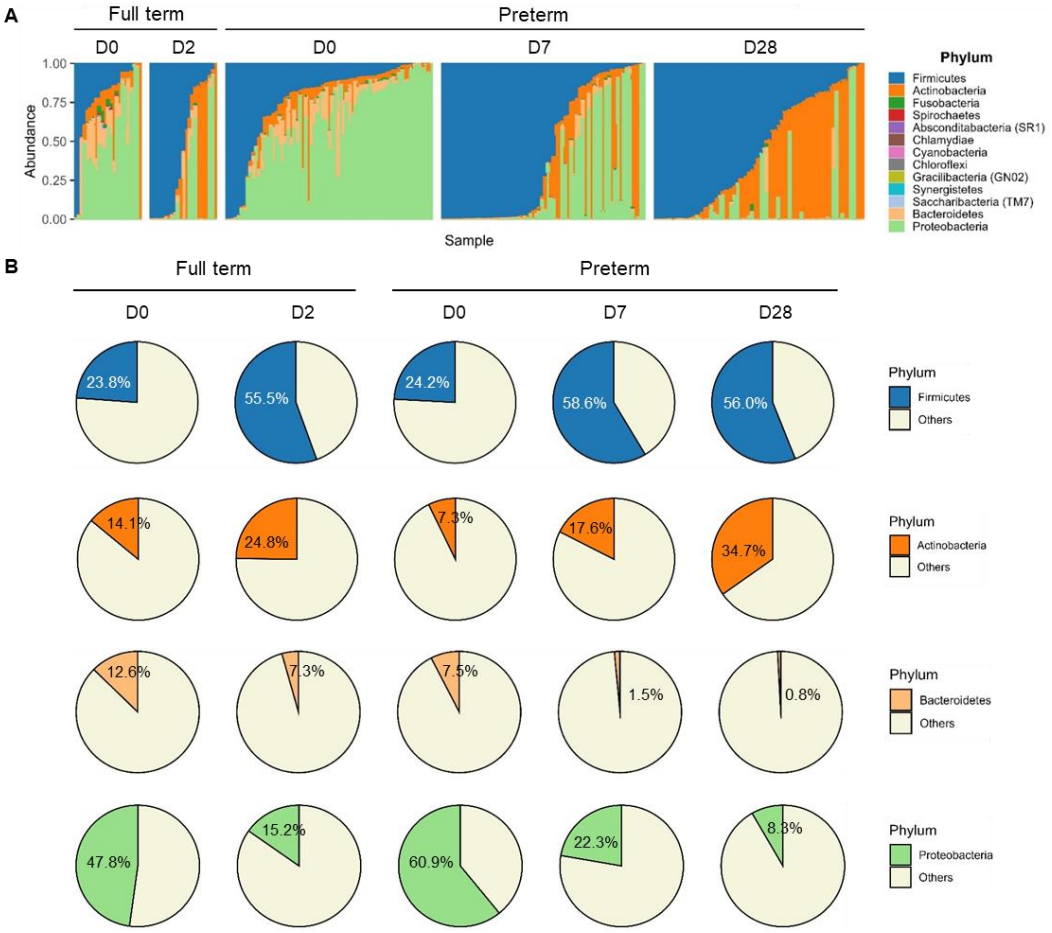

Figure 8. Oral microbiome phylum level and pie chart trends in full term and preterm newborns. (A) Bar graph representing the relative abundance of bacterial phyla in the oral microbiome of full term and preterm newborns at D0, D2, D7, and D28. The height and width of each colored block indicate the proportion of each phylum. (B) Pie chart summarizing the relative abundance of the four major bacterial phyla (*Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*) at each time point. The percentage within each slice represents the phylum's contribution to the overall microbiome composition

3.5. Comparison of the representative microbiome found in full term and preterm newborns over time

The analysis presents bacterial community distributions using a cladogram and LDA scores.

Among full term newborns, at D0 (green), *Proteobacteria*-related taxa such as *Betaproteobacteria*, *Burkholderiales*, and *Ralstonia* dominate. By full term D2 (orange), there was a striking shift toward *Firmicutes*, with *Bacillus*, *Staphylococcus*, and *Staphylococcaceae* becoming predominant. This rapid transition from *Proteobacteria* to *Firmicutes*, especially *Staphylococcus* species, occurred within the first 2 days after birth (Figure 9A).

Preterm newborns demonstrated similar trends but with slightly different timing. At preterm D0 (purple), the microbiota was dominated by early colonizers such as *Proteobacteria*, including *Betaproteobacteria*, *Burkholderiales*, and *Ralstonia*. By preterm D7 (pink), the bacterial community composition shifted quickly toward *Firmicutes*, particularly *Staphylococcus* and *Staphylococcaceae*. This rapid change underscored how preterm newborns transition from a *Proteobacteria*-dominated microbiota to one dominated by *Firmicutes*, particularly *Staphylococcus* species, within the first week of life (Figure 9B).

Between preterm D0 and D28, preterm newborns experienced a more gradual progression. Initially, the microbiota at D0 (purple) was dominated by *Proteobacteria*-related taxa, such as *Burkholderiales* and *Ralstonia*. By preterm D28 (light green), the composition evolved into *Firmicutes* and *Actinobacteria*, with *Rothia*, *Micrococcaceae*, and *Streptococcaceae* becoming the key taxa. This gradual shift from *Proteobacteria* to a more diverse microbiota highlighted the dynamic evolution of microbial communities during the first month, with the emergence of species such as *Rothia* and *Streptococcus* (Figure 9C).

