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ORIGINAL RESEARCH

Changes in the Gut Microbiota of Neonates with Hyperbilirubinemia Reaching Phototherapy Thresholds

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Purpose: The gut microbiota plays a crucial role in bilirubin metabolism in neonates. The phototherapy threshold assesses the need for clinical intervention in neonatal hyperbilirubinemia (NH). This study aimed to investigate gut microbiota alterations in neonates with NH meeting the phototherapy threshold.

Patients and Methods: A total of 75 neonates with NH who met the phototherapy threshold (NH group), and 67 healthy neonates (control group) were included. Fecal samples were collected within one hour before initiating phototherapy for 16S rRNA gene sequencing and bioinformatic analysis.

Results: In contrast to healthy controls, the NH group showed significantly higher Shannon ($p<0.001$) and abundance-based coverage estimator ($p<0.05$) indices, as well as significant differences in both unweighted and weighted UniFrac values ($p<0.001$ for each). In addition, linear discriminant analysis effect size revealed significant taxonomic shifts in the gut microbiota of the NH group at multiple levels, including phylum, class, order, family, and genus. Among the key differential genera, the abundance of *Streptococcus* ($p<0.001$) was significantly reduced, whereas *Escherichia* ($p<0.001$) and *Klebsiella* ($p<0.001$) were markedly enriched.

Conclusion: Neonates meeting the phototherapy threshold exhibit altered gut microbiota composition, characterized by increased diversity, richness, and an elevated abundance of opportunistic pathogenic genera. These results offer valuable preliminary insights into the gut microbiome changes associated with NH requiring phototherapy.

Keywords: neonatal hyperbilirubinemia, phototherapy threshold, gut microbiota, neonate, 16S rRNA

Introduction

Neonatal hyperbilirubinemia (NH) is a predominant metabolic disorder, particularly during the first week of life, which affects 8–11% of full-term infants and is even more common among preterm infants.[1,2] Without timely treatment, NH may progress to acute bilirubin encephalopathy and kernicterus, which can significantly impair neurological development and cause lifelong disabilities.[3,4]

Bilirubin is a byproduct of heme catabolism, primarily produced from the breakdown of red blood cells. Normally, unconjugated bilirubin is transported to the liver, conjugated with glucuronic acid by UDP-glucuronosyltransferase 1, and excreted into the bile and intestine.[1] Recent evidence has highlighted the role of the gut microbiota in modulating bilirubin metabolism.[5] Specifically, gut bacteria influence the deconjugation and reabsorption of bilirubin through the activity of microbial enzymes such as β-glucuronidase.[6] Certain microbial populations also regulate bile acid metabolism, intestinal transit time, and barrier function, all of which can impact the efficiency of bilirubin clearance.[7–10] Dysbiosis or delayed colonization in neonates may therefore disrupt this delicate balance, contributing to the onset, persistence, or exacerbation of NH. Consequently, oral probiotics have been extensively adopted as an adjunct therapy

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for NH in clinical practice.[5,11–13] However, recent studies have shown that specific probiotics, including *Bacillus clausii, Saccharomyces boulardii, Bifidobacterium animalis subsp. lactis* BB-12, and *Lactobacillus reuteri* DSM 17938, may not provide therapeutic benefits for NH.[14,15] These findings suggest that the therapeutic efficacy of probiotics for NH may be strain-specific.

Phototherapy is widely acknowledged as a non-invasive and highly effective first-line therapy for NH, yet emerging evidence indicates that the procedure itself can reshape the gut microbiota. One study, for example, documented a post-phototherapy reduction in overall microbial diversity, along with enrichment of beneficial genera and depletion of opportunistic taxa.[16] However, the baseline microbiome before phototherapy remains poorly characterized. Profiling this pre-treatment community captures the gut environment at the point of clinical intervention and could inform the development of microbiota-targeted adjuncts, such as strain-specific probiotics, to complement phototherapy. Therefore, we employed 16S rRNA gene sequencing to characterize the gut microbiota in neonates just before they required phototherapy, and the resulting data provide valuable preliminary insights into the microbial changes associated with NH at this pre-treatment stage.

