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Longitudinal analysis of oral microbiome changes during the neonatal period in full-term and preterm newborns

Taeyang Lee[a,b], Hyun-Yi Kim[c], Jung Ho Han[d], Jeong Eun Shin[d], Na-Young Song[e,f,g], Won-Yoon Chung[e,f,g] and Chung-Min Kang[a,b]

[a]Department of Pediatric Dentistry, Yonsei University College of Dentistry, Seoul, Republic of Korea; [b]Oral Science Research Center, Yonsei University College of Dentistry, Seoul, Republic of Korea; [c]NGeneS Inc., Ansan-si, Republic of Korea; [d]Department of Pediatrics, Yonsei University College of Medicine, Seoul, Republic of Korea; [e]Department of Oral Biology, Yonsei University College of Dentistry, Seoul, Republic of Korea; [f]Department of Applied Life Science, The Graduate School, Yonsei University, Seoul, Republic of Korea; [g]BK21 Four Project, Yonsei University College of Dentistry, Yonsei University, Seoul, Republic of Korea

ABSTRACT

Background: The neonatal period is critical for oral microbiome establishment, but temporal patterns in preterm newborns remain unclear. This study examined longitudinal microbiome changes in full-term and preterm newborns and assessed perinatal and clinical influences.

Methods: Oral swabs were collected from 98 newborns (23 full-term, 75 preterm). Samples were obtained at birth and Day 2 for full-term, and at birth, Day 7, and Day 28 for preterm newborns. 16S rRNA gene sequencing was used to analyze microbial diversity, taxonomic shifts, and virulence-related genes.

Results: Preterm newborns showed persistently lower α-diversity and delayed succession compared with full-term newborns. Full-term infants transitioned rapidly from Proteobacteria-dominant to Firmicutes- and Actinobacteria-rich communities, while preterm infants maintained Proteobacteria longer. Diversity in preterm newborns was significantly affected by gestational age, birthweight, delivery mode, feeding type, and β-lactam exposure. Breastfeeding supported more stable diversity, whereas cesarean delivery and formula feeding reduced diversity. Functional profiling revealed greater abundance of virulence-associated genes in preterm newborns, suggesting differences in early host–microbe interactions.

Conclusions: Preterm newborns exhibit delayed oral microbiome development, influenced by multiple modifiable factors. Supportive strategies, such as breastfeeding and prudent antibiotic use, may help foster microbial stability and potentially reduce infection risk in this vulnerable population.

KEY MESSAGES

- Preterm newborns exhibit delayed and less diverse oral microbiome development compared to full-term newborns, with persistent dominance of Proteobacteria and enrichment of virulence-related genes.
- Environmental exposures, including delivery mode, feeding type, and antibiotic use, exert a greater influence on early oral microbial composition than biological maturity alone.
- Targeted early-life interventions – such as promoting breastfeeding and minimizing unnecessary antibiotic exposure – may help establish a more resilient and health-promoting oral microbiome in preterm newborns.

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Oral microbiome; preterm newborns; microbial diversity; virulence genes; neonatal health

Introduction

The World Health Organization defines preterm birth as birth before 37 weeks of gestation [1]. The preterm birth rate is 9.9% globally and 6.8% in East Asia [2]. Factors such as preterm labor, premature rupture of membranes, maternal age, genetic predisposition, and infections contribute to the increasing incidence of preterm births [3]. Advances in neonatal care have improved survival rates; still, preterm newborns remain

CONTACT Chung-Min Kang kangcm@yuhs.ac Department of Pediatric Dentistry, Yonsei University College of Dentistry, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea

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vulnerable to complications, emphasizing the need for comprehensive medical and dental interventions [4,5].

The neonatal period is critical for establishing the oral microbiome, which plays a key role in immune development, metabolic function, and long-term health [6]. The oral microbiome, composed of bacteria, viruses, fungi, and archaea, is influenced by delivery mode, feeding practices, antibiotic use, and environmental exposure [7,8]. Imbalances in the microbiome are associated with higher risks of infections, inflammatory responses, and systemic diseases [9,10]. Studies suggest that the early colonization of microorganisms influences immune system priming and microbial stability, particularly in preterm newborns who experience delayed bacterial succession [11,12]. Compared to full-term newborns, preterm newborns harbor distinct microbial communities with a higher prevalence of opportunistic pathogens, predisposing them to respiratory infections and systemic inflammation [13].

Most microbiome research has focused on the gut, linking dysbiosis to necrotizing enterocolitis and sepsis [14]. However, emerging evidence highlights that oral microbiota, though less studied, may similarly reflect and impact systemic health. The mode of delivery affects early microbial colonization; vaginal delivery promotes maternal vaginal microbiota transfer, while cesarean section (C/S) newborns acquire skin-associated microbes [15]. Breastmilk feeding fosters microbial stability, whereas antibiotics disrupt microbial composition, though their effects on the oral microbiome remain understudied [16]. Additionally, factors such as prolonged hospitalization, invasive procedures, and antimicrobial stewardship policies in neonatal intensive care units (NICUs) have been shown to influence the microbial landscape. Recent findings suggest that environmental exposures in NICUs and frequent medical interventions further shape the microbial landscape of preterm newborns, increasing their risk of dysbiosis and related morbidities [17]. The limited studies on the neonatal oral microbiome highlight the need for further investigation into how external factors influence early microbial development [18].

Preterm newborns exhibit delayed microbial stabilization, with lower microbial diversity and increased presence of opportunistic pathogens [11,13]. While studies have linked oral microbiota to respiratory and gastrointestinal health [12,19], few have explored its development in preterm newborns. The oral microbiome is a microbial reservoir that may influence systemic health, yet its role in preterm newborns remains underexplored [7,20].

Despite growing interest in neonatal microbiome research, the temporal dynamics of the oral microbiome remain poorly understood, especially in preterm newborns. While previous studies have explored gut microbiota development, few have investigated how host-related factors (e.g. gestational age, birthweight) and environmental exposures (e.g. delivery mode, feeding practices, antibiotic use) jointly shape the establishment and maturation of the oral microbiota during the neonatal period. To date, no comprehensive longitudinal studies have directly compared the oral microbial trajectories of full-term and preterm newborns across multiple time points. Given the clinical relevance of microbial dysbiosis in early life, identifying modifiable factors that influence oral microbial colonization may enable targeted strategies to support immune development and reduce infection risk in vulnerable neonates.

We hypothesized that the oral microbiome in preterm newborns would exhibit delayed maturation and reduced diversity compared to that of full-term newborns, and that modifiable environmental exposures would have a stronger impact on early oral microbiome development than biological maturity alone.

Materials and methods

Study population

The study included 23 full-term and 75 preterm newborns admitted to the neonatal ward or the NICU of Severance Hospital. The study included 23 full-term and 75 preterm newborns admitted to the neonatal ward or the NICU of Severance Hospital. Preterm newborns were classified into three subgroups based on gestational age: extremely preterm (EP, < 28 weeks), very preterm (VP, 28–31 weeks), and moderate-to-late preterm (32–36 weeks). Among the preterm group, 21 were EP, 40 were VP, and 14 were moderate-to-late preterm.

The inclusion criteria were as follows: full-term newborns born at Severance Hospital at a gestational age of 37 weeks and preterm newborns admitted to the NICU or neonatal ward at a gestational age of 32 weeks.

The exclusion criteria included newborns with congenital anomalies or other disabilities diagnosed during the preterm newborn screening test and high-risk newborns with a low probability of surviving beyond 1 month. The guardians of all study participants consented to the collection of oral samples and medical records from the children and mothers (Supplemental table S1).