The microbiota differences between D7 and D28 in preterm newborns were also notable. At preterm D7 (pink), *Proteobacteria*, *Staphylococcaceae*, *Staphylococcus*, and *Ralstonia* dominated, reflecting the characteristics of an early-stage microbiota centered on *Proteobacteria*. By preterm D28 (light green), the microbial community became significantly more diverse, with *Actinobacteria* (e.g., *Rothia*) and *Firmicutes* (e.g.,

Lactobacillales and *Streptococcaceae*) emerging as the dominant groups. Taxa such as *Micrococcaceae*, *Streptococcus*, and *Staphylococcus* are particularly prominent, signaling a transition from a simpler *Proteobacteria*-centric microbiota to a complex and diverse microbial ecosystem. These findings highlighted the dynamic reorganization of preterm newborns' microbiota, with increasing complexity and diversity during the first month (Figure 9D).

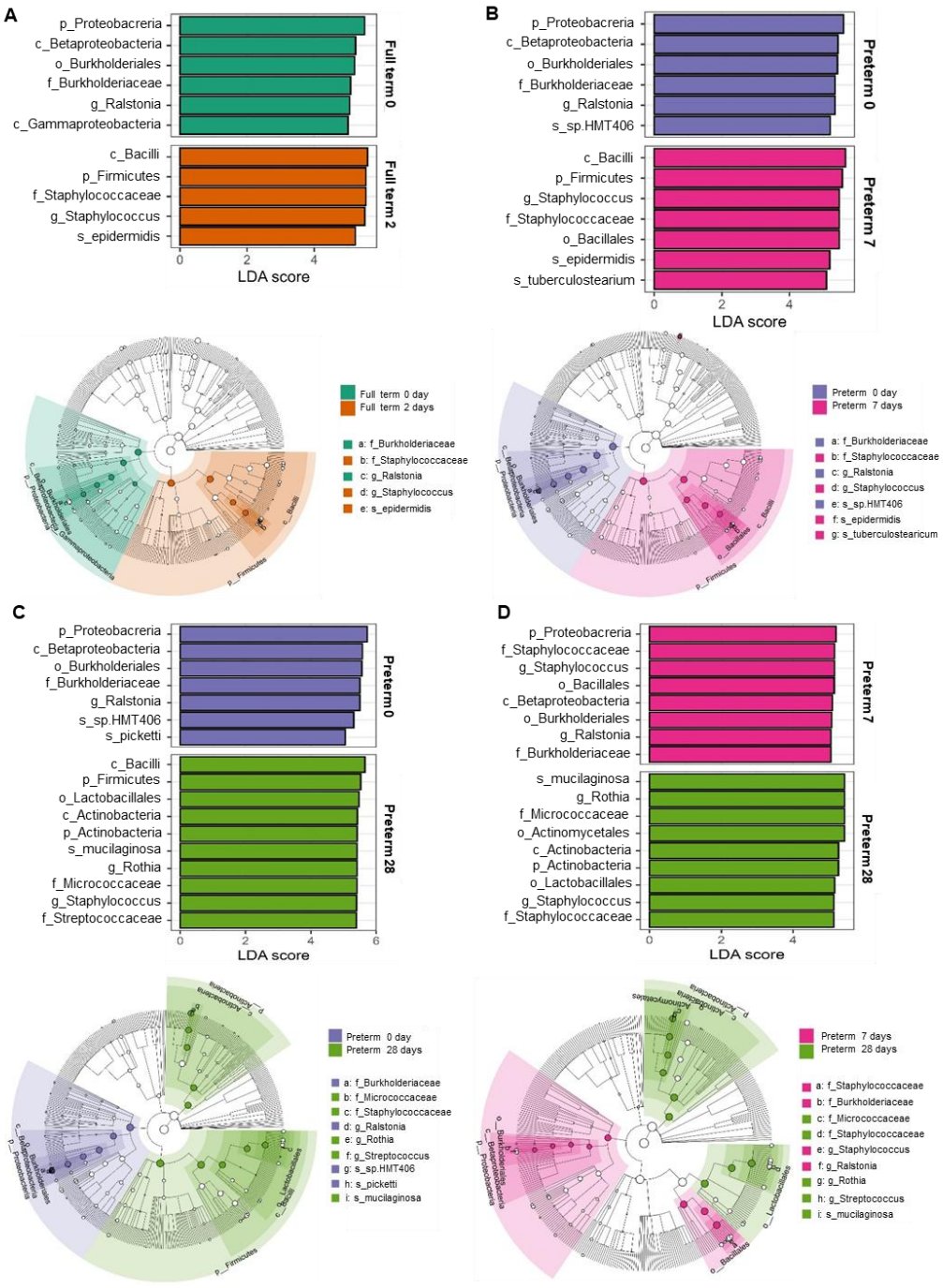

Figure 9. Oral microbiome cladograms and LEfSe in full term and preterm newborns. (A) Full term newborns comparing D0 (green) and D2 (orange). (B) Preterm newborns comparing D0 (purple) and D7 (pink). (C) Preterm newborns comparing D0 (purple) and D28 (light green). (D) Preterm newborns comparing D7 (pink) and D28 (light green)

3.6. Functional potential and gene expression dynamics of the oral microbiome in full term and preterm newborns

3.6.1. Major pathogen-disease-gene associations in full term newborn interactions

This analysis compared the microbial and gene-level differences between full term newborns on D0 and D2, focusing on the most prominently observed pathogen-disease-gene associations at each time point.

The heatmap identified the dominant pathogen-disease-gene associations at D0 and D2 in full term newborns (Figure 10A). In full term D0, *Escherichia coli* (*E. coli*) was the most prominent pathogen observed, showing strong associations with hemolytic uremic syndrome (HUS), urinary tract infection (UTI), and neonatal meningitis, as evident from the large number of associated genes and their distinct red shading. By full term D2, *Streptococcus pneumoniae* (*S. pneumoniae*) emerged as the most prominent pathogen, particularly associated with pneumococcal pneumonia and meningitis, as indicated by the orange shading and high genetic involvement.