Materials and Methods

Study Population

Neonates diagnosed with NH at the Affiliated Suqian Hospital of Xuzhou Medical University, who met the phototherapy thresholds between December 2022 and June 2023, were enrolled in the NH group. The control group comprised healthy neonates born at the same hospital during the study period, selected without individual (one-to-one) gestational-age matching. Neonates in the NH group met the following inclusion criteria: (1) diagnosis of NH based on the revised clinical practice guideline;[17] (2) gestational age between 37 and 42 weeks; (3) birth weight ranging from 2500–4000 g; and (4) age at admission ≤7 days. The exclusion criteria were as follows: (1) presence of congenital genetic, metabolic, immunological, or gastrointestinal disorders; (2) evidence of fetal distress, neonatal hypoxic-ischemic encephalopathy, or hypoglycemia at birth; (3) diagnosis of bacteremia or other systemic infections; and (4) maternal use of antibiotics or probiotics within one month before delivery. The control group was also subjected to the same exclusion criteria.

This study was approved by the Ethics Committee of the Affiliated Suqian Hospital of Xuzhou Medical University (Approval No. 2021022). Before enrollment, a dedicated specialist from our department conducted individualized discussions with each neonate's guardian to ensure full comprehension of the study's objectives and procedures, after which written informed consent was obtained.

Fecal DNA Extraction

Stool samples were collected within one hour before initiating phototherapy by trained and qualified nurses following neonatal defecation, with the assistance of a glycerin suppository when necessary. A sterile spoon was used to collect 3–5 g of stool, which was then transferred into three sterile cryogenic tubes (triplicate aliquots) and stored in a −80 °C freezer for future use. Sterile gloves were worn throughout the procedure, and all instruments were thoroughly disinfected in advance.

After collection, stool samples were snap-frozen at –80 °C and stored for no longer than four weeks before processing. DNA was extracted batchwise in sets of 24 samples using the MagPure Stool DNA KF Kit B (MAGEN, Guangzhou, China) in a class II laminar-flow biosafety cabinet that had been UV-sterilized for 30 min prior to each run. DNA concentration and purity were quantified with a Thermo NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA), yielding 20–100 ng/μL and A260/A280 ratios between 1.8 and 2.0. DNA integrity was assessed by 1% agarose gel electrophoresis to confirm the presence of distinct bands without signs of degradation or contamination.

16S rRNA Gene Sequencing

The library was constructed using the HotStarTaq Master Mix (QIAGEN, Hilden, Germany), and the bacterial 16S rDNA V4 region was amplified using forward and reverse primers, 515F (5′-GTGCGCAGCMGCCGGTAA-3′) and 806R (5′-

GACTACHVGGGTWTCTAAT-3′). Polymerase chain reaction (PCR) amplification was performed in a 50 μL reaction mixture containing 30 ng of template DNA and fusion primers. The cycling conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 30 cycles of 95 °C for 15s, 56 °C for 15s, and 72 °C for 45s, with a final extension at 72 °C for 5 min. The PCR products were purified using Hieff NGS DNA Selection Beads (YEASEN, Shanghai, China). The validated libraries were sequenced on an Illumina MiSeq platform (BGI, Shenzhen, China) according to the manufacturer's standard protocol, producing 2×300 bp paired-end reads.

Bioinformatics Analysis

The raw sequencing data were filtered and processed to generate high-quality clean data using the following steps: (1) Reads matching the primers were trimmed using Cutadapt version 2.6 to remove primer sequences and adapter contamination, retaining only the target region fragments. (2) A sliding window approach was applied to remove low-quality sequences using a window length of 30 bp. If the average quality score within the window was lower than 20, the sequence was truncated at the beginning of the window. Sequences that were reduced to less than 75% of their original length after trimming were excluded from the analysis. (3) Sequences containing ambiguous bases (denoted by N) were discarded. (4) Low-complexity reads (defined as those with 10 consecutive identical bases from ATCG) were also discarded. The remaining reads were considered clean data.

The paired-end reads obtained from sequencing were first assembled into single sequences (tags) using FLASH version 1.2.11, based on their overlapping regions. Assembly parameters included a minimum overlap of 15 bp and an allowed mismatch rate of 10% in the overlapping region. Subsequently, the assembled tags were grouped into operational taxonomic units (OTUs) using USEARCH version 7.0.1090. The main process included the following steps: (1) Clustering with UPARSE at a 97% similarity threshold to identify representative sequences for each OTUs. (2) Removal of chimeric sequences resulting from PCR amplification using UCHIME version 4.2.40, with the Gold Database version 20110519 as the reference. (3) All tags were mapped back to the OTUs representative sequences using the usearch_global method to construct an OTUs abundance table for each sample.

