Computational and Structural Biotechnology Journal 27 (2025) 937–945

Contents lists available at ScienceDirect

Computational and Structural Biotechnology Journal

journal homepage: www.elsevier.com/locate/csbj

Research article

Altered heme metabolism and hemoglobin concentration due to empirical antibiotics-induced gut dysbiosis in preterm infants

Seung Hyun Kim [a,b,1], Min-Jin Kwak [c,1], Jae Kyoon Hwang [a,1], Jihyun Keum [d], Hee Yeon Jin [e], Chan-Yeong Lee [f], Rahul Sadashiv Tanpure [f], Yong Joo Kim [a], Jeong-Kyu Hoh [d], Jae Yong Park [e], Woojin Chung [g], Byong-Hun Jeon [f,*], Hyun-Kyung Park [a,*]

[a] Department of Pediatrics, Hanyang University College of Medicine, Seoul 04763, South Korea
[b] Department of Pediatrics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 06351, South Korea
[c] Emerging Pathogens Institute, University of Florida, Gainesville, FL 32611, USA
[d] Department of Obstetrics and Gynecology, Hanyang University College of Medicine, Seoul 04763, South Korea
[e] Division of Microbiome, Int-Gen Company, Seoul 04799, South Korea
[f] Department of Earth Resources and Environmental Engineering, Hanyang University, Seoul 04763, South Korea
[g] Department of Civil & Energy System Engineering, Kyonggi University, Suwon 16227, South Korea

ARTICLE INFO

Keywords:
Antibiotics
Preterm infants
Gut microbiome
16S rRNA gene
Illumina sequencing
PICRUSt2

ABSTRACT

Background: High-risk infants are usually treated with empirical antibiotics after birth, regardless of the evidence of infection; however, their gut microbiome and metabolome have seldom been studied. This study investigated the influence of antibiotic exposure on the gut microbiome and associated metabolic pathways in term and preterm infants.

Methods: Thirty-six infants within 10 days of birth who were admitted to a neonatal intensive care unit/newborn nursery unit were divided into four groups based on maturity (gestational age) and use of empirical antibiotics. Genomic DNA was extracted from the fecal samples and underwent high-throughput 16S rRNA amplicon sequencing using the Illumina platforms. Taxonomic classification, diversity analysis, and metagenomic function prediction were performed.

Results: Preterm infants with empirical antibiotics showed a significantly decreased population of *Firmicutes* ($p = 0.003$) and an increased population of *Proteobacteria* ($p < 0.001$) compared to other groups. At the genus level, the populations of *Raoultella* ($p = 0.065$) and *Escherichia* ($p = 0.052$) showed an increased trend. The change in microbial composition was correlated with increased heme biosynthesis and decreased hemoglobin levels.

Conclusion: Collectively, our finding suggested that empirical antibiotic exposure in preterm infants alters the gut microbiome, potentially leading to adverse health outcomes. This dysbiosis may affect heme metabolism, increasing the risk of anemia in these vulnerable infants. Therefore, antibiotic use should be carefully tailored to minimize potential harm.

1. Introduction

Recently, the neonatal gut microbiome has attracted considerable attention. At birth, neonates have a low diversity and abundance of gut microbiome that can be easily disturbed depending on their maturity and type of care received, including antibiotic use [1–3]. The difficulty in distinguishing symptoms in preterm infants from actual infection risk has resulted in exposure to empirical antibiotics early in life. Consequently, perturbation of the gut microbiome in early life may affect both short- and long-term health outcome [4–6]. The effects of antibiotic therapy on the gut microbiome of term and preterm infants and on gut microbial metabolism are of emerging interest.

Microbial colonization is influenced at birth by the uterine environment, mode of delivery, type of feeding, and antibiotic administration [7–9]. The overuse of antibiotics early in life can disrupt the gut–brain axis, which could have lifelong consequences on health.

* Corresponding authors.
E-mail addresses: bhjeon@hanyang.ac.kr (B.-H. Jeon), neopark@hanyang.ac.kr (H.-K. Park).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.csbj.2025.03.009
Received 11 November 2024; Received in revised form 4 February 2025; Accepted 4 March 2025
Available online 5 March 2025

Recent studies using a multi-omics approach have demonstrated that antibiotic intervention in preterm infants perturbs the early life gut microbiome, metabolome, and inflammatory environment in ways that may be consequential to health and development [9–11]. However, the effects of antibiotic exposure on the gut microbiome and metabolites in preterm infants remain underexplored, and differences between preterm and term infants in microbiome composition due to the use of antibiotics have seldom been studied.

The impact of antibiotics on the gut microbiome has been revealed in greater depth through the application of high-throughput sequencing technologies in both animal and human trials [12,13]. In this study, we explored the impact of early empirical antibiotic use on microbial composition in term and preterm infants to understand how antibiotic treatment influences microbiome development in the early perinatal period. This study investigates changes in the gut microbiome within the first 10 days of life in infants considering maturity and use of empirical antibiotics. We utilized high-throughput amplicon sequencing for comprehensive analysis of the gut microbiome. Furthermore, inference of metabolic pathways and investigation of relevant laboratory test results were conducted based on changes in the gut microbiome.

2. Methods

2.1. Study population

We prospectively enrolled 36 infants (21 term and 15 preterm infants) admitted to the newborn nursery unit (NB) or neonatal intensive care unit (NICU) of Hanyang University Hospital, Seoul, Korea, between September 2021 and January 2022. Written informed consent was obtained from the parents of all the infants. This study was approved by the Institutional Review Board of Hanyang University Medical Center (IRB No. 2021–03–017). All methods were performed in accordance with standard human research ethics guidelines (Declaration of Helsinki) and regulations. The term infant is defined as a neonate born at or after 37 weeks of gestational age (GA), and a preterm infant is born before 37 weeks of GA. Fecal samples were collected within 10 days of birth. Stool samples were collected by a trained pediatrician and used to analyze the gut microbiome profiles of the infants. The study population was divided into four subgroups based on use of empirical antibiotics and GA: antibiotic-treated group of term infants (TA), antibiotic-treated group of preterm infants (PA), antibiotic-free group of term infants (TF), and antibiotic-free group of preterm infants (PF). The flow chart of this study is presented in Fig. 1. Infants in antibiotics-treated groups (TA, PA) received empirical antibiotics treatment in the absence of evidence of infection or unstable vital status. The empirical antibiotics regimen were ampicillin and gentamicin. Clinical data of the study population were obtained from the medical records. Hematological and biochemical parameters, including complete blood count with differential, were routinely conducted when infants were admitted to the NICU.

2.2. Fecal DNA extraction and quantification

Feces were collected and stored at –80 °C in a deep freezer. A DNeasy PowerSoil Pro Kit (QIAGEN, Germany) was used for genomic DNA extraction from the samples according to the manufacturer's instructions. The concentration and purity of the genomic DNA were analyzed using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA).