When full term D2 was reached, *S. pneumoniae* became the dominant pathogen in the pathogenic microbe network. Its associations with respiratory diseases such as pneumonia and systemic conditions such as meningitis are clearly highlighted. The key genes involved in these processes included *PspA* (pneumococcal surface protein A), which inhibits complement-mediated immune responses to enhance bacterial survival; *nanA* (neuraminidase), shared with *E. coli*, which facilitates host cell adhesion and invasion; *lytA* (Lysin A), an autolysin that promotes toxin release; and *Pde1* (Phosphodiesterase 1), involved in biofilm formation for chronic infections. These genetic interactions demonstrated *S. pneumoniae*'s growing influence on neonatal health as full term newborns progress to D2 (Figure 10B).

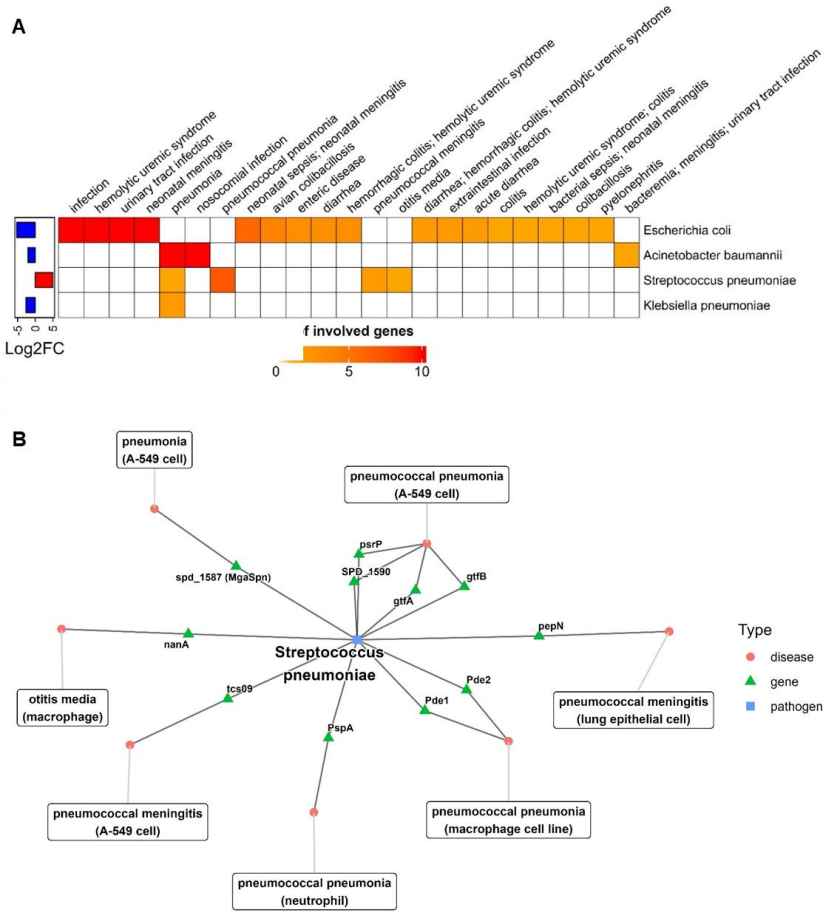

Figure 10. Pathogen-disease-gene associations in full term newborns on D0 and D2. (A) Heatmap showing the dominant pathogen-disease-gene associations in full term newborns on D0 and D2. The color gradient represents $\log_2$ fold changes ($\log_2$FC), with red indicating an increase and blue indicating a decrease in gene associations. The number of genes involved is represented by the intensity of the orange shading. Pathogens include *E. coli*, *Acinetobacter baumannii*, *S. pneumoniae*, and *Klebsiella pneumoniae*. (B) Pathogenic microbe network for D2, highlighting *S. pneumoniae* as the dominant pathogen. The network links genes to diseases such as pneumonia, meningitis, and otitis media. Key genes include *PspA*, *nanA*, and *Pde1*, which are critical in *S. pneumoniae*'s pathogenic mechanisms. The relationships between pathogens, genes, and diseases illustrate the dynamic shifts in microbial interactions between D0 and D2

3.6.2. Major pathogen-disease-gene associations of interactions in preterm newborns 0 and 7 days

This analysis compared the microbial and gene-level differences between preterm newborns on D0 and D7, focusing on pathogen-disease-gene associations.

In preterm D0 newborns, *E. coli* was highly associated with HUS, UTI, and neonatal meningitis. In preterm D7 newborns, *S. pneumoniae* and *Serratia marcescens* (*S. marcescens*) were more prominent, especially in association with pneumococcal pneumonia and other respiratory infections (Figure 11A).

By preterm D7, the network showed a transition toward *S. pneumoniae* and *S. marcescens*, with these pathogens becoming more involved in diseases such as pneumonia and systemic infections (Figure 11B). The *S. pneumoniae* key genes, such as *PspA* and *nanA*, are linked to diseases such as pneumonia, otitis media, and meningitis. These genes are critical for bacterial survival, host adhesion, and immune evasion.