Taxonomic annotation of OUT representative sequences was performed using RDP Classifier version 2.2, with a confidence threshold of 0.6. The annotation results were filtered based on the following criteria: (1) OTUs without taxonomic annotations were discarded and (2) OTUs annotated with species outside the target taxonomic group of the study were also removed. The remaining OTUs were included in subsequent analyses.

Statistical Analyses

Statistical analyses were performed using SPSS version 27.0. All statistical tests were conducted using a two-tailed approach with a significance level of $p < 0.05$. Normally distributed continuous variables were reported as mean ± standard deviation ($\bar{x} \pm SD$), whereas non-normally distributed data were described as median (interquartile range, Q2 [Q1, Q3]). Between-group differences were assessed using the *t*-test for normally distributed variables and the Wilcoxon test for non-normally distributed variables. Categorical variables were presented as proportions (%) and evaluated using Pearson's chi-square test. All statistical analyses were performed in accordance with the guidelines for statistical reporting in medical research.

Results

Characteristics of Neonates

A total of 160 neonates were screened. Eighteen were excluded: 10 for gestational age < 37 weeks (preterm), 4 for birth weight > 4 000 g (large for gestational age), 2 for birth weight < 2 500 g (small for gestational age), and 2 for congenital disorders. The remaining 142 term neonates met all criteria and were included in the analysis (75 NH, 67 controls). Demographic and clinical characteristics, including sex distribution, age, gestational age, birth weight, delivery method, and feeding type, were similar between the two groups, with no statistically significant differences (all $p > 0.05$, Table 1).

Table 1 Neonates Characteristics

	NH Group (n = 75)	Control Group (n = 67)	*P*-value
Gender			0.118
Men (n)	51 (68.0%)	37 (55.2%)	
Women (n)	24 (32.0%)	30 (44.8%)	
Age (days)	3.06 ± 0.57	3.30 ± 0.61	0.124
Gestational age (weeks)	38.27 ± 2.43	38.75 ± 1.97	0.173
Birth weight (g)	3346.6 ± 319.78	3384.44 ± 286.54	0.294
Mode of delivery			0.489
Vaginal delivery	49 (65.3%)	40 (59.7%)	
Cesarean section	26 (34.7%)	27 (40.3%)	
Feeding method			0.084
Breastfeeding	54 (72.0%)	39 (58.2%)	
Formula feeding	21 (28.0%)	28 (41.8%)	

Notes: Data are n (%) and mean±SD.

Alpha and Beta Diversity

All 142 collected stool samples—75 from the NH group and 67 from controls—passed quality control, with one NH specimen qualifying after re-extraction from a backup aliquot. A total of 451 OTUs were detected: 103 were unique to the NH group (Table S1), 124 were unique to the control group (Table S2), and 112 were shared between the two groups (Table S3) (Figure 1). The Shannon index (Figure 2A) was significantly higher in the NH group (1.09 [0.77, 1.44]) than that in the control group (0.62 [0.27, 1.08], $p < 0.001$). Similarly, the abundance-based coverage estimator (ACE) index (Figure 2B) was significantly elevated in the NH group (22.0 [18.5, 25.0]) than that in the control group (20.0 [15.5, 24.5], $p < 0.05$). The unweighted UniFrac values in the NH and control groups were 0.37 (0.29, 0.45) and 0.43 (0.32, 0.55), respectively (Figure 3A), while the weighted UniFrac values were 0.36 (0.22, 0.48) and 0.23 (0.09, 0.43), respectively (Figure 3B). These differences were statistically significant ($p < 0.001$).

Composition and Abundance of Gut Microbiota

Partial least squares discriminant analysis (PLS-DA) revealed a clear separation between the NH and control groups, indicating distinct microbial community structures (Figure 4). The microbial composition was analyzed at both the phylum and genus levels.

At the phylum level (Figure 5A), the NH group primarily comprised four phyla: *Pseudomonas* (47.13%), *Bacillota* (33.70%), *Actinomycetot*a (12.10%), and *Bacteroidetes* (6.95%). Conversely, the control group mainly comprised three phyla: *Bacillota* (75.54%), *Pseudomonadota* (19.41%), and *Actinomycetota* (4.30%).