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 [D0] and within 48 h [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. Specifically, the posterior region of the tongue, buccal mucosa, alveolar ridge, and palate were gently swabbed using a sterile cotton swab. Samples were stored in OMNIgene·ORAL OMR-110 collection kits (DNA Genotek, Canada) for DNA preservation.

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), fetal polymorphism (singletons vs. twins), advanced maternal age (< 35 vs. ≥35 years), pregnancy method (artificial vs. natural), and mode of delivery (C/S vs. NSVD).

Medications for preterm newborns were evaluated based on their route of administration (oral vs. non-oral). The medications assessed included ubacillin (ampicillin/sulbactam), claforan (cefotaxime), meropenem, Oneflu, penbrex (ampicillin), and ceftazidime.

Antibiotic and oral medication exposure

Antibiotic administration was confirmed through electronic medical records and was fully documented in individual neonatal case report forms. All antibiotics were administered intravenously (IV) and diluted in normal saline, then delivered using 30–50 cc syringes via peripheral venous access (e.g. hand or foot). The dosing regimen varied based on the clinical condition, typically involving 2–3 doses per day at 8–12-hour intervals. Duration of treatment generally ranged from 3 to 7 days, extending up to 2 weeks in certain cases. As full-term newborns were generally healthy and discharged shortly after birth, antibiotic use was minimal in this group. In contrast, antibiotic administration was common among preterm newborns due to their higher risk of early-onset infections, and treatment was typically initiated within the first days of life.

Oral medication use was defined as the administration of at least one non-antibiotic agent, and included commonly used supplements such as vitamin D3, Alvityl, Galtase, Neocaf Sol, FerrumKid, and Brufen. These agents were typically administered by mixing with expressed breast milk or formula. Although oral medication use was not included in downstream statistical analyses due to heterogeneity in agents and administration patterns, it was documented for descriptive comparison purposes (Supplemental table S1).

DNA extraction, polymerase chain reaction amplification, and sequencing

Genomic DNA was extracted using the FastDNA Spin Kit (MP Biomedicals, USA) following the manufacturer's instructions. Polymerase chain reaction amplification targeted the V3–V4 regions of the 16S rRNA gene using Illumina NexTera primers. The reaction conditions included an initial denaturation at 95°C (3 min), 25 cycles (95°C for 30 s, 55°C for 30 s, 72°C for 30 s), and a final extension at 72°C (5 min). A secondary amplification step (eight cycles) was performed for barcode attachment. Polymerase chain reaction products were verified via 1% agarose gel electrophoresis, visualized with a Gel Doc system (Bio-Rad, USA), and purified using ProNex® Size-Selective Purification System (Promega, UK). DNA

concentration was assessed using the PicoGreen assay (Molecular Probes, Invitrogen, USA). The sequencing was performed at CJ Bioscience, Seoul, Korea, using the Illumina MiSeq platform (Illumina, USA).

Bioinformatics and data analysis

Raw sequence reads underwent quality filtering ($Q < 25$) using Trimmomatic v0.32 [21]. Taxonomic classification was conducted using EzBioCloud and the Expanded Human Oral Microbiome Database (eHOMD v15.1). All analyses were performed using CJ Bioscience's EzBioCloud 16S-based MTP platform.

Sequence processing and amplicon sequence variant (ASV) table construction

Raw paired-end reads were processed using dada2 (v1.32.0) in R [22]. Reads were trimmed (forward: 280 bp, reverse: 260 bp) and filtered for expected errors (> 4 forward, > 6 reverse). ASV inference was performed using the dada2 algorithm, and taxonomy was assigned using a Naive Bayesian classifier with a bootstrap confidence threshold of 50. The ASV table recorded the frequency of each variant across samples.

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 [23] (version 1.48.0). 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 [24,25].

Linear discriminant analysis effect size (LEfSe) was performed using the microbiomeMarker R package [26] (version 1.10.0). Heatmaps were generated using the ComplexHeatmap package [27] (version 2.20.0), and all other plots were created with the ggplot2 package (version 3.5.1).

Statistical analysis

Multivariate dispersion analysis and pairwise permutation-based analysis of variance for β-diversity were conducted using the vegan R package [28] (version 2.6–8). Pairwise t-tests were used to evaluate differences in α-diversity between groups. 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 non-significant differences ($p > 0.05$).

All microbiome statistical analyses were conducted in the R environment (version 4.4.1; R Foundation for Statistical Computing, Vienna, Austria).

Results

To test our hypothesis, we evaluated whether preterm newborns exhibit delayed oral microbiome maturation and reduced diversity compared to full-term newborns, and whether environmental factors play a dominant role over biological maturity. Our findings support this hypothesis, demonstrating significant differences in microbial diversity, taxonomic succession, and functional gene profiles between the two groups, largely driven by modifiable clinical exposures.

Temporal dynamics of α- and β-diversity in the oral microbiome

To evaluate the early developmental trajectory of the oral microbiome, a longitudinal study design was applied, as shown in Figure 1(a). Oral swabs were obtained from full-term newborns ($n = 23$) at birth (Day 0 [D0]) and within 48 h (Day 2 [D2]), reflecting typical discharge timelines. In contrast, preterm newborns ($n = 75$), who typically remain in neonatal care for longer periods, were sampled at birth (D0), 1 week (D7), and 1 month (D28). Sampling and storage procedures were standardized to ensure consistency between groups.