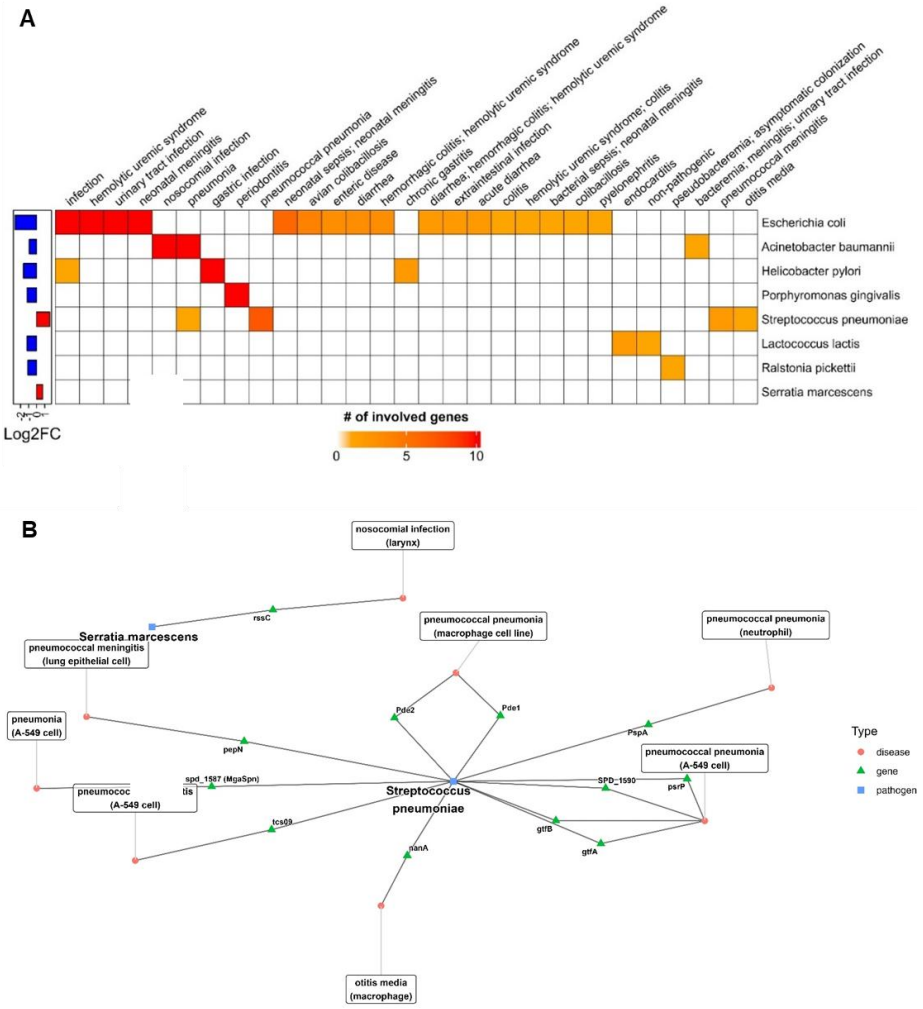

Figure 11. Pathogen-disease-gene associations in preterm newborns on D0 and D7. (A) Heatmap showing log_2FC in gene expression between preterm D0 and D7 newborns, highlighting pathogen-disease associations. Key observations include *E. coli* (D0) associated with HUS and UTI and *S. pneumoniae* and *S. marcescens* (D7) linked to pneumococcal pneumonia and respiratory infections. (B) Pathogenic microbe network at D7, showing a transition to *S. pneumoniae* and *S. marcescens* as the dominant pathogens. The network links key genes such as *PspA* and *nanA* to respiratory diseases such as pneumonia, otitis media, and meningitis. The relationships between pathogens, genes, and diseases reflect the dynamic changes in the microbial ecosystem during the first week of life in preterm newborns

3.6.3. Major pathogen-disease-gene associations of interactions in preterm newborns 0 and 28 days

This study compared the differences in the microbiome and genetic levels between preterm newborns on D0 and D28, focusing on pathogen-disease-gene associations.

The heatmap displays the log_2FC in gene expression between D0 and D28 in preterm newborns, highlighting the dynamic shifts in pathogen-disease associations over time (Figure 12A). The most prevalent pathogen in preterm D0 newborns is *E. coli*. *E. coli* is strongly associated with HUS, neonatal meningitis, and UTI. *Ralstonia pickettii* (*R. pickettii*) was abundant in D0 and is associated with infections, including *pseudobacteremia* and asymptomatic colonization. In preterm D28, *S. pneumoniae* and *Staphylococcus aureus* (*S. aureus*) are more prominent, particularly associated with pneumococcal pneumonia, skin infections, food poisoning, and respiratory disease.

The pathogenic network visualized the relationships between pathogens, genes, and diseases, emphasizing the shifts from D0 to D28 (Figure 12B). The network shifted toward *S. pneumoniae* and *S. aureus* as the dominant pathogens. For *S. pneumoniae*, genes such as *PspA* and *nanA* are critical for adhesion and immune evasion, contributing to diseases such as pneumonia and meningitis. *S. aureus* expresses genes such as *hla* (*α-hemolysin*) and *scpA* (*staphylococcal cysteine proteinase A*) and is closely related to skin infections and toxic shock syndrome (TSS).