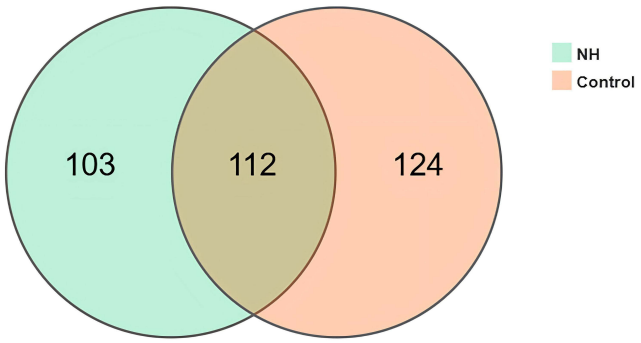

Figure 1 Number of unique and shared OTUs between the two groups.
Abbreviation: OTUs, operational taxonomic units.

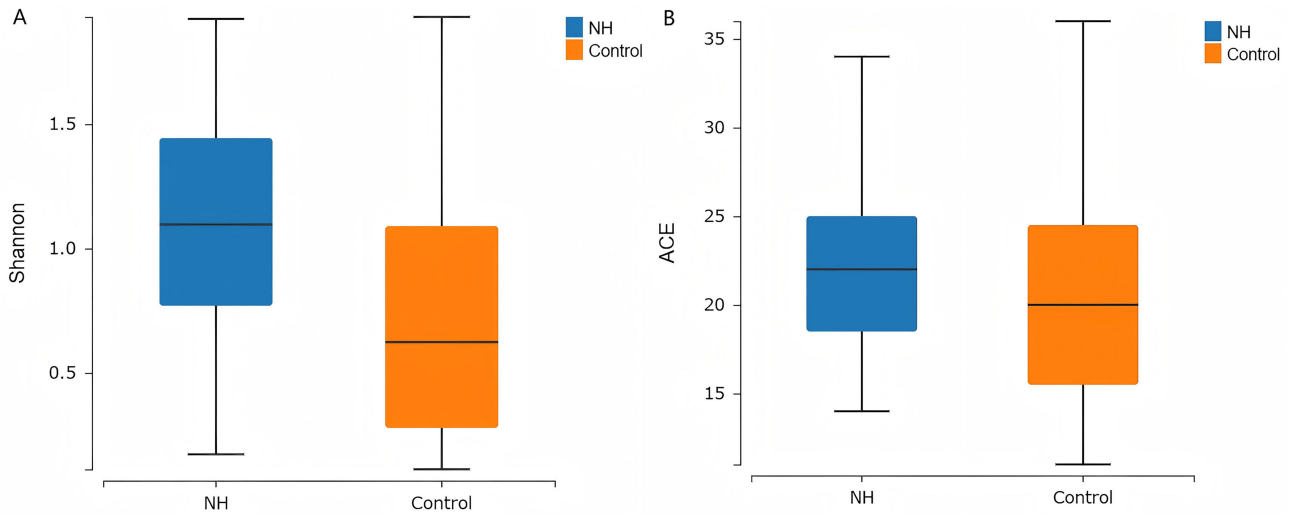

Figure 2 Shannon (**A**) and ACE (**B**) indices in α-diversity analysis.
Abbreviation: ACE, abundance-based coverage estimator.