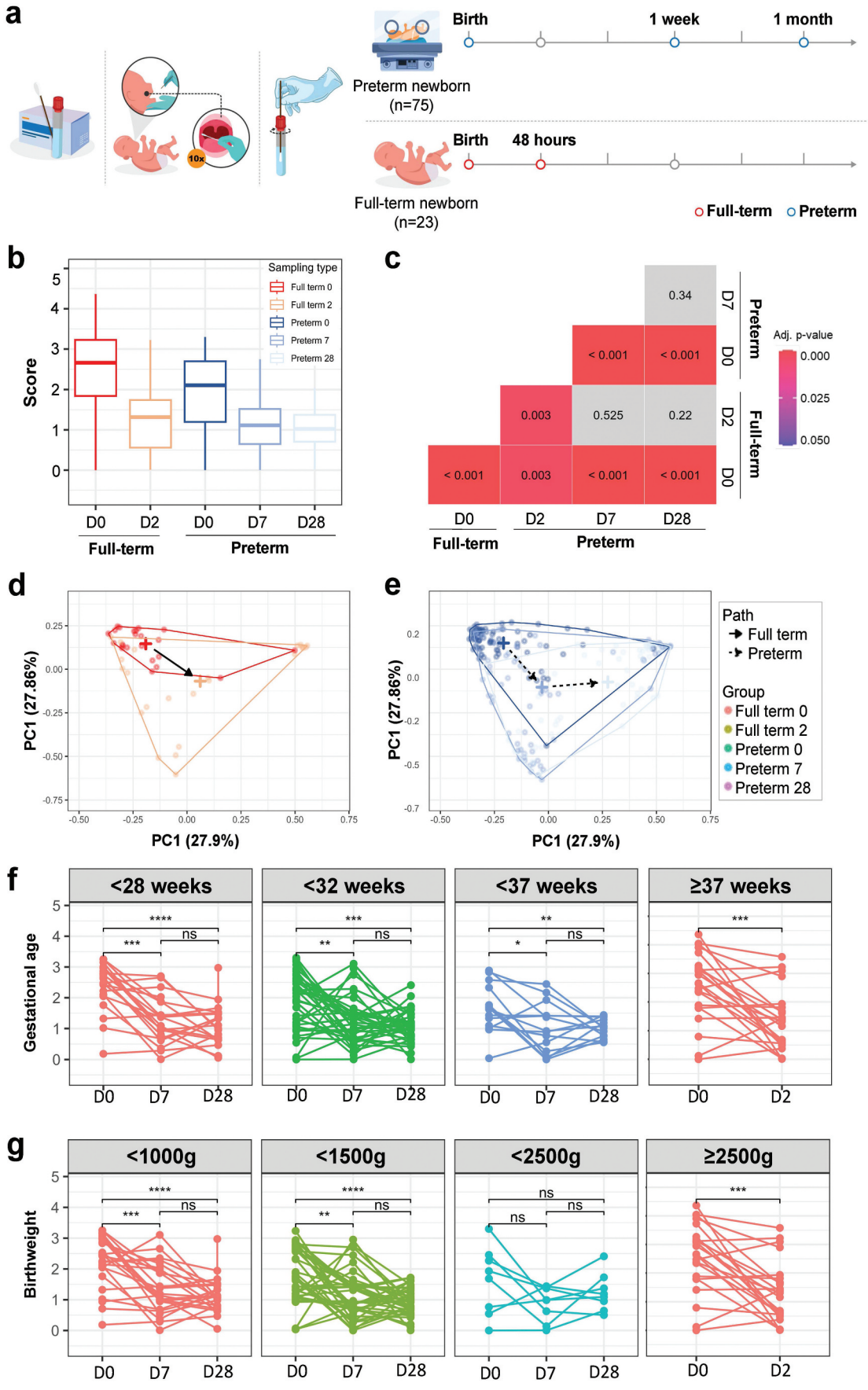

Figure 1. Longitudinal analysis of oral microbiome diversity in full-term and preterm newborns. (a) Schematic overview of the study design and sampling time points. Oral swabs were collected at birth (D0) and 48 h (D2) for full-term newborns, and at birth (D0), 7 days (D7), and 28 days (D28) for preterm newborns. Red indicates full-term newborns, and blue indicates

Analysis of α-diversity revealed a temporal decline in microbial diversity in both full-term and preterm groups (Figure 1(b)). In full-term newborns, the reduction from D0 to D2 was significant ($p < 0.01$). In preterm newborns, diversity decreased significantly from D0 to D7 and from D0 to D28 ($p < 0.001$). Notably, α-diversity remained consistently lower in preterm compared to full-term newborns at all time points. These differences were statistically validated through pairwise t-tests, with false discovery rate-adjusted *p*-values presented in the heatmap (Figure 1(c)).

The β-diversity analysis using principal coordinates revealed distinct temporal microbial trajectories (Figures 1(d,e)). In full-term newborns (Figure 1(d)), samples collected at D0 clustered tightly, while D2 samples were more dispersed, indicating a rapid diversification within 48 h. In preterm newborns (Figure 1(e)), samples exhibited a broader, more gradual dispersion across D0, D7, and D28, suggesting delayed and protracted microbiome development. The arrows between centroids indicate the directional transition of microbial communities over time in each group. This temporal instability in preterm newborns was observed over time.

Impact of gestational age and birthweight on microbial diversity

Microbial diversity was further examined according to gestational age and birthweight through stratified analyses (Figures. 1f,g). Extremely preterm (EP, < 28 weeks), very preterm (VP, 28–31 weeks), and moderate-to-late preterm (MLP, 32–36 weeks) newborns showed more pronounced reductions in α-diversity over time compared to those born at MLP (Figure 1(f)). Notably, EP newborns exhibited the most significant diversity decreases across all time points (D0, D7, and D28; $p < 0.001$), while those born at EP and MLP also showed significant but less steep declines ($p < 0.01$ and $p < 0.05$, respectively). Full-term newborns demonstrated smaller yet significant changes between D0 and D2 ($p < 0.01$).

Similarly, stratification by birthweight revealed that extremely low birthweight newborns (ELBW, < 1,000 g) experienced substantial decreases in α-diversity over time ($p < 0.001$; Figure 1(g)). Newborns weighing Very low birth weight (VLBW, < 1,500 g) also exhibited significant reductions, though to a lesser extent ($p < 0.01$). In contrast, those with birthweight ≥ 2,500 g showed more modest but still significant diversity changes between D0 and D2 ($p < 0.01$).

Factors shaping early oral microbiome composition

Stratified analyses based on fetal polymorphism, maternal age, and pregnancy method revealed distinct trajectories in microbial diversity (Figure 2(a)). Among full-term newborns, α-diversity significantly decreased from D0 to D2 in single births ($p < 0.01$), whereas twins did not exhibit significant changes. In contrast, preterm newborns showed consistent reductions in α-diversity over time regardless of polymorphism status, with significant decreases observed between D0 and D7 and between D0 and D28 ($p < 0.001$).

Maternal age was also associated with oral microbiome diversity. Newborns of mothers < 35 years showed significant declines in α-diversity across all time points in both groups, with particularly pronounced reductions in preterm newborns ($p < 0.001$). Similarly, in newborns of mothers ≥35 years, α-diversity significantly decreased over time, most notably in the preterm group ($p < 0.05$).

The pregnancy method influenced microbial diversity as well. Among full-term newborns, those conceived naturally exhibited significant diversity loss between D0 and D2 ($p < 0.05$). In preterm newborns, both artificial and natural conception were associated with significant reductions in α-diversity over time ($p < 0.01$).

preterm newborns. (b) Box plots showing α-diversity (Shannon index) at each sampling time point. (c) Heatmap showing pairwise comparisons (t-test) of α-diversity across groups, with FDR-adjusted p-values. Significant comparisons are highlighted in red. (d – e) Principal coordinate analysis (PCoA) plots based on β-diversity metrics, illustrating temporal trajectories of microbial community composition in full-term (d) and preterm (e) newborns. (f) Temporal changes in α-diversity stratified by gestational age groups (< 28 weeks, < 32 weeks, < 37 weeks, and ≥37 weeks). (g) Temporal changes in α-diversity stratify by birthweight groups (< 1,000 g, < 1,500 g, < 2,500 g, and ≥ 2,500 g). Statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$; ns, not significant.

