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Characterization of gut microbiota signatures in Indian preterm infants with necrotizing enterocolitis: a shotgun metagenomic approach

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Introduction: Necrotizing enterocolitis (NEC) is an inflammatory bowel disease that primarily affects preterm infants. Predisposing risk factors for NEC include prematurity, formula feeding, anemia, and sepsis. To date, no studies have investigated the gut microbiota of preterm infants with NEC in India.

Method: In the current study, shotgun metagenomic sequencing was performed on fecal samples from premature infants with NEC and healthy preterm infants (n = 24). Sequencing was conducted using the NovaSeq X Plus platform, generating 2 × 150 bp paired-end reads. The infants were matched based on gestational age and postnatal age.

Result: The median time to NEC diagnosis was 9 days (range: 1–30 days). Taxonomic analysis revealed a high prevalence of *Enterobacteriaceae* at the family level, with the genera *Klebsiella* and *Escherichia* particularly prominent in neonates with NEC. No statistically significant differences in alpha or beta diversity were observed between stool samples from infants with and without NEC. Linear regression analysis demonstrated that *Enterobacteriaceae* were significantly more abundant in stool samples from infants with NEC than without NEC (q < 0.05). Differential abundance analysis using Linear Discriminant Analysis Effect Size (LEfSe) identified *Klebsiella pneumoniae* and *Escherichia coli* as enriched in the gut microbiota of preterm infants with NEC. Functional analysis revealed an increase in genes associated with lipopolysaccharide (LPS) O-antigen, the type IV secretion system (T4SS), the L-rhamnose pathway, quorum sensing, and iron transporters, including ABC transporters, in stool samples from infants with NEC.

Conclusion: The high prevalence of *Enterobacteriaceae* and enrichment of LPS O-antigen and T4SS genes may be associated with NEC in Indian preterm infants.

KEYWORDS

Necrotizing enterocolitis, gut microbiota, preterm infants, shotgun metagenomics, LPS O-antigen, TLR4, type IV secretion system (T4SS), India

Introduction

Necrotizing enterocolitis (NEC) is a devastating inflammatory disease that significantly contributes to morbidity and mortality in preterm infants. The incidence of NEC is approximately 7% (95% CI: 6–8%) among very low birth weight (VLBW) infants (Alsaied et al., 2020). Risk factors for NEC, such as formula feeding, maternal and neonatal antibiotic exposure, and premature birth, have been shown to induce alterations in gut microbiota (Thänert et al., 2020). The immature intestines of preterm infants, when exposed to inappropriate bacterial colonization alongside a hyperactivated immune system, may drive the development of NEC (Mara et al., 2018). Dysbiosis of the gut microbiota has been observed in individuals with NEC even beyond five years of age (Magnusson et al., 2024). The severity of dysbiosis correlates with the stage of NEC and is more pronounced in patients who undergo surgical treatment (Magnusson et al., 2024). These findings demonstrate how the sequelae of NEC lead to persistent perturbations in the gut microbiota of affected children.

Despite two decades of extensive research using next-generation sequencing techniques, the specific microbial communities responsible for the development of NEC have not yet been identified. Several factors contribute to this challenge, including heterogeneity among studies, such as variations in the timing of sample collection, duration of clinical presentations, and differences in sequencing methods—ranging from targeting variable regions of the 16S rRNA gene to whole-genome bacterial sequencing. Additionally, the gut microbiota of preterm infants is influenced by environmental factors related to hospital admission, antibiotic use, feeding methods, medications, and other supportive interventions (Thänert et al., 2024). During hospitalization, feed intolerance, sepsis, and other comorbidities commonly co-occur with NEC, further contributing to alterations in the gut microbiota. Probiotics have been shown to restore dysbiosis in infants, thereby reducing the incidence of NEC (Samara et al., 2022). Disruptions in the gut microbiota during the early weeks of life in preterm infants are associated with an increased risk of NEC.

Several studies conducted in high-income countries have established an association between the microbiome and NEC (Thänert et al., 2020; Tarracchini et al., 2021); however, data from low- and middle-income countries remain scarce. Investigating the gut microbiota is valuable not only for identifying microbial biomarkers but also for developing bacterial-mediated therapies, such as fecal microbiota transplantation and bacteriophage treatment. In this study, we employed a shotgun metagenomic approach to characterize the gut microbiome and identify functional metabolic pathways as the primary and secondary outcomes in fecal samples from preterm infants with NEC.