2.3. Library preparation and sequencing

Sequencing libraries were prepared according to Illumina 16S Metagenomic Sequencing Library protocols. DNA were polymerase chain reaction (PCR)-amplified, and the cycle conditions for the first PCR were as follows: heat activation at 95 °C for 3 min; 25 cycles of 30 s at 95 °C, 10 s at 78 °C, 60 s at 50 °C, and 60 s at 72 °C; followed by a final extension at 72 °C for 5 min. The universal primer pairs with Illumina adapter overhang sequences used for amplification were as follows: V3-F:5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and V4- R:5′-GTCTCGTGGGCTCGGAGATGTGTATA

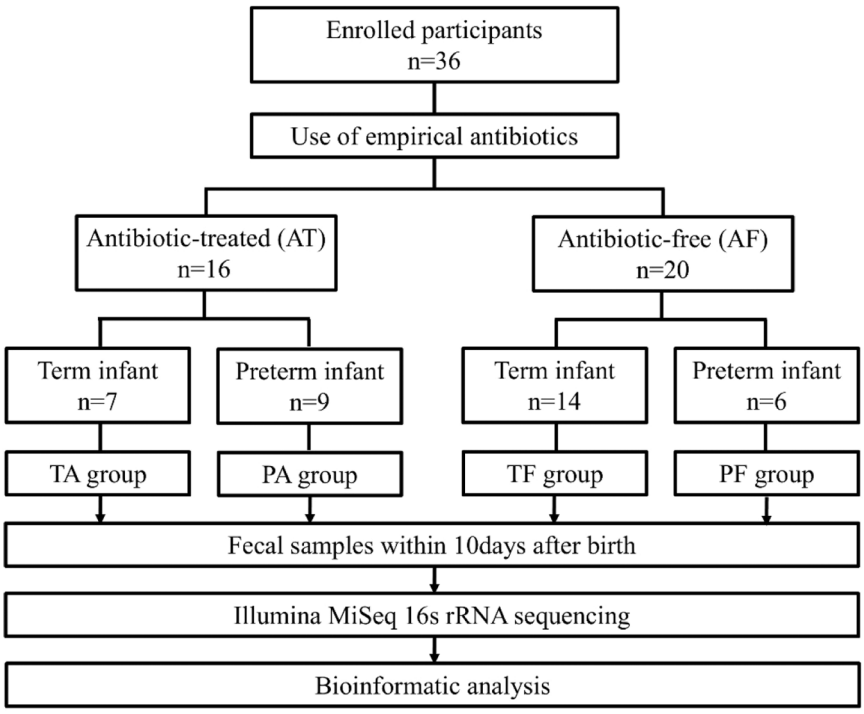

Fig. 1. Flow chart of the study. Enrolled infants were divided into four groups based on the use of empirical antibiotics and maturity (gestational age). TA, term infant with empirical antibiotics; PA, preterm infant with empirical antibiotics; TF, term infant without empirical antibiotics; PF, preterm infant without empirical antibiotics.

AGAGACAGGACTACHVGGG TATCTAATCC-3'. The cycle conditions for the second PCR were the same as those for the first, and the products were purified using AMPure XP beads. To verify the size of the PCR-enriched fragments, template size distribution was assessed using a TapeStation D1000 Screen Tape (Agilent Technologies, Waldbronn, Germany). Targeted amplicon sequencing was performed on the Illumina MiSeq platform using standard Illumina 16S Metagenomic Sequencing Library protocols to obtain FASTQ data, with read counts ranging from 121,470 to 609,040 [14].

2.4. FASTQ quality control and bioinformatics analysis

Illumina sequencing data were obtained and demultiplexed using each barcode. Paired-end reads were merged, and chimeric reads were eliminated. The QIIME2 platform was utilized for Illumina V3–V4 data analysis. OTUs were clustered with 99 % sequence identity using the vsearch cluster-features-de-novo method from the q2-vsearch plugin and annotated with the SILVA 138.1 NR99 database. The generated taxonomy was tabulated from the phylum to the genus level, and relative abundance values were calculated. To measure gut microbial diversity, we employed various indices including ACE, Chao 1, Shannon, and Simpson. ACE and Chao1 indices were used to estimate species richness, as they are particularly sensitive to rare taxa, which are crucial for understanding the microbial diversity in the context of antibiotic-induced dysbiosis. Shannon and Simpson indices were used to assess both richness and evenness, providing a balanced perspective on microbial community structure. Beta diversity was assessed using weighted UniFrac distances and calculated to generate a principal coordinate analysis plot. Statistical differences between the gut microbial taxa were confirmed by analyzing the composition of microbiomes with bias correction (ANCOM-BC). The predicted metagenomic function of the gut microbiome was analyzed using the phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt2) [15], and the predicted gene counts of the bacterial communities were compared with the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways database [16].

2.5. Statistical analysis

Statistical System 9.4 (SAS Institute, Cary, NC, USA) and R statistical software version 4.6.3 (R Foundation) were used to analyze the data in this study. The chi-square test, Mann Whiteny *U* test, and one-way analysis of variance (ANOVA) were performed to investigate clinical characteristics and to analyze laboratory test results. The correlation between hematological and biochemical parameters and gut microbial taxonomy was estimated by Pearson's correlation analysis. Statistically significant differences were defined as $p < 0.05$. In the microbiome analysis, statistical significance was set at an adjusted P (Q value) < 0.05.

3. Results

3.1. Baseline characteristics

The 36 infants were categorized into four groups based on empirical antibiotic use and maturity (GA) as follows: TA group (n = 7), TF group (n = 14), PA group (n = 9) and PF group (n = 6). The characteristics of participants that can influence gut microbial composition are shown in Table 1. Prior to analysis of gut microbiome composition, a univariate analysis was performed to compare factors likely to influence the gut microbiome composition in the perinatal period including GA, birth weight (BW), sex, mode of delivery, maternal antibiotic use, and type of feeding (Table 1). Except for significant differences in GA and BW between term and preterm infant groups, most factors showed no significant differences between the groups.

Table 1
Characteristics of the participants.

	TF (n = 14)	TA (n = 7)	*p*-value	PF (n = 6)	PA (n = 9)	*p*-value
GA (weeks)	38.6 ± 1.2	38.9 ± 0.8	0.489	35.0 ± 2.2	34.9 ± 1.1	0.949
BW (gram)	3071.4 ± 253.5	3300.0 ± 349.2	0.102	2336.7 ± 720.4	2305.3 ± 353.6	0.912
Male, n (%)	10 (71.4)	2 (28.6)	0.159	4 (66.7)	4 (44.4)	0.608
C-section, n (%)	12 (85.7)	4 (57.1)	0.280	4 (66.7)	6 (66.7)	1.000
Maternal AB, n (%)	2 (14.3)	1 (14.3)	1.000	0 (0.0)	2 (22.2)	0.486
Feeding, n (%)	14 (100.0)	7 (100.0)		6 (100.0)	9 (100.0)	
BM, n (%)	0 (0.0)	2 (28.6)	0.100	3 (50.0)	2 (22.2)	0.329

TF, term infant without empirical antibiotics; TA, term infant with empirical antibiotics; PF, preterm infant without empirical antibiotics; PA, preterm infant with empirical antibiotics; GA, gestational age; BW, birth weight; C-section, cesarean section; Maternal AB, maternal antibiotic use before delivery; EN, enteral feeding before sampling; BM, breast milk for enteral feeding.