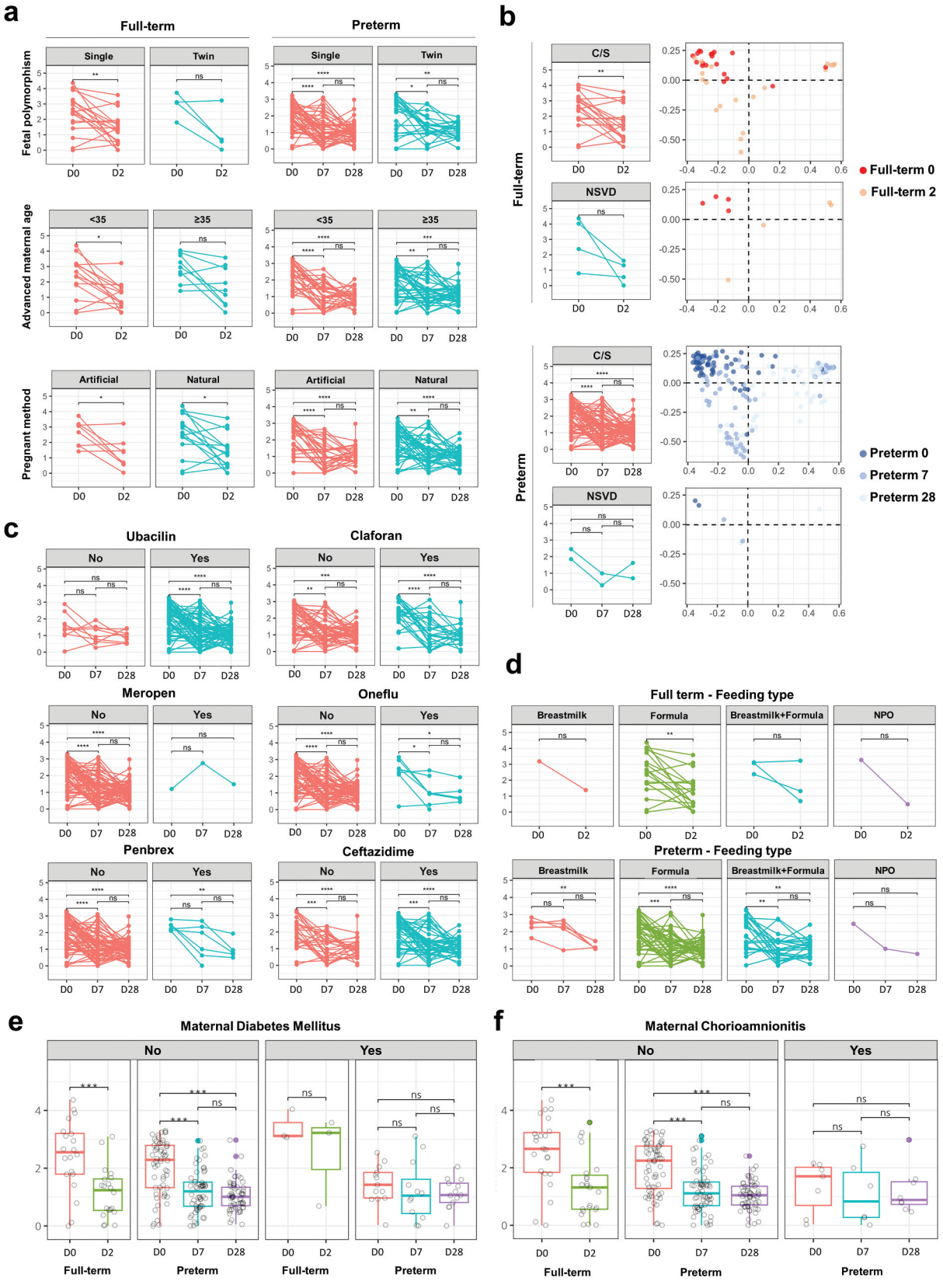

Figure 2. Factors influencing early oral microbiome diversity in full-term and preterm newborns. (a) Temporal changes in α-diversity (Shannon index) stratified by fetal polymorphism (single vs. twin), advanced maternal age (< 35 vs. ≥35 years), and pregnancy method (natural vs. artificial) in full-term and preterm newborns. (b) Principal coordinate analysis (PCoA) plots of β-diversity according to delivery mode [cesarean section (C/S) vs. normal spontaneous vaginal delivery (NSVD)] in full-term and preterm newborns. (c) Temporal changes in α-diversity based on oral administration of antibiotics among

The delivery mode was associated with distinct microbial trajectories (Figure 2(b)). In full-term newborns delivered via C/S, α-diversity significantly declined from D0 to D2 ($p < 0.01$), whereas those born through normal spontaneous vaginal delivery (NSVD) showed no significant changes. In preterm newborns, C/S was linked to notable diversity reductions between D0 and D7 and D0 and D28 ($p < 0.0001$), while NSVD-delivered newborns maintained relatively stable diversity across all time points. Principal coordinates analysis further supported these findings, revealing distinct β-diversity clustering patterns by delivery mode.

Oral antibiotics and feeding type affect microbial diversity in preterm newborns

The relationship between oral antibiotic administration and microbial diversity was assessed in preterm newborns (Figure 2(c)). Significant reductions in α-diversity were observed in newborns administered Ubacillin and claforan from D0 to D28 ($p < 0.001$). Ceftazidime also led to consistently decreased diversity regardless of administration status ($p < 0.001$), with no significant difference between D7 and D28.

Oneflu® was associated with reduced diversity across all time points ($p < 0.05$), with a diminished effect over time. For penbrex(ampicillin), α-diversity significantly decreased between D0 and D28 in both administered ($p < 0.01$) and non-administered ($p < 0.001$) groups. In contrast, newborns who were administered meropenem did not show significant changes, while non-recipients showed marked reductions in diversity ($p < 0.001$). Across all antibiotics, reductions in diversity varied by drug type and time point, with notable effects observed for β-lactam (penicillin and cephalosporin) antibiotics.

Feeding type was also associated with oral microbial diversity (Figure 2(d)). In full-term newborns, those fed with formula exhibited a significant decline in diversity between D0 and D2, while breastfed and mixed-fed newborns did not show significant changes. Among preterm newborns, all feeding types (breastmilk, formula, and mixed) were associated with significant reductions in α-diversity from D0 to D28 ($p < 0.01$). Although a decline in diversity was observed in the nothing per oral group, the change was not significant.

In both full-term and preterm newborns of mothers without diabetes, α-diversity significantly decreased over time ($p < 0.01$), with the most pronounced reductions observed in preterm newborns (Figure 2(e)). In contrast, no statistically significant changes in α-diversity were observed among newborns of diabetic mothers in either group. Likewise, among newborns of mothers without chorioamnionitis, α-diversity significantly declined over time, particularly in preterm newborns ($p < 0.01$) (Figure 2(f)). However, in newborns exposed to chorioamnionitis, no significant differences in diversity were observed across time points.

Taxonomic shifts in early oral microbial composition

Temporal shifts in oral microbiota composition at the phylum level were examined to delineate differences between full-term and preterm newborns (Figure 3(a)). In full-term newborns, *Firmicutes* were the most abundant phylum, increasing from 23.8% at D0 to 55.5% at D2. A simultaneous decrease in *Proteobacteria* was observed, from 47.8% to 15.2%, suggesting rapid microbial succession shortly after birth. *Actinobacteria* increased from 14.1% to 24.8%, while *Bacteroidetes* declined slightly. In preterm newborns, the microbial transition was more gradual. *Firmicutes* rose from 24.2% at D0 to 56.0% by D28. *Actinobacteria* increased from 7.3% to 34.7% over the same period, while *Proteobacteria* sharply declined from 60.9% to 8.3%. *Bacteroidetes*, which initially accounted for 7.5%, nearly disappeared by D28 (0.8%) (Figure 3(b)). These findings indicate a delayed but eventually comparable maturation of the microbial community in preterm newborns compared to their full-term peers.

preterm newborns. 'Yes' indicates antibiotic use; 'no' indicates no antibiotic use. (d) Temporal changes in α-diversity according to feeding type (breastmilk, formula, breastmilk + formula, and nothing per oral [NPO]) in full-term and preterm newborns. (e) Comparison of α-diversity by maternal diabetes mellitus status (yes vs. No) in full-term and preterm newborns. (f) Comparison of α-diversity by maternal chorioamnionitis status (yes vs. No) in full-term and preterm newborns. Statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$; ns, not significant.