Methods

Participant recruitment

This prospective observational cohort study was conducted from September 2022 to January 2025 at a tertiary care neonatal unit in

Northern India. Preterm infants born between 26 and 32 weeks of gestation, with a birth weight of less than 1500 grams, and admitted to the NICU were recruited. Participants' demographic information and clinical details were collected prospectively until discharge or death using a standardized proforma. NEC was diagnosed according to the Bell staging criteria and classified as cases. Healthy preterm infants matched for gestational and postnatal age were recruited as controls. The study was approved by the Institutional Ethics Committee (IEC) of the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, and was conducted in accordance with the Declaration of Helsinki.

Sample collection

The fecal samples from infants were collected in sterile containers during the first month of life as described in previous studies (Devarajalu et al., 2024; Devarajalu et al., 2025). Fecal samples from control subjects were collected on days of life matched to those of the NEC group. Participants were recruited after obtaining written informed consent from one of the parents. Stool samples were stored in an ultra-low temperature freezer (-80 °C) until further processing.

Whole genome sequencing

Genomic DNA was isolated from approximately 100 milligrams of fecal specimens using the QIAamp PowerFecal Pro DNA Kit (Qiagen Inc., Germany). The purity of the extracted DNA was assessed by 1% agarose gel electrophoresis. DNA concentration was determined using the Qubit DNA High Sensitivity (HS) assay (Invitrogen). A paired-end DNA library was prepared using the Twist EF Library Prep Kit (Illumina, Inc., USA). Briefly, the DNA was first fragmented to the desired size, then end-repaired and mono-adenylated at the 3' end in a single enzymatic reaction. Next, adapters were ligated to the DNA fragments using a T4 DNA ligase-based reaction. Following ligation, the fragments were prepared as substrates for PCR-based indexing in the subsequent step. During PCR, barcodes were incorporated using unique primers for each sample, enabling multiplexing. All prepared libraries were assessed for fragment distribution using a 5300 Fragment Analyzer system (Agilent Technologies, Inc., CA, United States). The resulting libraries were pooled and diluted to achieve optimal loading concentrations. Finally, the pooled libraries were loaded onto a NovaSeq X Plus system (Illumina, Inc., CA, United States) to generate 150 bp paired-end reads. The average read count of the samples is 22.8 million. Sequencing statistics are shown in [Supplementary Table 1](#).

Sequencing and mapping

The adapter sequences were trimmed using the fastq-mcf tool (version 1.04.803). The trimmed reads were then aligned to the human genome (hg19) using BWA (version 0.7.12) to remove

human contamination. The remaining unaligned reads were *de novo* assembled with MEGAHIT (version 1.2.9). The resulting assembled genome was used for open reading frame (ORF) prediction and annotation with Prodigal (version 2.6.3). Taxonomic classification was performed using datasets from the National Center for Biotechnology Information (NCBI), and functional pathways were identified from the predicted ORFs using SEED protein classification in MEGAN 6 (Huson et al., 2016).

Microbiome composition and statistical analysis

Alpha diversity metrics, including observed counts and the Shannon index, were calculated using the Phyloseq package (McMurdie and Holmes, 2013). Differences in alpha diversity between groups were assessed using the Wilcoxon rank-sum test. Beta diversity metrics were computed with the Vegan package. A permutational analysis of variance (PERMANOVA) was performed using the *adonis2* function from the pairwise package to identify variation between groups by fitting Bray-Curtis and Jaccard distance matrices separately, incorporating covariates, with 999 permutations (Oksanen et al., 2001).

Differential bacterial abundance between variables was analyzed using Linear Discriminant Analysis (LDA) Effect Size (LEfSe) with an LDA threshold of 4, implemented through the MicrobiomeMarker package (Cao et al., 2022). Additionally, a linear regression model employing the LinDA and MaAsLin2 packages was used to identify taxa and functional pathways associated with necrotizing enterocolitis (NEC) (Mallick et al., 2021; Zhou et al., 2022). Multiple testing corrections, Benjamini-Hochberg (BH) was applied, with a significance threshold of $q < 0.05$. Welch's t-test was conducted for differential functional analysis using STAMP software (Parks et al., 2014). Correlation and network analyses were performed using the Network Construction and Comparison for Microbiome Data (NetCoMi) package (Peschel et al., 2021). All statistical analyses were conducted using R software (version 4.4.2), and figures were generated using the ggplot2, pheatmap, and MicrobiomeStat packages.

Results

Baseline demographic characteristics

The baseline demographic information is presented in Table 1. The NEC and control groups were matched for gestational and postnatal ages. One sample (NEC) was excluded from downstream analysis due to low sequencing read quality.