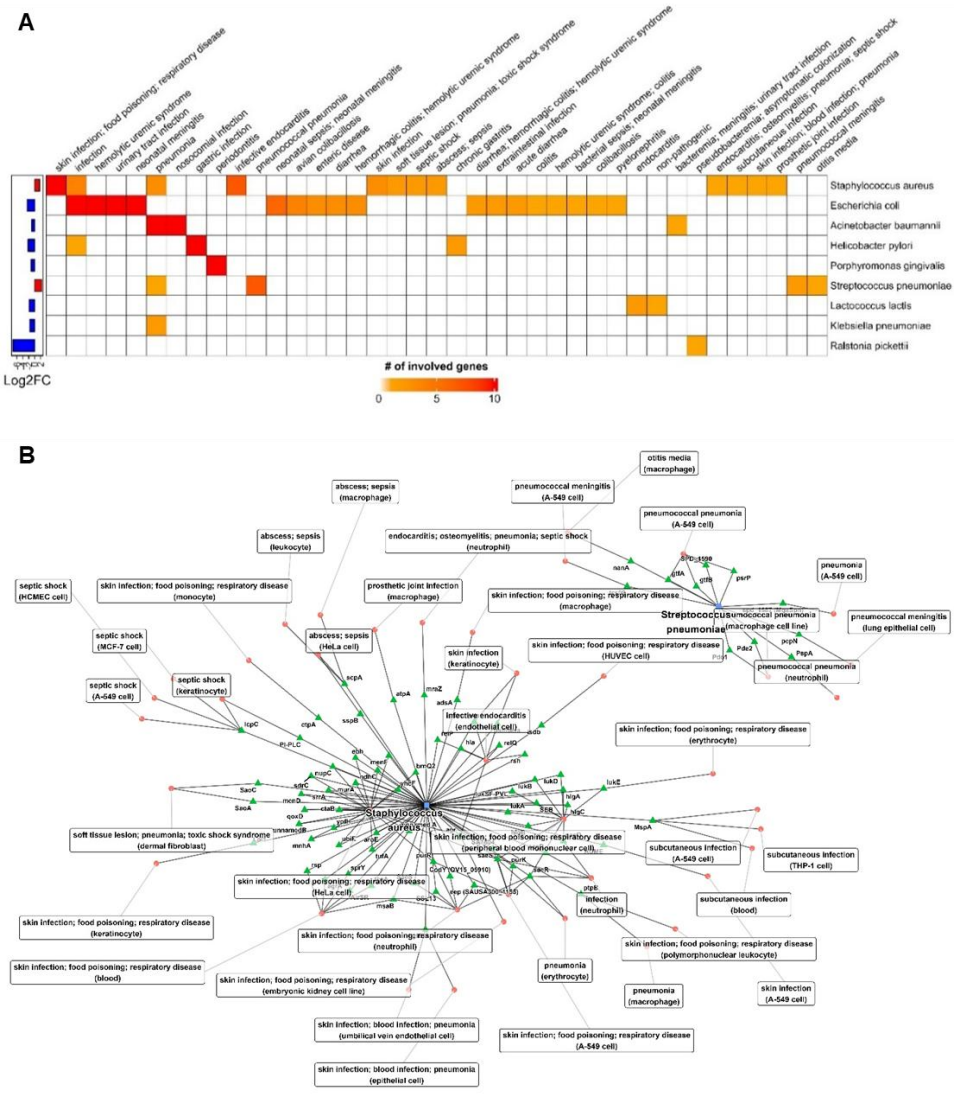

Figure 12. Pathogen-disease-gene associations in preterm newborns on D0 and D28. (A) Heatmap illustrating the $\log_2$FC in gene expression between preterm newborns on D0 and D28. Pathogens are represented on the right, with their associated diseases on the top. *E. coli* is strongly associated with neonatal meningitis and UTI on D0, whereas *S. aureus* becomes more prominent on D28, linked to skin infections and TSS. (B) Pathogenic microbe network visualizes the relationships among pathogens, genes, and diseases. Nodes are color-coded: green triangles represent genes, red circles represent diseases, and blue squares represent pathogens.

4. Discussion

This study focused on the differences and changes in the oral microbiome of full term and preterm newborns and on the influence of multiple factors, such as gestational age, mode of delivery, feeding type, antibiotic use, and perinatal period. Both groups showed decreased total microbial abundance over time, whereas microbial diversity increased significantly, in preterm newborns experiencing more pronounced and delayed changes. Delayed colonization and increased susceptibility to opportunistic pathogens were observed. These findings emphasized the importance of the early postnatal period in shaping the neonatal oral microbiota, particularly in preterm newborns. By understanding the factors influencing these microbial shifts, this study aimed to provide insights into strategies for optimizing early microbiota development and improving neonatal health outcomes.

The observed increase in diversity over time, as reflected in α-diversity metrics, indicated a gradual maturation of the oral microbiome in full term and preterm newborns, although the progression rate differed between the two groups. α-Diversity, which measures the richness and evenness of microbial communities within individual samples, decreased on D2 compared to D0 in full term newborns. This is likely because the microbiota begins to transition from diverse environmental bacteria (e.g., those acquired from the hospital or external contact) to host-associated bacteria (e.g., those that are well-suited to the oral cavity, skin, or gut of the newborn), consistent with studies showing that the microbiota stabilizes earlier in full term newborns, as environmental bacteria are replaced by host-associated taxa (La Rosa et al., 2014; Selway et al., 2023).

Preterm newborns showed significant differences in α-diversity between D0 and later time points (D7 and D28), reflecting delayed colonization and maturation. This delayed transition was further supported by changes in β-diversity, which measures the differences in microbial composition between samples or groups. Preterm newborns displayed greater β-diversity over time, indicating increased variability in the composition of their oral microbiome. This suggested that whereas individual samples (α-diversity) from preterm newborns showed delayed maturation, the overall group exhibited diverse trajectories in microbial colonization, reflecting the instability and heterogeneity of their microbiota during early life.

Preterm newborns are more susceptible to infections with opportunistic pathogens such as *Staphylococcus* and *Enterobacteriaceae* because of their delayed microbiota development. This finding aligned with previous studies linking a high prevalence of opportunistic pathogens in preterm populations (Pammi et al., 2017). The combination of reduced α-diversity and increased β-diversity in preterm newborns underscored the critical importance of early microbial stabilization.

Gestational age and birthweight emerged as significant factors influencing microbial diversity, with preterm newborns (<28, <32, and <37 weeks) and those with low birthweight (<1,000 and <1,500 g) showing greater and more dynamic changes over time compared to full term newborns (≥37 weeks and ≥2,500 g). Full term newborns exhibited a moderate decrease in microbial diversity, reflecting a more stable microbiome maturation process. Conversely, preterm newborns experienced delayed colonization and marked alterations in microbial diversity, consistent with their immaturity of the immune system and prolonged medical interventions requiring prolonged NICU stays (Pammi et al., 2017; Arrieta et al., 2014).