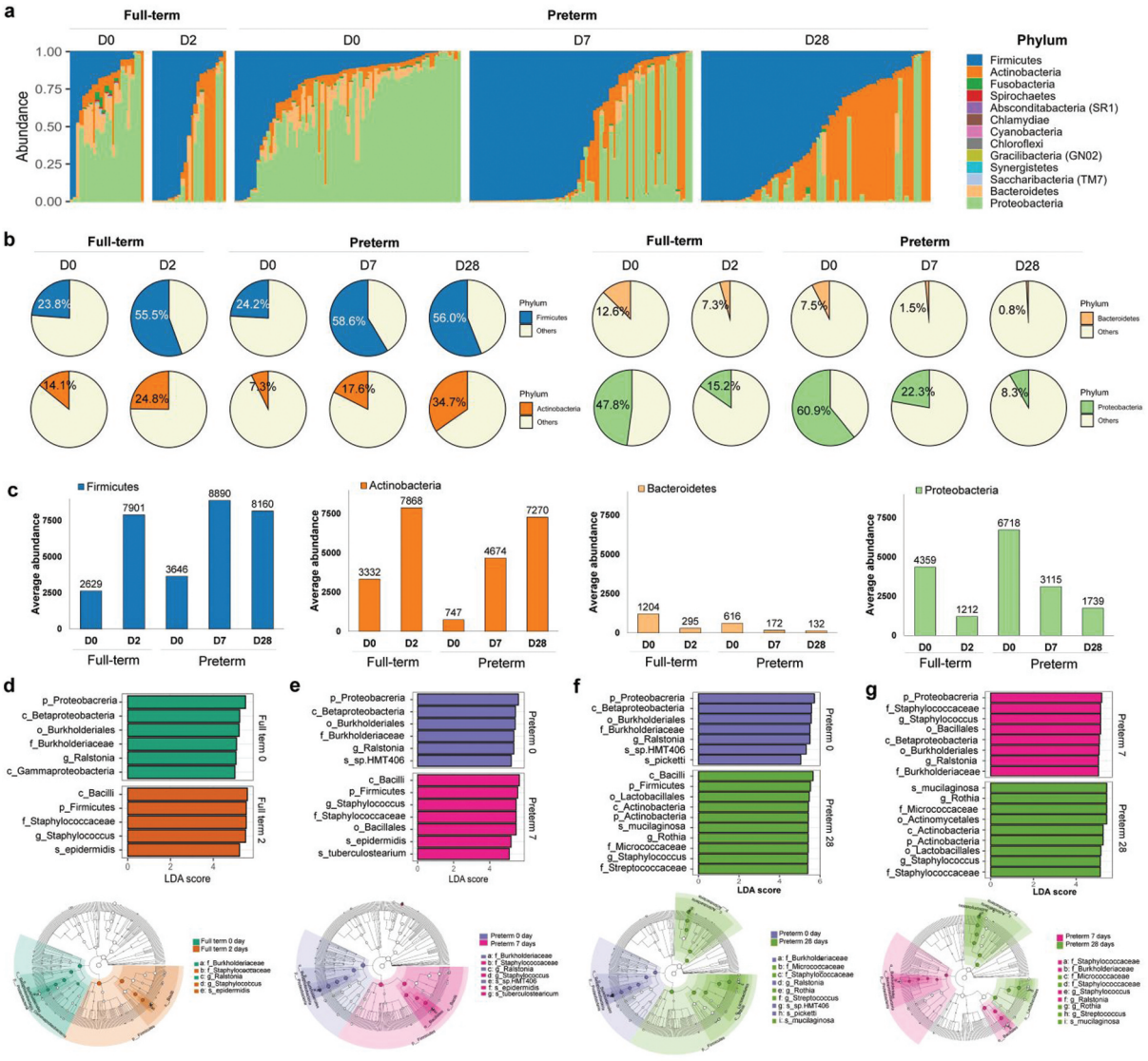

Figure 3. Temporal changes in oral microbiota composition in full-term and preterm newborns. (a) Stacked bar plots showing phylum-level relative abundance of oral microbiota at each sampling time point in full-term (D0, D2) and preterm (D0, D7, D28) newborns. Phylum-level color coding: *Firmicutes* (blue), *Actinobacteria* (orange), *Bacteroidetes* (beige), *Proteobacteria* (green). (b) Pie charts summarizing the relative abundance of dominant phyla (*Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*) at each time point. (c) Bar graphs showing the phylum-level absolute abundance (based on 16S rRNA gene copy number) of *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* in full-term and preterm newborns over time. (d – g) Taxonomic biomarker comparison using linear discriminant analysis effect size (LEfSe) plots across longitudinal time points in full-term and preterm newborns: (d) Full-term newborns at D0 and D2. (e) Preterm newborns at D0 and D7. (f) Preterm newborns at D0 and D28. (g) Preterm newborns at D7 and D28. Consistent with clinical immaturity, preterm newborns showed early enrichment of *Ralstonia*, *Burkholderiaceae*, and *Staphylococcus*, while normalization over time was marked by increasing abundance of *Streptococcus* and *Rothia*. Each LEfSe plot shows taxa enriched at different time points based on linear discriminant analysis (LDA) scores.

Absolute abundance patterns of the dominant bacterial phyla were assessed over time in both full-term and preterm newborns (Figure 3(c)). In full-term newborns, the absolute abundance of *Proteobacteria* markedly declined from 4,359 at birth (D0) to 1,212 by Day 2 (D2), while Bacteroidetes similarly decreased from 1,204 to 295. In contrast, *Firmicutes* exhibited a substantial increase from 2,629 to 7,901, and Actinobacteria rose from 3,332 to 7,861 during the same period. These findings indicate a rapid shift in microbial composition immediately after birth.

In preterm newborns, a more gradual transition was observed. The abundance of *Proteobacteria* decreased from 6,713 at D0 to 1,739 at D28, and Bacteroidetes declined steadily from 616 to 132.

Meanwhile, *Firmicutes* increased from 3,646 at D0 to 8,890 at D7 and remained high (8,160) at D28, and *Actinobacteria* showed a consistent upward trend from 747 to 7,270. These temporal dynamics reflect delayed but progressive microbial succession in preterm newborns, ultimately approaching levels comparable to those of full-term newborns by the end of the first month of life.

LEfSe analysis was performed to identify the differentially abundant taxa associated with specific time points and groups (Figures 3(d–f)). At D0, *Proteobacteria*-related taxa, including *Burkholderiales* and *Ralstonia*, dominated in both groups. By D2 in full-term newborns and D7 in preterm newborns, *Firmicutes* such as *Staphylococcus* and *Staphylococcaceae* became predominant, reflecting a rapid community shift. By D28, preterm newborns exhibited further diversification with the enrichment of *Actinobacteria* (e.g. *Rothia*, *Micrococcaceae*) and *Firmicutes* (e.g. *Lactobacillales*, *Streptococcaceae*), signaling a transition toward a more stable and functionally complex microbiota. The distinct succession patterns between full-term and preterm newborns emphasize the influence of postnatal age and developmental maturity on early oral microbial colonization.

To further assess how perinatal factors influenced microbial composition, we examined the absolute abundance of dominant bacterial phyla (Firmicutes, Actinobacteria, Bacteroidetes, and Proteobacteria) across clinical subgroups, including gestational age, birthweight, delivery mode, feeding type, and fetal polymorphism (Supplementary Fig. S1). Among preterm newborns, cesarean delivery and formula feeding were generally associated with lower abundance of beneficial phyla such as Firmicutes and Actinobacteria over time. In contrast, vaginal delivery and breastmilk feeding were linked to increased abundance of these taxa. These observations support the notion that perinatal clinical factors contribute to shaping early oral microbial succession in both diversity and composition.

Functional microbial profiles and pathogen-gene-disease associations

To investigate the potential pathogenic impact of early oral microbiota, we explored pathogen-gene-disease networks through heatmap and network-based analyses (Figures 4(a–f)). These analyses revealed dynamic shifts in disease-related microbial signatures over time, with marked differences between full-term and preterm newborns.

In full-term newborns, *Escherichia coli* was the most prominent pathogen at D0, showing strong associations with neonatal meningitis, urinary tract infection, and hemolytic uremic syndrome, supported by the expression of multiple virulence-related genes (Figure 4(a)). By D2, *Streptococcus pneumoniae* emerged as the dominant species, linked to respiratory and systemic conditions such as pneumococcal pneumonia and meningitis. Key virulence factors, including pneumococcal surface protein A (pspA), neuraminidase A (nanA), and autolysin (lytA), were enriched, suggesting enhanced immune evasion and host colonization (Figure 4(b)).