Microbial richness and diversity

Microbial richness, assessed using alpha diversity, showed no significant differences in observed operational taxonomic units

(OTUs) and Shannon index between infants with NEC and the control group (Figure 1). Alpha diversity indices did not differ significantly when analyzed against other covariates, including mode of delivery (vaginal vs. LSCS), sex (male vs. female), birth weight (<1000 g vs. >1000 g), and gestational age (<28 weeks vs. 28–32 weeks). Furthermore, no statistically significant differences or effect size variations were observed between NEC and control groups for beta diversity, as measured by both Bray-Curtis and Jaccard distance metrics (Figure 2). PERMANOVA analysis revealed that mode of delivery accounted for a greater variance in beta diversity than other covariates within our cohort (Table 2).

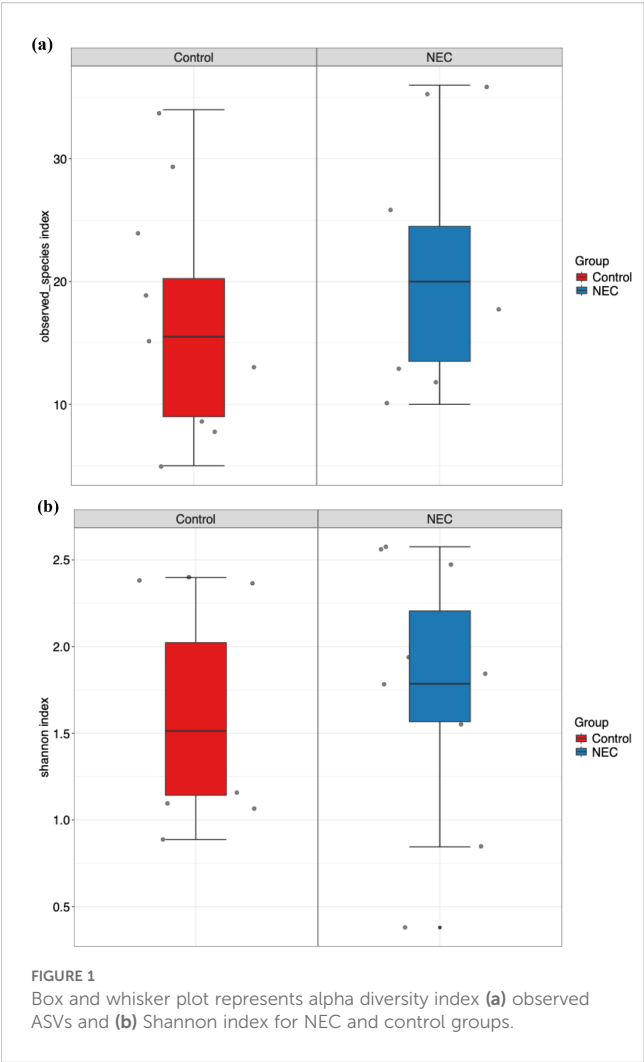
Taxonomic composition and differential abundance

The taxonomic composition at the phylum level indicated that *Pseudomonadota*, *Bacillota*, and *Actinomycetota* were the predominant phyla during the first week of life. An increased abundance of *Pseudomonadota* was observed in preterm infants with NEC, accounting for 47% of the total phyla (Figure 3a). The two major families identified in the gut of preterm infants during the first week were *Enterobacteriaceae* and *Enterococcaceae*, which together comprised 60% of the total taxonomic families (Figure 3b). The abundance of *Enterobacteriaceae* was significantly higher in the