Feeding practices differed between full term and preterm newborns. In full term newborns, formula feeding was associated with a slight decrease in microbial diversity between D0 and D2, whereas breastmilk feeding, breastmilk + formula feeding, and NPO resulted in minimal changes and a stable microbial community. In contrast, preterm newborns exhibited more pronounced differences across feeding types. Breastmilk feeding was associated with a stable diversity over time (D28), reinforcing its protective role in maintaining microbial balance. Formula feeding showed a significant decrease in diversity, particularly between D0 and D7, highlighting its potential to disrupt the microbial community during this critical period. Breastmilk + formula feeding in preterm newborns also decreased diversity, although to a lesser extent than formula feeding alone. NPO in preterm newborns showed minimal changes in diversity, suggesting that microbial diversity was limited in the absence of oral feeding. Although the small sample size in the full term group limited direct comparisons between full term and preterm newborns, previous studies consistently demonstrated that breastmilk contains bioactive components, such as prebiotics, which promote the growth of beneficial bacteria (e.g., *Firmicutes* and *Actinobacteria*) (Bode, 2012; Pannaraj et al., 2017; Bäckhed et al., 2015). Based on these findings, breastmilk feeding played a critical role in fostering a diverse and stable microbiota during the early stages of oral microbiota formation, particularly in preterm newborns more vulnerable to microbial dysbiosis.

Fetal polymorphism, maternal age, and pregnancy method significantly influenced the microbiome diversity. Diversity decreased over time in singletons and twins, but no significant differences were observed between these groups. This suggested that shared maternal and environmental factors during pregnancy may play a more critical role in shaping the microbiome than fetal polymorphism itself. Maternal age showed a variable effect, particularly in preterm newborns.

Preterm newborns born to younger mothers (<35 years) exhibited greater variation in microbial diversity compared to those born to older mothers (≥35 years), potentially reflecting differences in the maternal immune system that develop with age. Studies indicated that the transfer of certain immune components, such as immunoglobulin A (IgA), may change with increasing maternal age, and these components are crucial in stabilizing the neonatal microbiome during early life (Walker, 2017; Korpela & de Vos, 2018). Although further research is needed to fully understand the variation in microbial diversity across maternal age, existing studies support the idea that immune factors such as IgA play a pivotal role in shaping and stabilizing the early microbiome of newborns.

The method of conception also affected microbial diversity, with naturally conceived newborns showing a greater decrease in diversity compared to those conceived via artificial reproductive technology. Full term newborns exhibited a more significant decrease in diversity than preterm newborns, whereas preterm newborns showed a faster decrease in diversity between D0 and D7 than between D7 and D28. Naturally conceived newborns form their initial microbial communities through direct contact with the maternal vaginal or gut microbiome during delivery, leading to higher initial diversity that decreases as the community becomes more specialized around specific beneficial bacteria. In contrast, newborns conceived via artificial reproductive technology may have limited direct exposure to maternal microbiomes and may instead be more influenced by the hospital environment. This could result in less dramatic initial changes in diversity and a slower stabilization process. These findings highlighted the multifaceted impact of maternal and perinatal factors on establishing and developing the neonatal microbiome.

The mode of delivery was a significant determinant of microbial diversity. Although NSVD maintained a stable diversity, no significant differences were observed compared to C/S. Newborns delivered via NSVD acquire beneficial microorganisms from the mother's vaginal and fecal microbiota (e.g., *Lactobacillus*, *Bacteroides*, and *Firmicutes*), which play a critical role in shaping their initial microbiome. In contrast, newborns delivered by C/S are deprived of exposure to these beneficial microorganisms and are instead colonized by

less beneficial microorganisms or potential pathogens from the mother's skin (e.g., *Staphylococcus* and *Corynebacterium*) or the hospital environment (Dominguez-Bello et al., 2010). Consequently, C/S may negatively affect establishing and stabilizing the neonatal microbiome, making it more challenging to form a balanced and beneficial microbiome than NSVD.

Antibiotics significantly disrupted microbial diversity, particularly in preterm newborns. Medications such as claforan and ceftazidime were associated with marked reductions in microbial diversity, highlighting the vulnerability of the microbiota to broad-spectrum antibiotics during the neonatal period. Interestingly, the impact of some antibiotics varied, emphasizing the need for careful management to balance the prevention of infections with the preservation of microbial diversity. The findings reinforced the critical role of cautious antibiotic use in the early postnatal period to minimize the long-term risks of dysbiosis and related complications.

The phylum *Firmicutes* significantly shape the oral microbiome, particularly through bacteria such as *Streptococcus* and *Lactobacillus*, which contribute to oral biofilm formation during early colonization. These bacteria metabolize carbohydrates to produce acids that can lead to enamel demineralization, but they are also essential for promoting a balanced microbiome and maintaining oral health (Magoc et al., 2021). In full term newborns, the relative abundance of *Firmicutes* increased significantly between D0 and D2, with taxa such as *Streptococcaceae* and *Staphylococcaceae* dominating the initial microbiome, as highlighted in the cladogram. In preterm newborns, however, *Firmicutes* became prominent only by D28, suggesting a delayed establishment of this crucial group.

The phylum *Actinobacteria*, which includes *Rothia* and *Actinomyces*, plays a key role in the maturation of the oral microbiome by breaking down complex polysaccharides and contributing to microbial stability (Arrieta et al., 2014; Kilian et al., 2016). In preterm newborns, *Actinobacteria* showed a pronounced increase from 7.3% at D0–34.7% at D28, emphasizing its role in stabilizing the oral microbiome. However, imbalances in certain *Actinomyces* species have been associated with oral diseases such as periodontitis (Chalmers et al., 2015), underscoring the need to closely monitor its development.