In preterm newborns, a similar transition occurred with delayed timing. At D0, *E. coli* predominated and was strongly associated with systemic diseases (Figure 4(c)). By D7, the dominant pathogens shifted to *S. pneumoniae* and *Serratia marcescens*, which are both linked to pneumonic and septic presentations. Associated genes included pspA and nanA, reinforcing their relevance in early neonatal infections (Figure 4(d)).

By D28, preterm newborns demonstrated further microbial maturation with the emergence of *Staphylococcus aureus* alongside *S. pneumoniae* (Figure 4(e)). These pathogens were connected to a broader disease spectrum, including skin infections, food poisoning, and toxic shock syndrome. Functionally, genes such as alpha-hemolysin (hla) and staphylococcal cysteine protease A (scpA) were involved in epithelial barrier disruption and immune modulation (Figure 4(f)).

Group-level microbial and functional disparities between full-term and preterm newborns

To explore the potential functional implications of microbial colonization, taxonomic biomarker analysis and network-based profiling were conducted to compare oral microbiota between full-term and preterm newborns (Figures 5(a–c), Supplementary Figs. S2 & S3).

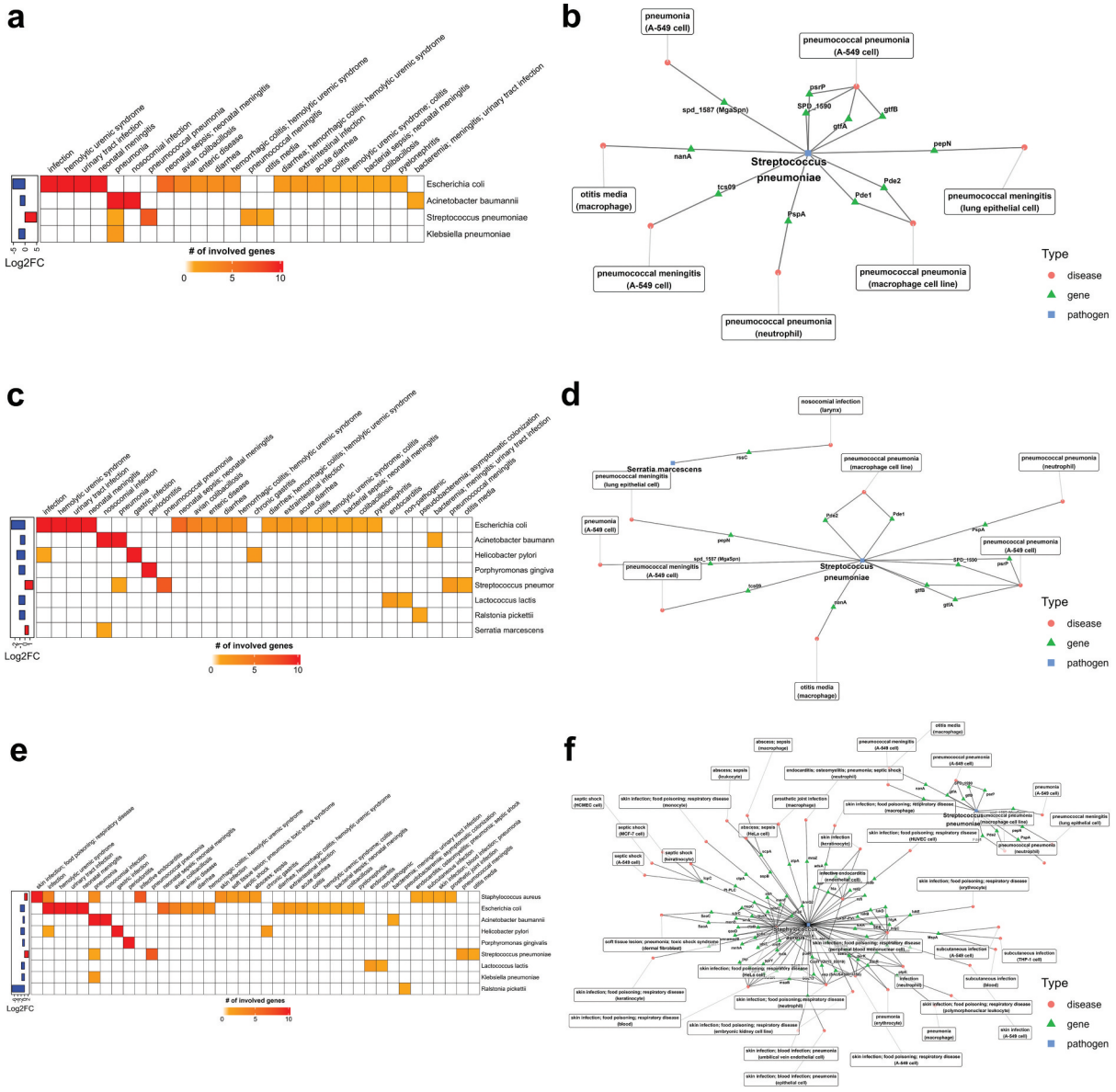

Figure 4. Temporal comparison of pathogen-gene-disease associations across sampling time points in full-term and preterm newborns. (a) Heatmap showing pathogen-disease associations based on virulent genes enriched at D2 compared to D0 in full-term newborns. Color intensity indicates the number of genes involved (red = higher number). (b) Network visualization illustrating disease – pathogen – gene relationships associated with *Streptococcus pneumoniae*-enriched genes at D2 in full-term newborns. (c) Heatmap showing pathogen – disease associations based on virulence genes enriched at D7 compared to D0 in preterm newborns. (d) Network visualization illustrating disease-pathogen-gene relationships associated with *Serratia marcescens*- and *Streptococcus pneumoniae*-enriched genes at D7 in preterm newborns. (e) Heatmap showing pathogen – disease associations based on virulence genes enriched at D28 compared to D0 in preterm newborns. (f) Expanded network visualization showing multiple pathogen-gene-disease connections associated with virulence gene enrichment at D28 in preterm newborns. In network diagrams, nodes represent diseases (squares), genes (triangles), and pathogens (circles).

LEfSe analysis (Figure 5(a)) identified distinct taxa enriched in each group. In full-term newborns, a broad array of *Bacteroidetes*, *Firmicutes* (*Clostridia*, *Lactobacillus*), and *Proteobacteria* taxa (*Zoogloea*, *Comamonadaceae*) were significantly enriched. In contrast, preterm newborns showed enrichment of potentially pathogenic taxa such as *Mycoplasma* and *Streptococcus pneumoniae*, suggesting delayed microbial maturation and increased susceptibility to infection.