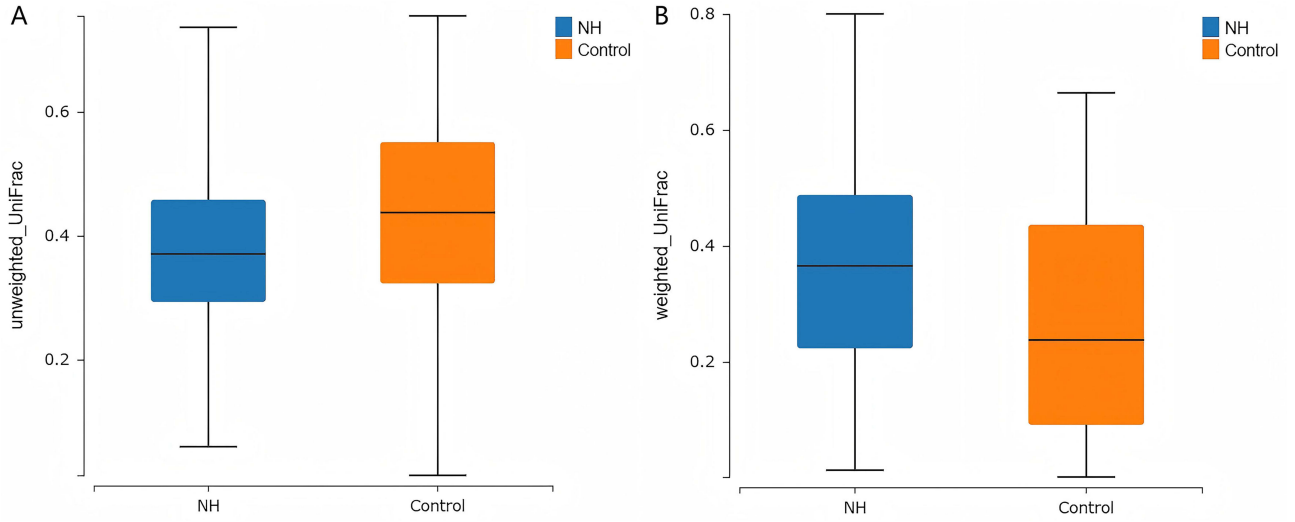

Figure 3 Unweighted UniFrac (**A**) and weighted UniFrac (**B**) values in β-diversity analysis.

At the genus level (Figure 5B), the 10 most abundant genera in the NH group were *Escherichia* (31.41%), *Streptococcus* (19.98%), *Klebsiella* (14.28%), *Bifidobacterium* (9.12%), *Enterococcus* (7.13%), *Veillonella* (3.88%), *Bacteroides* (3.37%), *Phocaeicola* (3.21%), *Rothia* (2.95%), and *Clostridium* (1.53%). The 10 most abundant genera in the control group were *Streptococcus* (59.50%), *Escherichia* (10.90%), *Enterococcus* (10.37%), *Klebsiella* (7.08%), *Bifidobacterium* (3.01%), *Veillonella* (3.16%), *Rothia* (1.27%), *Staphylococcus* (1.15%), *Phocaeicola* (0.67%), and *Clostridium* (0.33%).

Selection of Key Genera

An LDA threshold of 2.0 was set and genera with absolute LDA scores exceeding this value were considered to exhibit statistically significant differences. The gut microbiota composition in the NH group differed from that in the control group across multiple taxonomic levels (Figure 6), with variations observed in 5 phyla, 7 classes, 8 orders, 11 families, and 15 genera. The 10 most abundant genera, each representing over 5% of the relative abundance in either group, were

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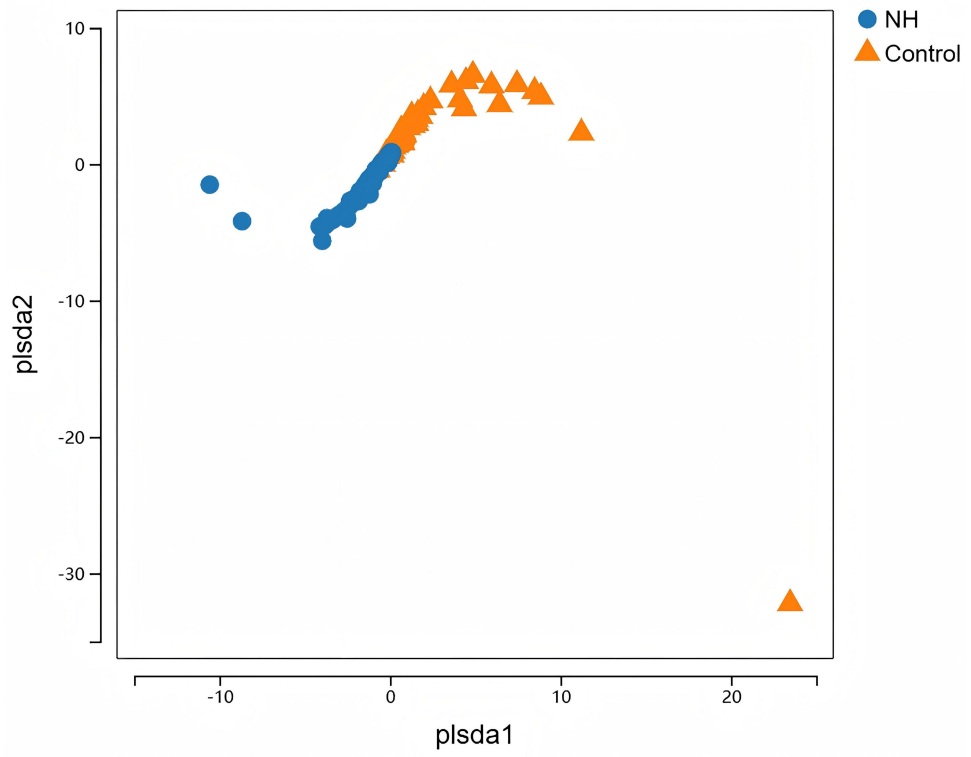

Figure 4 PLS-DA comparison of gut microbiota between the two groups.
Abbreviation: PLS-DA, partial least squares discriminant analysis.