3.2. Comparison of gut microbial compositions in TA, TF, PA, and PF groups

To understand the effects of antibiotics on gut microbial environments in term and preterm infants, microbiome analysis was conducted using 16S rRNA sequencing. At the phylum level, the population of *Firmicutes* ($p = 0.003$) demonstrated a significant increase in TF and PF groups but a decrease in the PA group (Fig. 2a), which instead showed an increased population of *Proteobacteria* ($p < 0.001$). At the genus level, *Raoultella* ($p = 0.065$) and *Escherichia* ($p = 0.052$) showed an increased trend in infants of the PA group (Fig. 2b). The diversity indices did not differ between groups (Fig. 2c-f). However, the gut microbial communities of PA group showed notable differences compared to the other groups (Fig. 2g).

3.3. Trends of hematological and biochemical parameters in TA, TF, PA, and PF groups

To investigate whether changes in gut microbiome composition among groups were associated with distinct hematological and biochemical differences in infants, we analyzed key parameters, including hemoglobin (Hb), hematocrit (Hct), total bilirubin, and direct bilirubin. Several parameters showed significant differences in the PA group. Specifically, Hb concentration (Fig. 2a, $p = 0.040$) and Hct levels (Fig. 2b, $p = 0.026$) were significantly lower in the PA group compared to the TF group. Additionally, serum total bilirubin levels were lower in the PA group than in the antibiotics-free groups (Fig. 2c; TF: $p = 0.050$; PF: $p = 0.043$). Moreover, serum direct bilirubin levels in the PA group were significantly lower than those in the TF group (Fig. 2d, $p = 0.050$).

3.4. Predicted metagenomic function difference in TA, TF, PA, and PF groups

Metagenomic functional analysis of the gut microbiome was performed using PICRUSt2 based on the KEGG pathways. Heme biosynthesis I (anaerobic) pathway was significantly increased in preterm infants compared to term infants (Fig. 4a, $p = 0.001$). Furthermore, gut microbiome of infants in the PA group showed significant increases in metabolic pathways related to heme metabolism, including heme biosynthesis II (anaerobic) (Fig. 4b, $p = 0.005$), heme biosynthesis from glutamate (Fig. 4c, $p = 0.003$), heme biosynthesis from glycine (Fig. 4d, $p = 0.018$), and heme biosynthesis from uroporphyrinogen (Fig. 4e, $p = 0.014$).

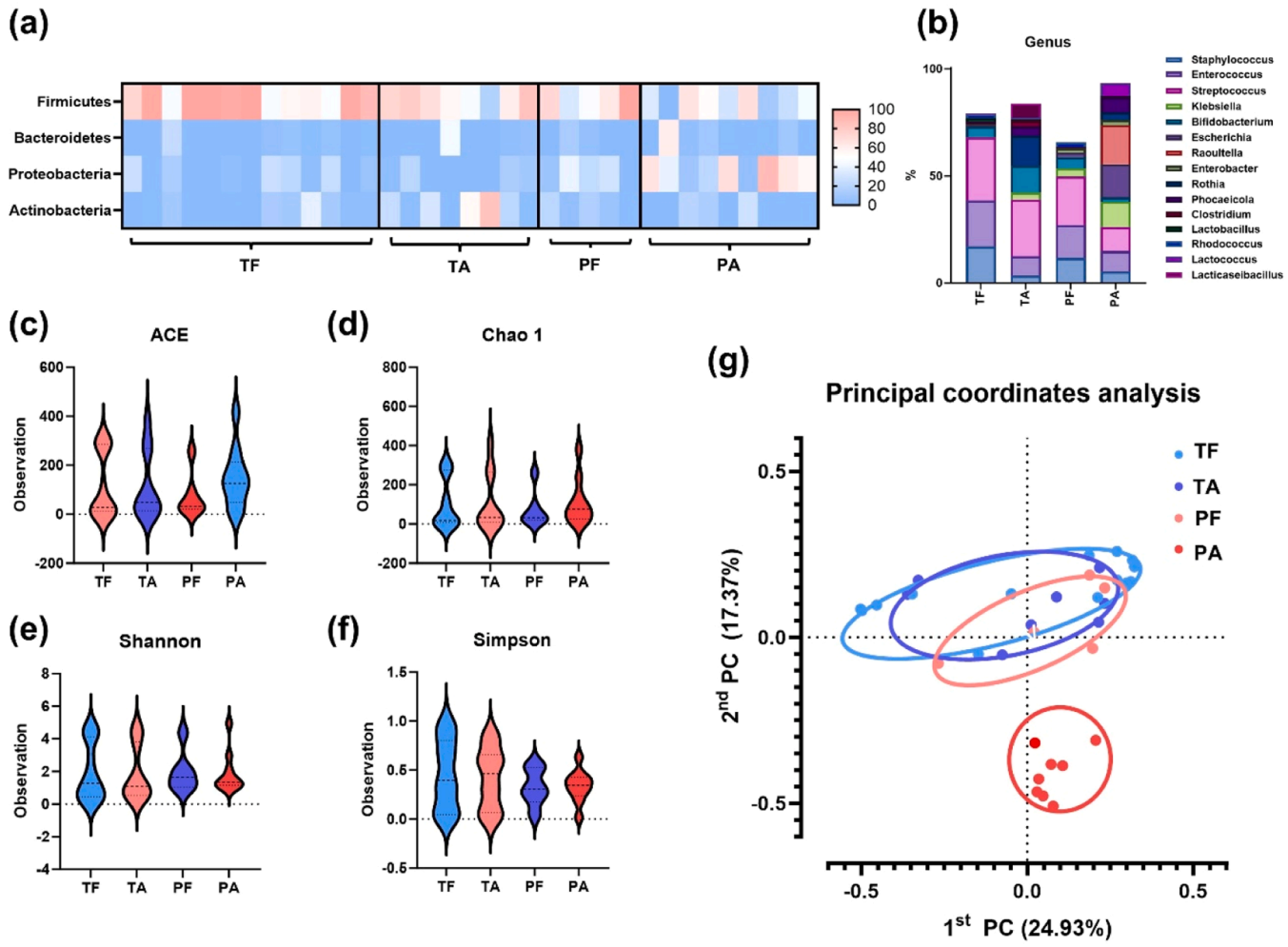

Fig. 2. Gut microbial populations and diversity of TA, TF, PA, and PF groups. (a) gut microbial composition at the phylum level (b) gut microbial composition at the genus level (c-f) α-diversity (ACE, Chao 1, Shannon, and Simpson) indices (g) principal coordinates analysis of gut microbial communities in TA, TF, PA, and PF groups. TA, term infant with empirical antibiotics; PA, preterm infant with empirical antibiotics; TF, term infant without empirical antibiotics; PF, preterm infant without empirical antibiotics.