A volcano plot analysis further highlighted group-specific differentially abundant species (Figure 5(b)). Full-term newborns were enriched in beneficial or commensal taxa, including *Lactobacillus*, *Staphylococcus*

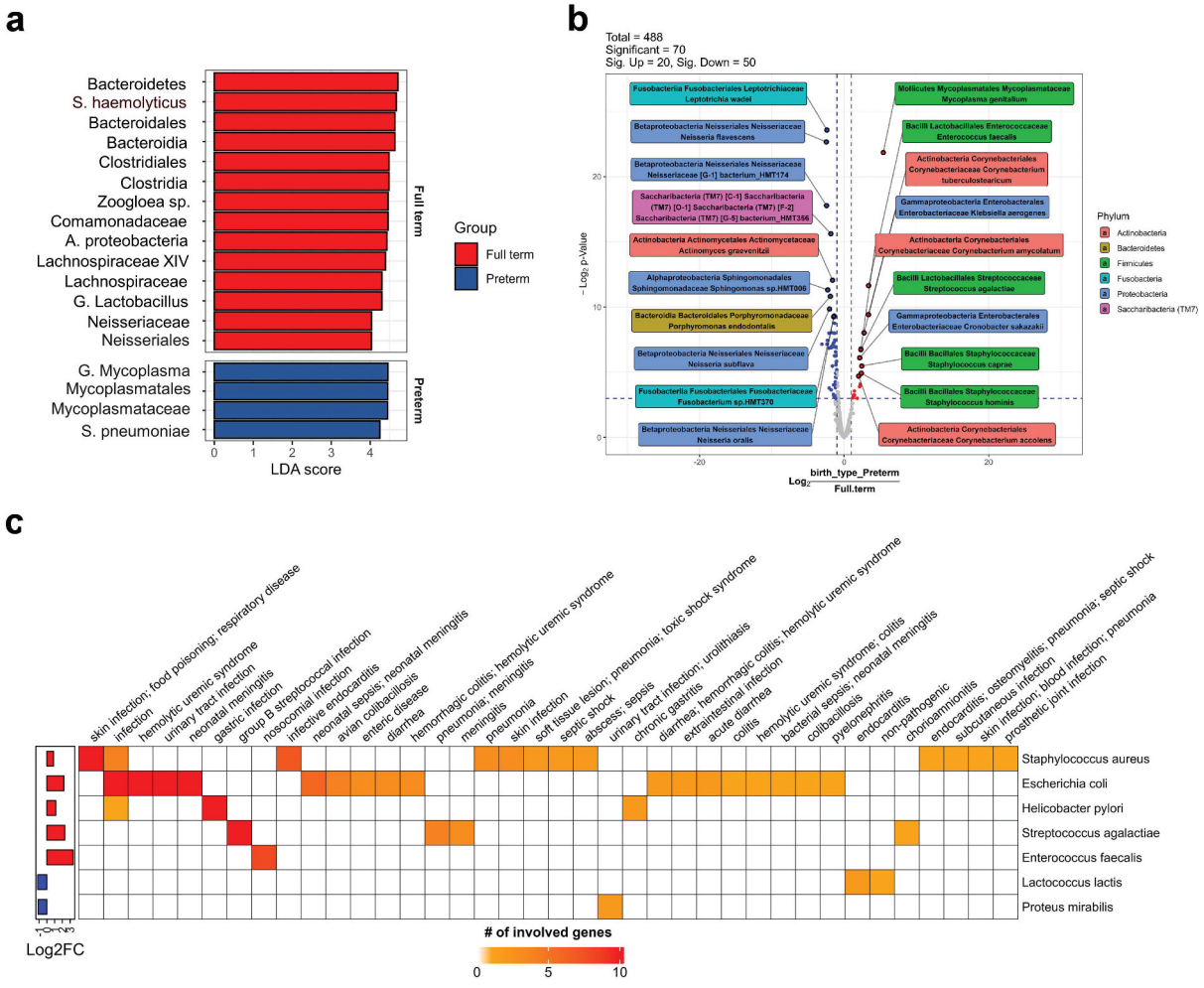

Figure 5. Overall comparison of pathogen-gene-disease associations between full-term and preterm newborns. (a) Linear discriminant analysis effect size (LEfSe) plot identifying taxa differentially abundant between full-term and preterm newborns based on linear discriminant analysis (LDA) scores. Red bars indicate taxa enriched in full-term newborns, and blue bars indicate taxa enriched in preterm newborns. (b) Volcano plot showing species-level differentially abundant taxa between full-term and preterm newborns. Significant taxa ($p < 0.05$) are highlighted by color according to phylum: *Actinobacteria* (yellow), *Firmicutes* (green), *Proteobacteria* (blue), *Bacteroidetes* (orange), and *Saccharibacteria* (purple). Taxa enriched in full-term newborns are located on the right, and those enriched in preterm newborns on the left. (c) Heatmap displaying pathogen – disease associations based on virulence genes enriched in preterm newborns compared to full-term newborns. Color intensity represents the number of associated genes (red = higher number).

caprae, and *Enterococcus faecalis*, while preterm newborns exhibited a higher relative abundance of taxa associated with dysbiosis and inflammation, such as *Mycoplasma genitalium* and *Corynebacterium* species. These shifts were predominantly observed in *Firmicutes* and *Proteobacteria* phyla.

To examine the pathogenic potential, a heatmap was constructed based on gene-level associations with known infectious diseases (Figure 5c). Full-term newborns showed stronger associations between pathogens such as *E. coli*, *S. pneumoniae*, and disease categories like meningitis, urinary tract infection, and foodborne illness. Preterm newborns, although less diverse, exhibited unique associations with pathogens, including *Staphylococcus aureus*, *Lactococcus lactis*, and *Proteus mirabilis*, which were linked to conditions such as skin infection, endocarditis, and toxic shock syndrome.

Supplementary network diagrams (Supplementary Figs. S2 & S3) visually mapped gene-disease-pathogen relationships. Supplementary Figure S2 shows dense connections between *E. coli* and numerous diseases via virulent genes, while *S. aureus* exhibited multiple links to respiratory and skin infections through genes like hla and scpA. Supplementary Figure S3 highlights focused gene-pathogen interactions in *P. mirabilis* and *L. lactis*, demonstrating their association with urinary and bloodstream infections, respectively. This cross-

sectional analysis underscored group-level disparities, with full-term newborns favoring beneficial taxa and preterm newborns exhibiting a pathogen-skewed profile.

These functional insights underscore the importance of early oral microbiome composition. While the enrichment of certain virulence-associated genes in preterm newborns suggests potential for increased infection susceptibility, it is also important to consider that microbial exposure during this period may play a role in immune training.

Overall, despite gradual microbial development over time, preterm newborns consistently displayed reduced diversity and functional profiles associated with increased disease risk compared to their full-term counterparts, highlighting distinct microbial developmental trajectories by birth status.

Discussion

Previous studies have shown that preterm newborns tend to develop their oral microbiomes more slowly and with lower microbial diversity [11,13]. Consequently, they may be more susceptible to colonization by harmful bacteria that can lead to infections [14]. A dominance of *Proteobacteria* and reduced abundance of beneficial commensals have been recognized as hallmarks of dysbiosis in preterm newborns [14]. These microbial imbalances are frequently associated with prolonged hospitalization and medical intervention [7,8].

In this study, we observed that preterm newborns maintained significantly lower α-diversity and greater β-diversity during the early postnatal period compared to full-term newborns, indicating a delayed and heterogeneous pattern of microbial development. Although relative abundance data showed a rapid transition toward *Firmicutes* and *Actinobacteria* in full-term newborns, absolute abundance data confirmed a significant increase in these phyla alongside a concurrent decline in *Proteobacteria*. In preterm newborns, the absolute abundance of *Firmicutes* and *Actinobacteria* also increased over time, indicating gradual microbial maturation, despite the persistent high relative proportion of *Proteobacteria* at early time points. These patterns may reflect delayed but ongoing microbial maturation rather than pathology. Indeed, early exposure to *Proteobacteria* may contribute positively to immune system development and mucosal adaptation. It is therefore important to recognize that the presence or prolonged dominance of certain microbial taxa, such as *Proteobacteria*, does not necessarily indicate a pathological state, but may instead reflect normal variability in early immune – microbial co-development.

Physiological immaturity is known to impact microbial establishment, particularly in newborns born before 28 weeks of gestation or with very low birthweight (< 1,000 g) [2]. Our findings reinforce this, as we observed the most pronounced reductions in diversity among extremely preterm and low-birthweight newborns, supporting the idea that underdeveloped immune and mucosal systems may impede colonization by beneficial microbes.