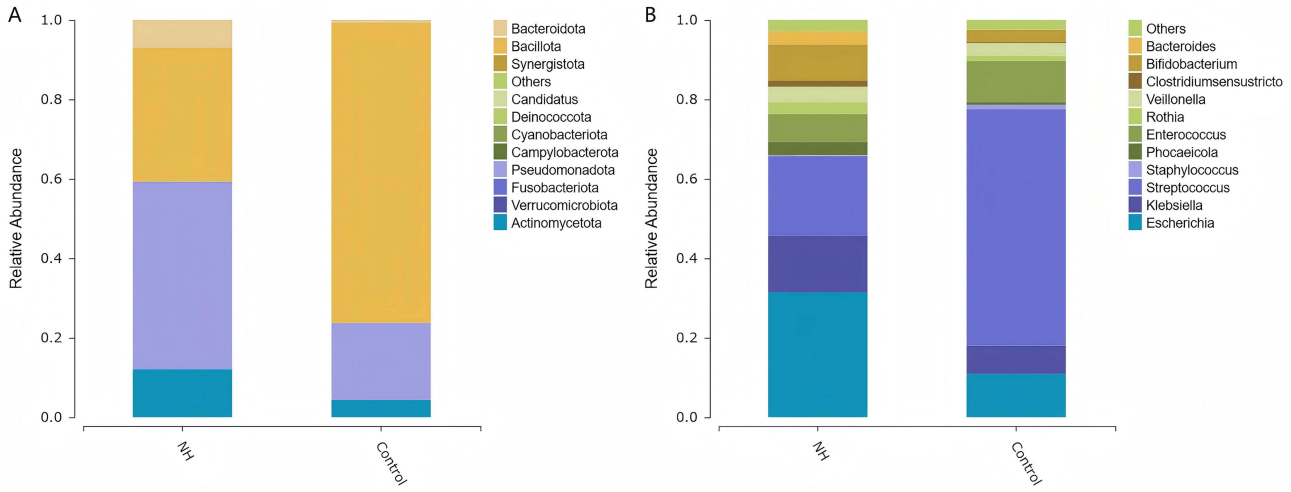

Figure 5 Relative abundance of major taxonomic groups in the gut microbiota. (**A**) Phylum level; (**B**) Genus level.

defined as the key genera. As shown in Figure 6, the abundance of *Streptococcus* ($p < 0.001$) was significantly reduced, whereas *Escherichia* ($p < 0.001$) and *Klebsiella* ($p < 0.001$) were significantly increased in the NH group.

Discussion

This study revealed significant perturbations in gut microbiota composition among neonates with hyperbilirubinemia who met the phototherapy threshold. Both microbial diversity and richness were markedly altered, indicating profound