Although *Firmicutes* formed the dominant group in the oral microbiome, *Bacteroidetes* also played an important role. This phylum significantly contributes to oral biofilm formation (dental plaque) (Aas et al., 2005). *Bacteroidetes* are particularly well-adapted to anaerobic (low oxygen) environments, such as the subgingival region, where

they are involved in biofilm development and maintenance (Lamont & Hajishengallis, 2015). A high *Firmicutes/Bacteroidetes* ratio was observed in full term and preterm newborns at the phylum level, reflecting an unbalanced microbiome common during the early stages of microbial colonization (Arrieta et al., 2014). In preterm newborns, *Bacteroidetes* persisted longer, reflecting a delayed stabilization process. Prolonged high levels of *Bacteroidetes* may contribute to local oral inflammation and potentially systemic inflammatory states (Kumar, 2013; Arrieta et al., 2014), emphasizing the need for careful monitoring to mitigate potential long-term health complications.

Proteobacteria initially dominated the oral microbiome in both groups, as seen at the phylum level and in the pie chart data, but the cladogram revealed contrasting temporal patterns. In full term newborns, *Proteobacteria* decreased rapidly and were replaced by beneficial taxa such as *Firmicutes*. However, in preterm newborns, *Proteobacteria* persisted long, reflecting delayed microbial stabilization. Genera such as *Neisseria* and *Haemophilus*—members of *Proteobacteria*—are commonly found in healthy oral environments but can cause infections when unchecked, particularly in preterm infants who are immunocompromised (Pammi et al., 2017; Kau et al., 2011). This persistence of *Proteobacteria* in preterm newborns may contribute to oral dysbiosis and an increased risk of opportunistic infections.

Overall, the establishment and stabilization of the oral microbiome differed significantly between full term and preterm newborns. Although full term newborns transitioned more rapidly to *Firmicutes*-and *Actinobacteria*-dominated communities, preterm newborns experienced delayed microbial transitions and prolonged dominance of *Proteobacteria*, increasing their susceptibility to infections and oral dysbiosis. These findings highlighted the importance of targeted interventions for timely microbiome stabilization in preterm newborns, which may help mitigate their vulnerability to infections and long-term health complications.

The oral microbiome of full term newborns showed a significant shift between D0 and D2, highlighted by the emergence of *S. pneumoniae* as the dominant pathogen by D2. At full term D0, *E. coli* was strongly associated with systemic infections such as HUS and neonatal meningitis. However, at full term D2, *S. pneumoniae* overtook *E. coli* and was more clearly associated with respiratory diseases, particularly pneumococcal pneumonia, and meningitis. These changes highlighted the dynamic nature of the early neonatal microbiome, with the early neonatal microbiome changing over time in response to environmental factors and exposures, with the respiratory pathogen *S. pneumoniae*

increasingly becoming dominant. The role of key genes, such as *PspA* and *nanA*, in facilitating host adhesion, immune evasion, and biofilm formation enhances the ability of *S. pneumoniae* to cause infection, allowing it to survive and become more dominant in the host environment (R. Lane, 2023). The microbiota changes in full term newborns are influenced not only by environmental factors but also by the genetic characteristics of specific pathogens, which play a key role in the establishment and disease initiation of pathogens in the respiratory tract of newborns.

In preterm newborns, *E. coli* was the dominant pathogen at D0 and was associated with UTI and neonatal meningitis. However, a shift occurred at D7, with *S. pneumoniae* and *S. marcescens* emerging as the dominant pathogens. *S. pneumoniae* was particularly associated with pneumococcal pneumonia and otitis media, whereas *S. marcescens* was associated with respiratory infections and systemic conditions. Network analysis revealed that key genes, such as *PspA* and *nanA*, of *S. pneumoniae* and virulence factors of *S. marcescens* drive the pathogenic potential. These results highlighted the vulnerability of preterm newborns to changes in the microbiota and the importance of early intervention to mitigate the risk of respiratory and systemic infections.

Comparison of D0 and D28 in preterm newborns revealed significant changes in the microbial community, with *S. pneumoniae* and *S. aureus* becoming dominant by D28. At preterm D0, *E. coli* and *R. pickettii* were abundant, with *R. pickettii* being associated with pseudobacteremia and asymptomatic colonization. By preterm D28, *S. aureus* was associated with skin infections, food poisoning, and TSS, whereas *S. pneumoniae* retained its association with respiratory disease. The emergence of *S. aureus* as the dominant pathogen highlighted its pathogenic potential, which is driven by genes such as *hla* and *scpA*. These genes play an important role in tissue invasion, immune regulation, and toxin production, contributing to conditions such as TSS (Natalia et al., 2024). Likewise, the persistence of *S. pneumoniae* highlights its adaptability and virulence, with genes such as *PspA* and *nanA* facilitating immune evasion and colonization. Overall, the neonatal oral microbiome underwent significant changes over time, influenced by intrinsic and extrinsic factors. Whereas full term newborns have a faster transition to respiratory pathogens, preterm newborns are more susceptible to systemic infections. During the first 28 days of life, *S. aureus* and *S. pneumoniae* emerge as important pathogens, the main causes of serious health problems in preterm newborns. The presence of these pathogens during the first month of life highlighted the need for preterm newborns to be protected from them.

Studying the genes of specific pathogens allows us to understand how pathogens adapt to neonatal hosts and trigger infections. This provides important information for developing disease prevention and treatment strategies. Genes such as *hla*, *scpA*, *PspA*, and *nanA* represent potential targets for therapeutic interventions to mitigate the risk of serious neonatal infections.

The limitations of this study include the small sample size in the full term group, which may restrict the generalizability of the findings. Although full term and preterm newborns were compared and categorized into age groups, it is important to note that they cannot be directly aligned on the same developmental timeline as full term newborns. The lack of stratification based on the exact gestational age at birth (e.g., <28 and 28–32 weeks) presents another limitation. Despite these limitations, this study offers a comprehensive view of the establishment and progression of the newborn oral microbiota. This is one of the few studies holistically comparing full term and preterm newborns while identifying significant differences influenced by multiple perinatal and postnatal factors. These findings highlight the distinct microbial trajectories in full term and preterm newborns, even with the constraints mentioned.