3.5. Correlation analysis between hematological and biochemical parameters and gut microbiome in TA, TF, PA, and PF groups

The relationship between hematological and biochemical parameters and key microbial taxa was analyzed using linear correlation analysis (Fig. 5a). Hb concentration showed a significant negative relationship with the population of *Proteobacteria* at the phylum level (Fig. 5b, $r^2 = 0.649$, $p < 0.001$) and that of *Klebsiella* at the genus level (Fig. 5c, $r^2 = 0.533$, $p = 0.002$). Similarly, serum Hct was negatively correlated with *Proteobacteria* at the phylum level (Fig. 5d, $r^2 = 0.843$, $p < 0.001$) and with *Klebsiella* at the genus level (Fig. 5e, $r^2 = 0.638$, $p = 0.001$). The populations of *Firmicutes* (Fig. 5f, $r^2 = 0.519$, $p = 0.042$) and *Streptococcus* (Fig. 5g, $r^2 = 0.638$, $p = 0.003$) were positively correlated with the serum total bilirubin levels. Also, the serum direct bilirubin levels had a positive relationship with the populations of *Firmicutes* (Fig. 5h, $r^2 = 0.732$, $p = 0.013$) and *Streptococcus* (Fig. 5i, $r^2 = 0.813$, $p < 0.001$) at the phylum and genus levels, respectively.

4. Discussion

The results of this study expand upon previous findings that early antibiotic use induces alterations in the early-life gut microbiome. We investigated the impact of early empirical antibiotic use on gut microbial communities in consideration of infant maturity, to understand how antibiotic treatment influences microbiome development during early life. Furthermore, we attempted to infer related metabolic pathways using PICRUSt2 and to assess the correlations with hematological and biochemical parameters. The change in microbial composition was pronounced when antibiotics were administered to the preterm infant group, suggesting a potential synergistic effect. A significant finding of this study is that antibiotic use eliminated the dominant bacteria in the gut microbiome and demonstrated a synergistic effect with prematurity in these changes. We predicted significantly upregulated microbial metabolic pathways from reference genomes closely related to the 16S rRNA data. Antibiotic-treated infants showed significantly upregulated gut microbial metabolic pathways, including flagellar assembly and biofilm formation. Although it is just a prediction, this analysis suggests how the gut microbiome responds to antibiotics.

Antibiotics are among the most frequently prescribed medications in NICUs, particularly for preterm infants [17]. Neonatal sepsis is a leading cause of complications and mortality in neonates, as they are highly vulnerable to bacterial infections. The primary pathogens responsible for early-onset sepsis in the neonatal period include Group B *Streptococcus*, *Escherichia coli*, and *Listeria monocytogenes*. To target these organisms, hospitalized infants, especially preterm infants, in the NICU commonly receive empirical antibiotic therapy consisting of ampicillin and aminoglycosides (gentamicin) [18]. It has been reported that the gut microbiome and its metabolites changed significantly after one week of

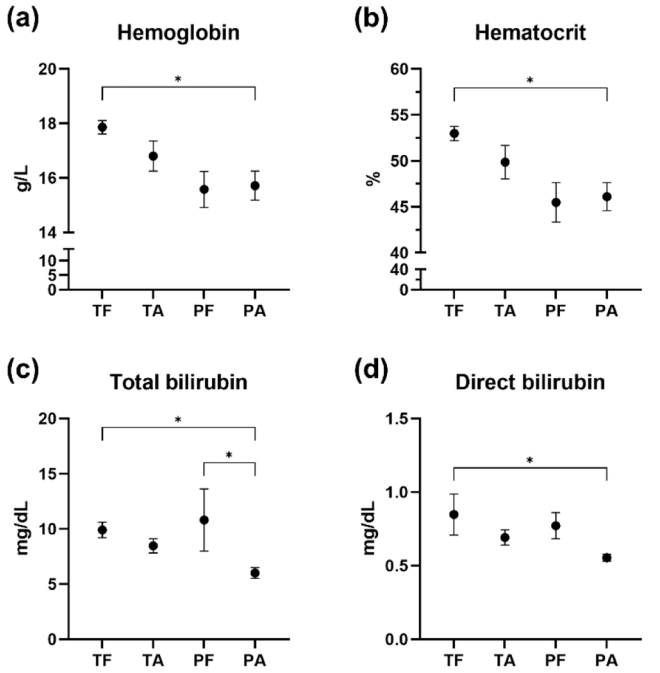

Fig. 3. Hematological and biochemical parameters. Hematological and biochemical parameters in TA, TF, PA, and PF groups. (a) hemoglobin (b) hematocrit (c) total bilirubin (d) direct bilirubin. TA, term infant with empirical antibiotics; PA, preterm infant with empirical antibiotics; TF, term infant without empirical antibiotics; PF, preterm infant without empirical antibiotics. Statistical significance is indicated as follows: *p ≤ 0.05, * *p ≤ 0.01.

antibiotic treatment in preterm infants, with an observed increase in the abundance of certain pathogenic bacteria [19]. Previous studies have shown that excessive antibiotic use during early life can disrupt the developmental trajectories during the critical period of gut microbial establishment and interactions. which may contribute to adverse outcomes, including late-onset sepsis (LOS), necrotizing enterocolitis (NEC), and even mortality in preterm infants [9,11,19–21]. The causative pathogens of LOS are often abundant in the gastrointestinal tract of infants, suggesting that the gut microbiome may play a role in the development of LOS [22]. Additionally, gut microbiome dysbiosis may influence neurodevelopment through the gut-brain axis, potentially playing a critical role in the development of neurodevelopmental impairment [23,24]. Gamma-aminobutyric acid (GABA) is essential for early brain development, and its levels are often reduced in preterm infants [25]. Recent studies suggest that antibiotic use may lead to a reduction in *Veillonella*, a genus positively correlated with GABA concentrations, potentially impacting neurodevelopment in neonates [9]. Increased abundance of *Proteobacteria*, which include more harmful than beneficial genera, is often used as a marker of microbial dysbiosis, accompanied by decreased abundance of *Firmicutes*. These changes, which may be induced by prolonged empirical antibiotic therapy, have been associated with an increased risk of NEC and LOS [10,26–29]. Similarly, in this study, antibiotics (ampicillin and gentamicin) administered to preterm infants were associated with a higher abundance of *Proteobacteria* compared to *Firmicutes* at the phylum level. At the genus level, *Raoultella* and *Escherichia* were predominant in the PA group. Notably, as observed in other study [19], *Escherichia*, which is one of the most common pathogens causing LOS in preterm infants, remained predominant despite the administration of empirical antibiotics that provide coverage against this pathogen. These findings offer a potential explanation for how the use of empirical antibiotics in the early neonatal period could influence the occurrence of LOS.