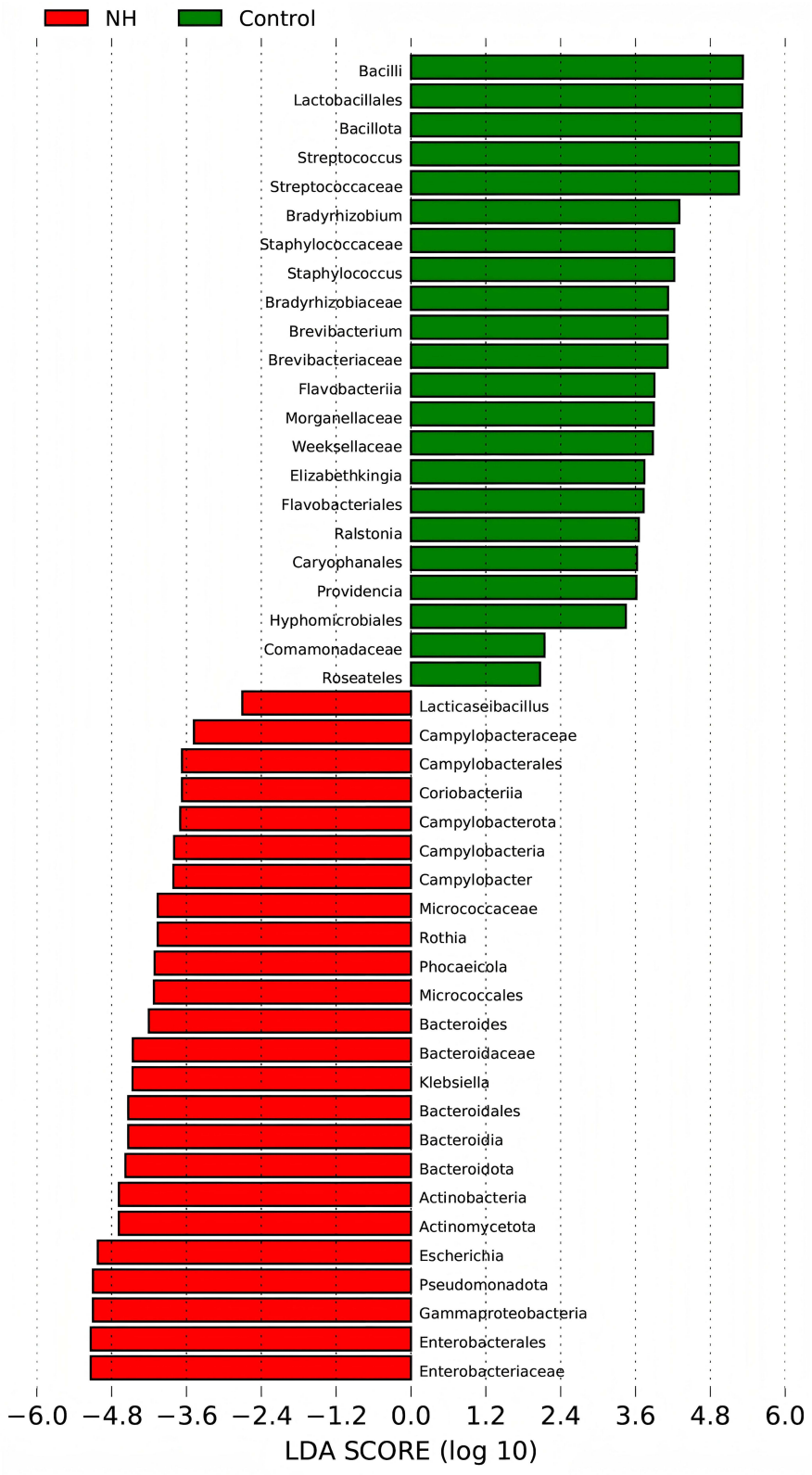

Figure 6 Taxa with absolute LDA scores exceeding the threshold of 2.
Abbreviation: LDA, linear discriminant analysis.

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dysbiosis, and key genus-level shifts included a pronounced decrease in *Streptococcus* alongside increases in *Escherichia* and *Klebsiella*.

The gut microbiota plays a critical role in bilirubin metabolism, as demonstrated in both animal models and clinical studies, particularly in the enterohepatic circulation.[7–10,18] In this study, we observed that the NH group exhibited higher gut microbiota diversity and richness, as indicated by the significantly increased Shannon and ACE indices, compared to healthy controls. Generally, higher microbial diversity and richness are hallmarks of a more complex and stable microbial ecosystem.[19] Although this observation may appear counterintuitive given the pathological state of NH, it may reflect adaptive changes in the gut microbiota in response to altered bilirubin metabolism. Because all faecal samples were obtained within one hour before phototherapy, the dysbiosis we report represents the untreated baseline and is therefore not confounded by therapy-induced shifts, offering a clearer view of disease-related microbial adaptation.

Studies have reported conflicting results regarding the relationship between gut microbiota diversity, richness, and NH. A study based on the NH guidelines established by the American Academy of Pediatrics in 2009 reported significantly reduced gut microbiota richness and diversity in the NH group than that in healthy controls.[20] In contrast, another study reported no changes in gut microbiota diversity in neonates with breast milk jaundice or neonatal cholestasis.[21] However, microbial richness decreases in breast milk jaundice and increases in neonatal cholestasis.[21] The findings of our study diverge from those of the aforementioned investigations, which may be attributed to differences in the study population characteristics or etiological subtypes of NH. This discrepancy underscores the importance of considering demographic factors and disease heterogeneity when interpreting alterations in gut microbiota in neonates with NH. Additionally, obstetric factors such as emergency versus elective birth and the delivery room environment may influence early microbial colonization, so future large-scale studies should control for these variables along with demographic characteristics and NH subtypes to clarify their individual contributions.