It has also been established that feeding practices and delivery methods shape early microbiome development. Breastmilk is known to support the growth of beneficial bacteria [15,29,30], while C/S limits the vertical transmission of maternal microbiota [15,29,30]. Our study found that breastfeeding was associated with relatively stable oral microbial diversity, while C/S and formula feeding were linked to more substantial diversity loss, especially in preterm newborns. These findings support the role of maternal and environmental exposures in modulating early microbial colonization.

Interestingly, a significant reduction in microbial diversity was observed over time in newborns of mothers aged ≥35 years, particularly among preterm newborns. This pattern may be partly explained by maternal or perinatal factors more commonly associated with advanced maternal age, such as higher rates of cesarean delivery, perinatal antibiotic exposure, or age-related changes in the maternal microbiota. These factors could have influenced early microbial colonization in neonates. While the precise mechanism remains unclear, these findings raise the possibility that maternal age may modulate early oral microbiome development, especially in vulnerable preterm populations.

Additionally, we explored the potential impact of maternal clinical conditions, including gestational diabetes and chorioamnionitis, on neonatal oral microbiome diversity. It is possible that neonates born to mothers with medical conditions received earlier or more intensive interventions – such as prophylactic antibiotics, delayed feeding initiation, or extended supportive care – that modulated early microbial succession. Furthermore, intrauterine microbial exposure in the context of chorioamnionitis or maternal

hyperglycemia may have preconditioned the neonatal mucosal environment, attenuating abrupt postnatal shifts. Alternatively, these findings could be influenced by sample size limitations or heterogeneous clinical variables within each subgroup.

Furthermore, while the current study did not identify a direct statistical association between maternal age and specific outcomes such as feeding practices or late-onset sepsis, it remains plausible that these intermediate variables may contribute to the observed diversity patterns. Further studies are needed to elucidate the potential mediating role of such clinical factors in the relationship between maternal age and neonatal oral microbiome development.

Antibiotic exposure is another critical factor influencing neonatal microbiota [16]. Our study expands on prior work by identifying that β-lactam antibiotics, particularly claforan(cefotaxime) and ceftazidime, significantly reduced microbial diversity. The effect size varied by antibiotic type, highlighting the importance of considering specific drug mechanisms and timing of administration. This suggests that preterm microbial development is not only vulnerable to disruption but also highly sensitive to medical intervention. Additionally, our findings support previous reports emphasizing that antibiotic exposure in early life, particularly β-lactam agents, can drastically alter the oral microbial environment, reducing diversity and favoring the emergence of opportunistic pathogens [16,31]. Frequent antibiotic use in NICU settings has been linked to long-term alterations in microbiota composition and increased susceptibility to systemic infections and inflammatory diseases [32,33]. Additionally, environmental and clinical factors in NICUs, including prolonged mechanical ventilation and parenteral nutrition, have been shown to hinder normal microbial colonization processes [34,35].

Functionally, the oral microbiota of preterm newborns showed increased expression of virulence-related genes such as *pspA*, *nanA*, *hla*, and *scpA*, which are known to facilitate immune evasion, compromise epithelial integrity, and promote systemic dissemination of pathogens [36–38]. This molecular profile is consistent with the elevated incidence of severe infections – such as sepsis, pneumonia, and necrotizing enterocolitis – commonly observed in this population [39]. Taxonomically, our results are consistent with previous reports identifying *Streptococcus*, *Staphylococcus*, and *Rothia* as dominant oral commensals in full-term newborns [5,40]. In contrast, preterm newborns in our study had *E. coli*, *Serratia marcescens*, and *S. aureus* as the dominant species, indicating a less mature and more infection-prone microbiome.

From a taxonomic perspective, our findings align with previous studies reporting *Streptococcus*, *Staphylococcus*, and *Rothia* as predominant oral commensals in full-term newborns [5,40]. In contrast, preterm newborns in our cohort were primarily colonized by opportunistic pathogens including *Escherichia coli*, *Serratia marcescens*, and *Staphylococcus aureus*, reflecting a delayed and dysbiotic colonization pattern. Cross-sectional comparisons further revealed that preterm newborns harbored less diverse microbial communities with a higher burden of pathogen-associated functional signatures compared to their full-term counterparts. Altogether, these results suggest that preterm newborns may be predisposed to early-life respiratory and systemic infections due to an immature and pathogen-enriched oral microbiome. In contrast, while full-term newborns exhibited rapid microbial shifts within the first 48 hours, their routine discharge limited the duration of follow-up. Therefore, interpretations regarding longer-term microbial development in this group should be made with appropriate consideration of this temporal constraint.

These findings should also be interpreted considering several limitations. First, although the overall cohort size was relatively larger than in previous studies, the small number of full-term newborns may have limited the statistical power for subgroup analyses. Second, the limited sampling window for full-term newborns (up to Day 2) restricts direct comparison with the longer-term microbial trajectories observed in preterm newborns sampled up to Day 28. The study was designed to characterize immediate postnatal shifts in microbial composition, rather than to provide paired longitudinal data. This limitation highlights the need for future research incorporating extended follow-up of full-term newborns to better capture long-term oral microbiome development. Third, while the analysis focused on antibiotic exposure, comprehensive data on non-antibiotic oral medications – such as nutritional, digestive, and metabolic supplements – were also collected from all participants. Although not included in the statistical models due to agent heterogeneity, these medications were commonly administered in preterm newborns and may have influenced early microbial dynamics. Therefore, future studies should investigate the role of oral supplement exposure in more detail. Finally, while 16S rRNA gene sequencing enabled broad

taxonomic and inferred functional analysis, it lacks the strain-level resolution and direct metabolic profiling offered by shotgun metagenomics. Therefore, the virulence gene predictions should be interpreted with caution.

Future multicenter studies employing larger and more diverse populations, combined with high-resolution metagenomic approaches, will be essential to validate and extend our findings and to better understand the mechanisms underlying early microbial dysbiosis in preterm newborns.

Conclusions

Our results suggest that in preterm newborns, the development of the oral microbiome is influenced more by external factors – such as the NICU environment, antibiotic use, and feeding practices – than by the newborn's biological maturity alone. The delayed and dysbiotic microbial trajectories observed in preterm newborns, coupled with the enrichment of virulence-associated genes, suggest a heightened vulnerability to systemic and respiratory infections during the neonatal period. These results highlight the importance of early-life interventions – such as promoting breastfeeding and limiting unnecessary antibiotic exposure – as potential strategies to foster a more balanced and resilient oral microbiome in preterm populations.

Author contributions

CRediT: **Taeyang Lee:** Formal analysis, Investigation, Methodology, Resources, Validation, Writing – original draft; **Hyun-Yi Kim:** Data curation, Investigation, Software, Validation; **Jung Ho Han:** Data curation, Resources, Supervision, Validation; **Jeong Eun Shin:** Data curation, Investigation, Resources, Validation; **Na-Young Song:** Investigation, Methodology, Supervision, Validation; **Won-Yoon Chung:** Investigation, Methodology, Supervision, Validation; **Chung-Min Kang:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Disclosure statement

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ORCID

Taeyang Lee http://orcid.org/0000-0002-2189-7692
Jung Ho Han http://orcid.org/0000-0001-6661-8127
Jeong Eun Shin http://orcid.org/0000-0002-4376-8541
Na-Young Song http://orcid.org/0000-0001-8658-7049
Won-Yoon Chung http://orcid.org/0000-0001-8428-9005
Chung-Min Kang http://orcid.org/0000-0001-7813-3741

Data availability statement

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical approval

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 and ethical guidelines, including the Declaration of Helsinki.

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