The regulatory function of the gut microbiome for bilirubin metabolism in enterohepatic circulation has been revealed in previous studies. The catabolism of bilirubin to urobilinogen mixtures, which are excreted in the urine and feces, is thought to be regulated by the gut microbiome [30]. In a previous study, when rats received oral antibiotics, urobilin was not produced and serum bilirubin levels increased [31]. A similar effect of antibiotics on bilirubin levels was observed in a human study [32]. The *Firmicutes* bacterial phylum is known to reduce bilirubin, and several studies have identified specific bacterial strains that can reduce bilirubin, including *Clostridium ramosum*, *Clostridium perfringens*, *Clostridium difficile*, and *Bacteroides fragilis* [30,33]. However, in the present study, positive correlations between the abundance of *Firmicutes* phylum and *Streptococcus* and serum bilirubin levels were observed. In studies on infants with hyperbilirubinemia, changes in the abundance of other bacterial species, such as *Bifidobacterium, Escherichia,* and *Klebsiella,* in the gut microbiome have been observed [33], and an increasing levels of bilirubin may have protective effects against the growth of pathogenic bacteria, such as group B *streptococcus* [34]. Considering these findings, further studies on the relationship between the bacterial strains that change with administration of antibiotics and serum bilirubin levels are needed.

In the human gastrointestinal tract, heme interacts with the gut microbiome, and its concentration has been associated with reduced gut microbiome diversity and changes of dominant species. Additionally, heme-induced epithelial hyperproliferation is modulated by the gut microbiome [35–37]. Iron is essential for bacterial survival, and host heme is a major source of iron acquisition for extracellular bacteria. Bacteria can synthesize heme from precursors shared with the host or lyse erythrocytes to release hemoglobin and extract heme [38–42]. Bacteria such as *Escherichia coli* can degrade hemoglobin through hemoglobin protease, facilitating the release of heme [43]. In the present study, Hb and Hct levels were significantly lower in the PA group. Although relatively lower Hb levels are common in preterm infants due to their lower iron stores compared to term infants [44,45], the PF group did not show significant differences in Hb and Hct levels compared to the term infant group, with only the PA group exhibiting significant differences. We hypothesized that the changes in gut microbiome composition induced by prematurity and antibiotics may lead to higher heme demands, resulting in increased heme biosynthesis by the gut microbiome in the PA group compared to term infants. *Proteobacteria* exhibit a smaller proportion of heme-auxotrophic bacteria compared to *Firmicutes* [46,47], and the increased heme biosynthesis observed in the PA group might be associated with the relative increase in *Proteobacteria*. Also, this increased heme biosynthesis may indicate that gut microbiome dysbiosis drives a greater need for heme acquisition from the host. Iron is an essential element, often leading to competition between the host and microbes [48]. Changes in the gut microbiome in a low-iron environment of preterm infants might induce host-microbe competition for iron and potentially contribute to the lower Hb and Hct levels observed in preterm infants. These findings highlight the potential link between gut microbiome dysbiosis and anemia in preterm infants. Also, the present study confirmed that an increased abundance of *Klebsiella* is associated with decreased Hb and Hct levels. The hemolytic activity of *Klebsiella* should be considered when interpreting these results [49].

There are several limitations in this study. Recovery of the gut microbiota after discontinuation of antibiotic treatment was not investigated. Fecal samples were collected within 10 days after birth. Therefore, the long-term effects of antibiotics on the gut microbiome could not be determined. This study was conducted with a limited number of samples from a single center. The gut microbiome of infants can also be influenced by environmental exposures and center policies, such as mother-infant bonding practices during early newborn care. Given that this study was performed in a single center, these factors must be considered when evaluating the generalizability of the findings. However, as the study was conducted on hospitalized infants exposed to the same environment, the effect of antibiotics on changes in the gut microbiome is unlikely to have been significantly impacted by these

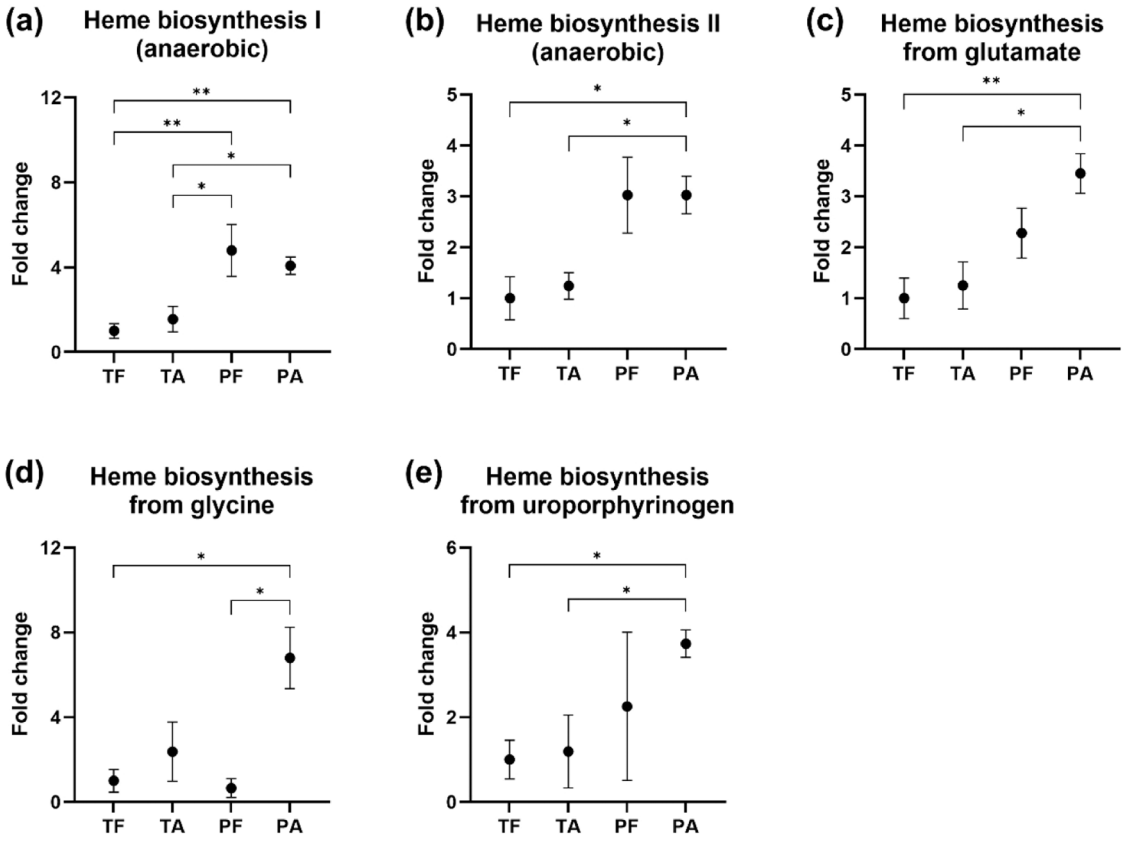

Fig. 4. The predicted metagenomic function of the gut microbiome. (a) gene expression level related to heme biosynthesis I (anaerobic) (b) gene expression level related to heme biosynthesis II (anaerobic) (c) gene expression level related to heme biosynthesis from glutamate (d) gene expression level related to heme biosynthesis from glycine (e) gene expression level related to heme biosynthesis from uroporphyrinogen. TA, term infant with empirical antibiotics; TF, term infant without empirical antibiotics; PA, preterm infant with empirical antibiotics; PF, preterm infant without empirical antibiotics. Statistical significance is indicated as follows: *p ≤ 0.05, * *p ≤ 0.01.

factors. This is supported by the consistency of the results of this study with those of previous research. Future larger, multicenter studies are needed to validate these results and improve their generalizability. Such studies would also facilitate subgroup analyses to investigate the influence of additional variables. Additionally, while this study identified significant correlations between gut microbiota changes and hematologic parameters, these findings do not establish causality. Although 16S rRNA sequencing and PICRUSt2 provide valuable insights into microbial composition and functional predictions, they are inherently limited in resolution compared to metagenomic or metabolomic approaches. Specifically, 16S rRNA sequencing cannot achieve strain-level resolution or provide direct evidence of functional activity, while PICRUSt2 predictions are based on inferred gene content rather than actual gene expression or metabolite production. To elucidate causal mechanisms, future research should employ germ-free or microbiome-transplant models. Moreover, multi-omics approaches, such as untargeted metabolomics, should be utilized to explore the gut microbiome's functional capacity and its metabolic interactions with the host.