Future studies should address these limitations by including larger sample sizes and stratifying preterm newborns according to their gestational age. Additionally, longitudinal studies that follow newborns beyond the neonatal period and into the later stages of childhood are necessary to investigate how early microbiota patterns influence long-term health outcomes. These studies could provide critical insights into potential interventions for optimizing microbiota development and improving health trajectories in preterm and full term newborns.

5. Conclusion

This study provided a comprehensive analysis of the dynamic changes in the oral microbiome of full term and preterm newborns during the early neonatal period, emphasizing the significant impact of clinical and environmental factors on microbial development. Results revealed that preterm newborns exhibit delayed microbial stabilization and a higher prevalence of opportunistic pathogens than full term newborns, underscoring their heightened vulnerability to infections during this critical developmental window.

Key determinants, such as gestational age, birthweight, delivery mode, feeding types, and antibiotic use were identified as influential factors shaping the oral microbiota. Breastmilk feeding demonstrated a protective role in promoting microbial diversity and balance, whereas C/S delivery was associated with delayed microbial stabilization and an increased risk of dysbiosis.

Functional and taxonomic analyses revealed distinct microbial trajectories between full term and preterm newborns. Full term newborns transitioned rapidly to a balanced microbiota dominated by beneficial taxa, such as *Firmicutes* and *Actinobacteria*. In contrast, preterm newborns experienced prolonged dominance of *Proteobacteria* and delayed colonization by *Firmicutes* and *Actinobacteria*, reflecting delayed microbial stabilization. Furthermore, the expression of virulence-related genes, such as *PspA* and *nanA* in *S. pneumoniae* and *hla* and *scpA* in *S. aureus* was associated with pathogenicity and a slower progression toward microbial balance in preterm newborns. These findings underscored the need for targeted interventions to mitigate the infection risks in this vulnerable population.

Full term newborns demonstrated a rapid transition to a stable microbiota with *S. pneumoniae* emerging as the dominant pathogen by D2, reflecting a natural shift toward respiratory-associated taxa. In contrast, preterm newborns exhibited delayed microbial stabilization, characterized by prolonged dominance of *E. coli* and *R. pickettii* at D0, followed by *S. marcescens*, *S. aureus*, and *S. pneumoniae* at later time points (D7 and D28). This delayed transition highlighted the increased susceptibility of preterm newborns to infections during early development.

In conclusion, this study underscored the importance of understanding early oral

microbiome development to inform interventions that optimize microbial health in newborns, particularly preterm populations. Future research should focus on longitudinal studies to explore the long-term health impacts of early microbiome patterns and develop targeted interventions to promote microbial balance in newborns. These insights can significantly improve neonatal care and reduce the burden of infections and long-term complications, particularly in preterm newborns.

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Abstract in Korean

만삭아와 조산아의 초기 구강미생물 변화에 대한 종단적 분석

신생아기는 구강 미생물이 형성되는 중요한 시기이다. 구강 미생물은 장기적인 건강에 큰 영향을 미치며, 특히 조산아는 미생물 안정화 지연과 불균형으로 인해 감염에 취약할 위험이 높다. 그러나 조산아의 구강 미생물 발달에 대한 연구는 아직 충분히 이루어지지 않았다. 따라서 본 연구는 만삭아와 조산아의 구강 미생물 변화를 시간에 따라 비교하고, 미생물 발달에 영향을 미치는 주요 임상 및 환경적 요인을 규명하고자 하였다.

본 연구에는 만삭아 23 명과 조산아 75 명이 포함되었다. 만삭아는 출생 후 0 일과 2 일에, 조산아는 0 일, 7 일, 28 일에 구강 면봉 샘플을 채취하였다. 구강 미생물의 구성과 다양성은 16S rRNA 유전자 시퀀싱을 통해 분석되었으며, 임신 기간, 출생 체중, 분만 방식, 수유 유형, 항생제 사용 등의 주요 요인이 구강 미생물에 미치는 영향을 평가하였다.

조산아는 만삭아에 비해 미생물 안정화가 지연되었으며, *Proteobacteria* 가 우세한 상태가 오래 지속되고 *Firmicute* 와 *Actinobacteri*a 의 정착이 늦게 이루어졌다. 만삭아는 *Firmicutes* 가 우세한 균형 잡힌 미생물 군집으로 빠르게 전환되었으며, 출생 후 2 일째에는 *Streptococcus pneumoniae* 가 주요 병원균으로 자리잡았다. 반면, 조산아는 출생 첫날 *Escherichia coli* 와 *Ralstonia pickettii* 가 우세를 보였고, 이후 7 일째와 28 일째에는 *Serratia marcescens*, *Staphylococcus aureus*, *Streptococcus pneumoniae* 로 전환되었다. 모유 수유는 미생물 다양성과 안정성을 증진시키는 반면, 제왕절개 분만은 미생물 안정화가 지연되고 불균형을 초래하였다. 기능 분석 결과, *PspA*, *nanA*, *hla*, *scpA* 와 같은 병원성과 관련된 유전자가 조산아의 병원성 증가에 기여하는 것으로 확인되었다.

본 연구는 임신 기간, 출생 체중, 분만 방식, 수유 유형, 항생제 사용이 신생아 구강 미생물 발달에 중요한 영향을 미칠 수 있다. 특히 조산아의 지연된 미생물 전환과 감염 취약성은 맞춤형 개입의 필요성을 강조하며, 초기 구강 미생물 발달과 장기적 건강 간의 연관성을 규명하기 위해 추가적인 장기 연구가 요구된다.

핵심되는 말 : 신생아, 조산아, 구강 미생물군, 구강 질환, 미생물 다양성, 신생아 건강, 미생물군 안정화, 종단 연구