Streptococcus is a genus of gram-positive cocci that is widely distributed in nature and various human and animal body sites, including the nasopharynx, gastrointestinal tract, respiratory tract, and reproductive system. The genus *Streptococcus* comprises numerous species and subspecies with diverse functions. Our study revealed a significant reduction in the relative abundance of *Streptococcus*, a key genus in the gut microbiota of neonates with NH. The decreased abundance of this genus may have several implications for the onset or progression of NH. First, certain commensal *Streptococcus* species may support mucosal health by outcompeting pathogens and secreting antimicrobial bacteriocins.[22] Although they are not major short-chain fatty-acid producers, some strains can generate small amounts of butyrate, a metabolite that reinforces tight junctions and helps maintain epithelial barrier integrity. Their reduction could potentially compromise protective functions, including the maintenance of an intestinal environment that suppresses β-glucuronidase-producing pathobionts (eg, *Escherichia, Clostridium*), thereby indirectly exacerbating bilirubin reabsorption and hyperbilirubinemia severity.[6] Second, given that Streptococcus species are involved in bile acid metabolism, their depletion may influence bilirubin processing and associated excretion pathways.[23,24] However, the precise mechanistic link between streptococcus reduction and NH pathogenesis warrants further investigation using targeted metagenomic and metabolomic analyses.

Conversely, our analysis demonstrated significant enrichment of *Escherichia* and *Klebsiella*, two *Enterobacteriaceae* genera, in the gut microbiota of the NH group. Both *Escherichia* and *Klebsiella* are clinically significant opportunistic pathogens capable of causing infections in multiple organs or systems, including the gastrointestinal tract, liver, bile ducts, and bloodstream.[25–28] These genera are particularly noteworthy in neonates because of their potential to cause severe clinical complications and their frequent association with nosocomial infections. Therefore, future research should explore whether clinicians should monitor gut-derived opportunistic pathogenic infections in neonates with hyperbilirubinemia requiring phototherapy. Additionally, when hyperbilirubinemia is infection-related, selecting antibiotics that inhibit *Escherichia* or *Klebsiella* may help reduce bilirubin levels.

β-glucuronidase (β-GD) is an important intestinal enzyme that plays a key role in bilirubin metabolism.[29,30] This enzyme is predominantly found in the gut microbiota, with *Escherichia* being one of the known producers of β-GD.[6] In a single-center prospective cohort study, Tang et al compared 50 neonates with hyperbilirubinemia to 30 age-matched, non-hyperbilirubinemic controls and reported a significant increase in the relative abundance of *Escherichia*, consistent with our findings.[31] Notably, their study further demonstrated that the elevated *Escherichia* levels significantly decreased following NH treatment. A positive correlation between *Escherichia* abundance and β-GD activity was maintained both before and after treatment.[31] These

observations suggest that *Escherichia* may contribute to bilirubin metabolism through β-GD activity and that a decrease in its abundance may hinder jaundice resolution.

However, this study has several limitations. First, to exclude the potential influence of prematurity and birth weight on the gut microbiota, we only included full-term neonates with birth weights ranging from 2.5–4.0 kg, which limits the applicability of these findings to preterm, low-birthweight, or large-for-gestational-age infants. Additionally, this was a non-randomized, small-sample investigation, and the study population was limited to residents of Jiangsu Province, China. Therefore, the results should be interpreted with caution.

Conclusion

Neonates who met the phototherapy threshold for hyperbilirubinemia exhibited marked gut-microbiota dysbiosis, with higher diversity and richness, a depletion of *Streptococcus*, and pronounced enrichment of the opportunistic genera *Escherichia* and *Klebsiella*. Future research should validate these findings in larger, multi-ethnic Asian cohorts and track microbiome dynamics at multiple time-points—before, during, and after phototherapy—to clarify causality and to guide targeted probiotic or antimicrobial interventions.

Abbreviations

NH, Neonatal hyperbilirubinemia; 16S rRNA, 16S ribosomal RNA; LEfSe, Linear discriminant analysis effect size; DNA, Deoxyribonucleic acid; PCR, Polymerase chain reaction; OTUs, operational taxonomic units; ACE, Abundance-based coverage estimator; PLS-DA, Partial least squares discriminant analysis; LDA, Linear discriminant analysis; β-GD, β-glucuronidase.

Data Sharing Statement

All raw reads were deposited in the National Center for Biotechnology Information Sequence Read Archive database (accession no. PRJNA1243920; https://www.ncbi.nlm.nih.gov/bioproject /PRJNA1243920).

Ethics Approval and Informed Consent

This study was approved by the Ethics Committee of the Affiliated Suqian Hospital of Xuzhou Medical University and informed consent was obtained from the patient's parents. All research was conducted in accordance with the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests in this work.

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