5. Conclusion

This study demonstrated dysbiosis in the early-life gut microbiome associated with empirical antibiotic exposure, especially emphasizing dysbiosis in antibiotic-treated preterm infants. The findings suggest that early antibiotic use may influence heme metabolism and hematologic parameters in infants. A better understanding of the adverse effects of antibiotic treatment on gut microbiota could lead to improved clinical practices for preterm infants. Empirical antibiotic use in preterm infants should be more carefully tailored to minimize potential harm. Utilizing early diagnostic tools, such as rapid microbiome analyses at the early neonatal period, could help guide targeted antibiotic therapy, reducing the risk of adverse outcomes and preserving infant health. Further studies involving large cohorts and longitudinal sampling are crucial for understanding the long-term impacts of current clinical practices on infant health.

CRediT authorship contribution statement

Park Hyun-Kyung: Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Kwak Minjin:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation. **Hwang Jae Kyoon:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Chung Woojin:** Funding acquisition, Conceptualization. **Jeon Byong-Hun:** Supervision, Funding acquisition, Data curation, Conceptualization. **Kim Seung Hyun:** Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Lee Chan-Yeong:** Software, Resources, Methodology, Investigation, Formal analysis. **Keum Jihyun:** Resources, Data curation, Conceptualization. **Jin Hee Yeon:** Software, Resources, Investigation, Formal analysis. **Hoh Jeong-Kyu:** Supervision, Resources, Funding acquisition, Conceptualization. **Park Jae Yong:** Project administration, Funding acquisition, Conceptualization. **Tanpure Rahul Sadashiv:** Software, Methodology, Investigation, Funding acquisition, Formal analysis. **Kim Yong Joo:** Supervision, Resources, Conceptualization.

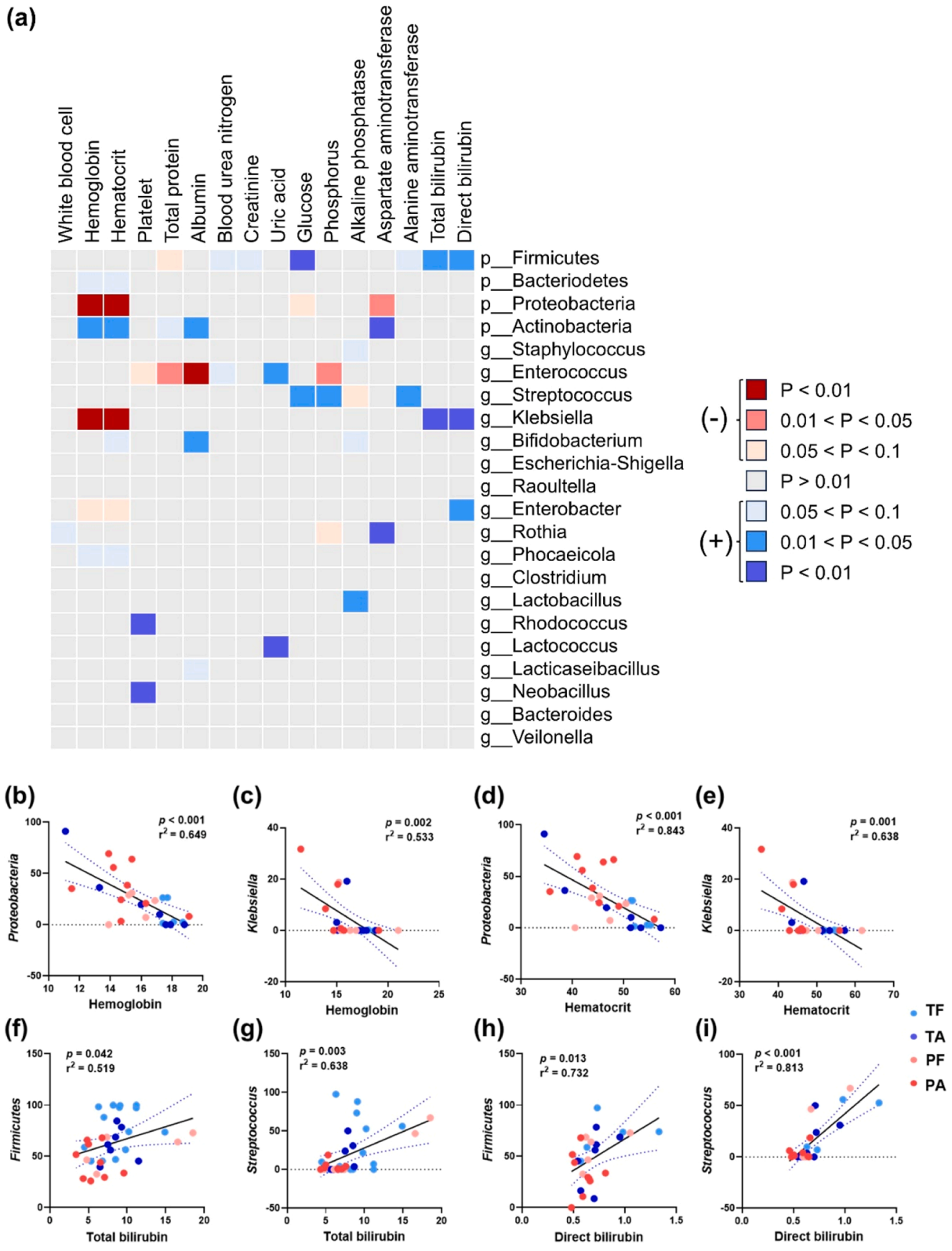

Fig. 5. The relationships between hematological and biochemical parameters and core bacteria were analyzed using linear correlation analysis. (a) correlations between parameters and the abundances of core bacteria. The colors were allotted according to significance. Red and blue colors represented negative and positive correlations, respectively (b) correlation between *Proteobacteria* and hemoglobin (c) correlation between *Klebsiella* and hemoglobin (d) correlation between *Proteobacteria* and hematocrit (e) correlation between *Klebsiella* and hematocrit (f) correlation between *Firmicutes* and total bilirubin. (g) correlation between *Streptococcus* and total bilirubin (h) correlation between *Firmicutes* and direct bilirubin (i) correlation between *Streptococcus* and direct bilirubin. TA, term infant with empirical antibiotics; PA, preterm infant with empirical antibiotics; TF, term infant without empirical antibiotics; PF, preterm infant without empirical antibiotics.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgments

We acknowledge our colleagues at Hanyang University MEB (Medicine-Engineering-Bio) Center. The authors gratefully acknowledge the first president of the Hanyang Inclusive Clinic for Developmental Disorders in Hanyang University College of Medicine for his helpful discussions, as well as CEO Gang Pyo Lee of int-Gen, Seoul, Republic of Korea, who conducted the microbiome and genetic research.

This research was funded by the Hanyang University Global Center for Developmental Disorders (HY-202300000002994), Genetic and Biochemical Analysis in Korean Neonates with Brain Injury (HY-201800000003037), the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (No. 2020R1A2C3004237, RS-2023–00219983), and int-Gen (HY-202200000001981).

Data availability statement

The raw 16S rRNA gene amplicon read files (.fastq format) used for V3–V4 hypervariable region (Illumina) are available in online repositories in the NCBI Sequence Read Archive (SRA) under accession number PRJNA1214324. The curated reference taxonomic sequences and the processed bioinformatic data of this study will be provided by the corresponding author upon request.

References

[1] Roubaud-Baudron C, Ruiz VE, Swan Jr AM, Vallance BA, Ozkul C, et al. Long-term effects of early-life antibiotic exposure on resistance to subsequent bacterial infection. mBio 2019;10(6). https://doi.org/10.1128/mBio.02820-19.
[2] Healy DB, Ryan CA, Ross RP, Stanton C, Dempsey EM. Clinical implications of preterm infant gut microbiome development. Nat Microbiol 2022;7(1):22–33. https://doi.org/10.1038/s41564-021-01025-4.
[3] Li H, Fu L, Chen X, Xu H, Jing Q, et al. Gut microbiota and metabolome description of antibiotic-treated neonates from parturients with intrauterine infection. Front Cell Infect Microbiol 2022;12:817832. https://doi.org/10.3389/fcimb.2022.817832.
[4] Bäckhed F, Roswall J, Peng Y, Feng Q, Jia H, et al. Dynamics and stabilization of the human gut microbiome during the first year of life. Cell Host Microbe 2015;17(5):690–703. https://doi.org/10.1016/j.chom.2015.04.004.
[5] Selma-Royo M, Calatayud Arroyo M, García-Mantrana I, Parra-Llorca A, Escuriet R, et al. Perinatal environment shapes microbiota colonization and infant growth: impact on host response and intestinal function. Microbiome 2020;8(1):167. https://doi.org/10.1186/s40168-020-00940-8.
[6] Senn V, Bassler D, Choudhury R, Scholkmann F, Righini-Grunder F, et al. Microbial colonization from the fetus to early childhood-a comprehensive review. Front Cell Infect Microbiol 2020;10:573735. https://doi.org/10.3389/fcimb.2020.573735.
[7] Shao Y, Forster SC, Tsaliki E, Vervier K, Strang A, et al. Stunted microbiota and opportunistic pathogen colonization in caesarean-section birth. Nature 2019;574(7776):117–21. https://doi.org/10.1038/s41586-019-1560-1.
[8] Stinson LF, Boyce MC, Payne MS, Keelan JA. The Not-So-Sterile Womb: evidence that the human fetus is exposed to bacteria prior to birth. Front Microbiol 2019;10:1124. https://doi.org/10.3389/fmicb.2019.01124.
[9] Russell JT, Lauren Ruoss J, de la Cruz D, Li N, Bazacliu C, et al. Antibiotics and the developing intestinal microbiome, metabolome and inflammatory environment in a randomized trial of preterm infants. Sci Rep 2021;11(1):1943. https://doi.org/10.1038/s41598-021-80982-6.
[10] Zou ZH, Liu D, Li HD, Zhu DP, He Y, et al. Prenatal and postnatal antibiotic exposure influences the gut microbiota of preterm infants in neonatal intensive care units. Ann Clin Microbiol Antimicrob 2018;17(1):9. https://doi.org/10.1186/s12941-018-0264-y.
[11] Chang HY, Chiang Chiau JS, Ho YH, Chang JH, Tsai KN, et al. Impact of early empiric antibiotic regimens on the gut microbiota in very low birth weight preterm infants: an observational study. Front Pedia 2021;9:651713. https://doi.org/10.3389/fped.2021.651713.
[12] Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci USA 2011;108 1(1):4554–61. https://doi.org/10.1073/pnas.1000087107.
[13] Fouhy F, Guinane CM, Hussey S, Wall R, Ryan CA, et al. High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. Antimicrob Agents Chemother 2012;56(11):5811–20. https://doi.org/10.1128/aac.00789-12.
[14] Cha T, Kim HH, Keum J, Kwak MJ, Park JY, et al. Gut microbiome profiling of neonates using nanopore minion and illumina Miseq sequencing. Front Microbiol 2023;14:1148466. https://doi.org/10.3389/fmicb.2023.1148466.
[15] Douglas GM, Maffei VJ, Zaneveld JR, Yurgel SN, Brown JR, et al. Picrust2 for prediction of metagenome functions. Nat Biotechnol 2020;38(6):685–8. https://doi.org/10.1038/s41587-020-0548-6.
[16] Kwak M-J, Tan PL, Oh JK, Chae KS, Kim J, et al. The effects of multispecies probiotic formulations on growth performance, hepatic metabolism, intestinal integrity and fecal microbiota in growing-finishing pigs. Anim Feed Sci Technol 2021;274:114833. https://doi.org/10.1016/j.anifeedsci.2021.114833.
[17] Xu Y, Milburn O, Beiersdorfer T, Du L, Akinbi H, et al. Antibiotic exposure prevents acquisition of beneficial metabolic functions in the preterm infant gut microbiome. Microbiome 2022;10(1):103. https://doi.org/10.1186/s40168-022-01300-4.
[18] Mukherjee D, Ryan RM. Postnatal Bacterial Infections. In: Martin RJ, Fanaroff AA, editors. Fanaroff and Martin's Neonatal-Perinatal Medicine. Philadelphia: Elsevier; 2024. p. 857–80.
[19] Zhu D, Xiao S, Yu J, Ai Q, He Y, et al. Effects of one-week empirical antibiotic therapy on the early development of gut microbiota and metabolites in preterm infants. Sci Rep 2017;7(1):8025. https://doi.org/10.1038/s41598-017-08530-9.
[20] Fjalstad JW, Esaiassen E, Juvet LK, van den Anker JN, Klingenberg C. Antibiotic therapy in neonates and impact on gut microbiota and antibiotic resistance development: a systematic review. J Antimicrob Chemother 2018;73(3):569–80. https://doi.org/10.1093/jac/dkx426.
[21] Ting JY, Roberts A, Sherlock R, Ojah C, Cieslak Z, et al. Duration of initial empirical antibiotic therapy and outcomes in very low birth weight infants. Pediatrics 2019;143(3). https://doi.org/10.1542/peds.2018-2286.
[22] Stewart CJ, Embleton ND, Marrs ECL, Smith DP, Fofanova T, et al. Longitudinal development of the gut microbiome and metabolome in preterm neonates with late onset sepsis and healthy controls. Microbiome 2017;5(1):75. https://doi.org/10.1186/s40168-017-0295-1.
[23] Nagpal J, Cryan JF. Microbiota-brain interactions: moving toward mechanisms in model organisms. Neuron 2021;109(24):3930–53. https://doi.org/10.1016/j.neuron.2021.09.036.
[24] Cryan JF, O'Riordan KJ, Cowan CSM, Sandhu KV, Bastiaanssen TFS, et al. The microbiota-gut-brain axis. Physiol Rev 2019;99(4):1877–2013. https://doi.org/10.1152/physrev.00018.2018.
[25] Kwon SH, Scheinost D, Lacadie C, Benjamin J, Myers EH, et al. Gaba, resting-state connectivity and the developing brain. Neonatology 2014;106(2):149–55. https://doi.org/10.1159/000362433.
[26] Wang Y, Hoenig JD, Malin KJ, Qamar S, Petrof EO, et al. 16s Rrna gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. ISME J 2009;3(8):944–54. https://doi.org/10.1038/ismej.2009.37.
[27] Mai V, Torrazza RM, Ukhanova M, Wang X, Sun Y, et al. Distortions in development of intestinal microbiota associated with late onset sepsis in preterm infants. PLoS One 2013;8(1):e52876. https://doi.org/10.1371/journal.pone.0052876.
[28] Arboleya S, Sánchez B, Milani C, Duranti S, Solís G, et al. Intestinal microbiota development in preterm neonates and effect of perinatal antibiotics. J Pedia 2015;166(3):538–44. https://doi.org/10.1016/j.jpeds.2014.09.041.
[29] Lu S, Huang Q, Wei B, Chen Y. Effects of B-lactam antibiotics on gut microbiota colonization and metabolites in late preterm infants. Curr Microbiol 2020;77(12):3888–96. https://doi.org/10.1007/s00284-020-02198-7.
[30] Hamoud AR, Weaver L, Stec DE, Hinds Jr TD. Bilirubin in the liver-gut signaling axis. Trends Endocrinol Metab 2018;29(3):140–50. https://doi.org/10.1016/j.tem.2018.01.002.
[31] Vítek L, Zelenka J, Zadinová M, Malina J. The impact of intestinal microflora on serum bilirubin levels. J Hepatol 2005;42(2):238–43. https://doi.org/10.1016/j.jhep.2004.10.012.
[32] Saxerholt H, Carlstedt-Duke B, Høverstad T, Lingaas E, Norin KE, et al. Influence of antibiotics on the faecal excretion of bile pigments in healthy subjects. Scand J Gastroenterol 1986;21(8):991–6. https://doi.org/10.3109/00365528608996410.
[33] Chen K, Yuan T. The role of microbiota in neonatal hyperbilirubinemia. Am J Transl Res 2020;12(11):7459–74.
[34] Hansen R, Gibson S, De Paiva Alves E, Goddard M, MacLaren A, et al. Adaptive response of neonatal sepsis-derived group B streptococcus to bilirubin. Sci Rep 2018;8(1):6470. https://doi.org/10.1038/s41598-018-24811-3.
[35] Monteagudo-Mera A, Shalunkhe A, Duhduh A, Walton GE, Gibson GR, et al. Impact of inorganic iron and haem on the human gut microbiota; an in vitro batch-culture approach. Front Microbiol 2023;14:1074637. https://doi.org/10.3389/fmicb.2023.1074637.
[36] Celis AI, Relman DA, Huang KC. The impact of iron and heme availability on the healthy human gut microbiome in vivo and in vitro. e113 Cell Chem Biol 2023;30(1):110–26. https://doi.org/10.1016/j.chembiol.2022.12.001.
[37] Ijssennagger N, Belzer C, Hooiveld GJ, Dekker J, van Mil SW, et al. Gut microbiota facilitates dietary heme-induced epithelial hyperproliferation by opening the mucus barrier in colon. Proc Natl Acad Sci USA 2015;112(32):10038–43. https://doi.org/10.1073/pnas.1507645112.
[38] Choby JE, Skaar EP. Heme synthesis and acquisition in bacterial pathogens. J Mol Biol 2016;428(17):3408–28. https://doi.org/10.1016/j.jmb.2016.03.018.
[39] Wang M, Wang Y, Wang M, Liu M, Cheng A. Heme acquisition and tolerance in gram-positive model bacteria: an orchestrated balance. Heliyon 2023;9(7):e18233. https://doi.org/10.1016/j.heliyon.2023.e18233.
[40] Runyen-Janecky LJ. Role and regulation of heme iron acquisition in gram-negative pathogens. Front Cell Infect Microbiol 2013;3:55. https://doi.org/10.3389/fcimb.2013.00055.

[41] Richard KL, Kelley BR, Johnson JG. Heme uptake and utilization by gram-negative bacterial pathogens. Front Cell Infect Microbiol 2019;9:81. https://doi.org/10.3389/fcimb.2019.00081.
[42] Perry WJ, Spraggins JM, Sheldon JR, Grunenwald CM, Heinrichs DE, et al. Staphylococcus aureus exhibits heterogeneous siderophore production within the vertebrate host. Proc Natl Acad Sci USA 2019;116(44):21980–2. https://doi.org/10.1073/pnas.1913991116.
[43] Otto BR, van Dooren SJ, Nuijens JH, Luirink J, Oudega B. Characterization of a hemoglobin protease secreted by the pathogenic escherichia coli strain Eb1. J Exp Med 1998;188(6):1091–103. https://doi.org/10.1084/jem.188.6.1091.
[44] Owusu-Ansah A, Letterio J, Ahuja SP. Red Blood Cell Disorders in the Fetus and Neonate. In: Martin RJ, Fanaroff AA, editors. Fanaroff and Martin's Neonatal-Perinatal Medicine. Philadelphia: Elsevier; 2024. p. 1520–52.
[45] Moreno-Fernandez J, Ochoa JJ, Latunde-Dada GO, Diaz-Castro J. Iron deficiency and iron homeostasis in low birth weight preterm infants: a systematic review. Nutrients 2019;11(5). https://doi.org/10.3390/nu11051090.
[46] Li J, Yang Z, Yuan W, Bao Z, Li MD. Heme metabolism mediates the effects of smoking on gut microbiome. Nicotine Tob Res 2024;26(6):742–51. https://doi.org/10.1093/ntr/ntad209.
[47] Rao F, Ji Q, Soehano I, Liang ZX. Unusual heme-binding pas domain from Yybt family proteins. J Bacteriol 2011;193(7):1543–51. https://doi.org/10.1128/jb.01364-10.
[48] Caza M, Kronstad JW. Shared and distinct mechanisms of iron acquisition by bacterial and fungal pathogens of humans. Front Cell Infect Microbiol 2013;3:80. https://doi.org/10.3389/fcimb.2013.00080.
[49] Pereira SC, Vanetti MC. Potential virulence of Klebsiella Sp. isolates from enteral diets. Braz J Med Biol Res 2015;48(9):782–9. https://doi.org/10.1590/1414-431x20154316.