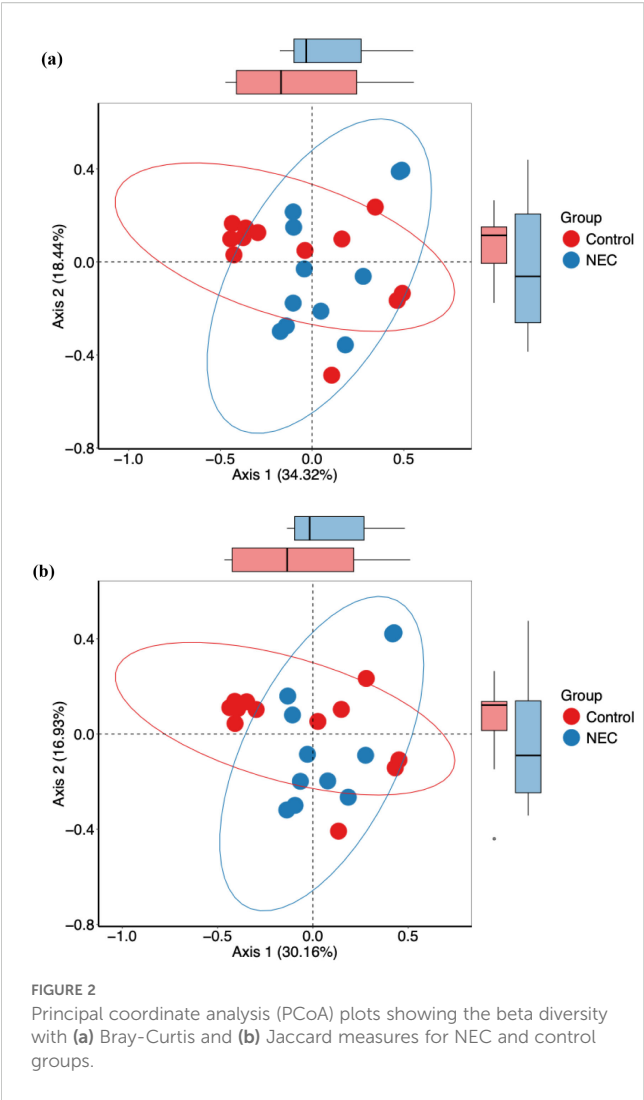
TABLE 1 Demographic details of subjects.

Variables	Control n=12	NEC n=12	P value
Gestational age (week), mean $\pm$ SD	30.5 $\pm$ 2.17	30.4 $\pm$ 1.75	0.829
Sex (male/female)	6/6	6/6	1
Birth weight (g), mean $\pm$ SD	1235 $\pm$ 311	1259 $\pm$ 289.4	0.978
No. (%) of mothers with PPRM	3 (25%)	2 (16.67%)	0.615
Mode of delivery (Vaginal/LSCS)	6/6 (50/50%)	6/6 (50/50%)	1
No. (%) of mothers with preeclampsia	4 (33.3%)	1 (8.3%)	0.314
No. (%) of mothers received antenatal corticosteroids	12 (100%)	11 (92%)	0.307
APGAR score at 1 min median (IQR)	7 (6–9)	6 (5–9)	0.922
APGAR score at 5 min median (IQR)	8 (7–9)	7 (6–9)	0.493
No. (%) of neonates receiving antibiotic	12 (100%)	12 (100%)	1
Day of life on which stool sample was collected	6 (3–20)	6 (3–20)	1
Age of diagnosis of NEC (days)	NA	9 (3–30)	NA
NEC associated deaths	NA	2 (16.7%)	NA

PPROM, Preterm Prelabor Rupture of Membranes; LSCS, Lower Segment Cesarean Section; SD, Standard deviation; IQR, Interquartile range; NA, Not applicable.



gut of infants with NEC than without NEC, as demonstrated by linear regression analysis (Table 3). There was an increased prevalence of the genera *Escherichia* and *Klebsiella* and a decreased prevalence of *Enterococcus* in preterm infants with NEC. Linear regression analysis indicated a significant increase in the abundance of the genus *Klebsiella* in NEC cases. Heatmap analysis revealed that either *Escherichia* or *Klebsiella* was present in 82% of NEC cases compared to 33.3% of controls (Figure 4). Krona plots showed that the abundance of *Klebsiella pneumoniae* in the



NEC and control groups was 24% and 8% of the total microbial OTUs, respectively, while *Escherichia coli* accounted for 21% in the NEC cases and 13% in controls (Figure 5). Differential abundance analysis using LEfSe revealed that *Klebsiella pneumoniae* and *Klebsiella quasipneumoniae* were more prevalent in infants with NEC, whereas unclassified *Acinetobacter* species were more abundant in the non-NEC group (Figure 6).

TABLE 2 PERMANOVA multivariate analysis performed based on Bray–Curtis dissimilarity distance.

Variables	Df	Sums of sqs	R2	F.model	Pr (>F)
Group (NEC Vs Control)	1	0.4336	0.0565	1.2584	0.217
Preterm (Extreme Vs Very)	1	0.2623	0.0342	0.7437	0.764
Mode of Delivery (Vaginal Vs LSCS)	1	0.6204	0.0809	1.8485	0.038
Gender (Male Vs Female)	1	0.4300	0.05607	1.2475	0.213
Birth weight (<1000 g Vs ≥ 1000 g)	1	0.3123	0.04073	0.8917	0.546

LSCS, Lower Segment Cesarean Section.

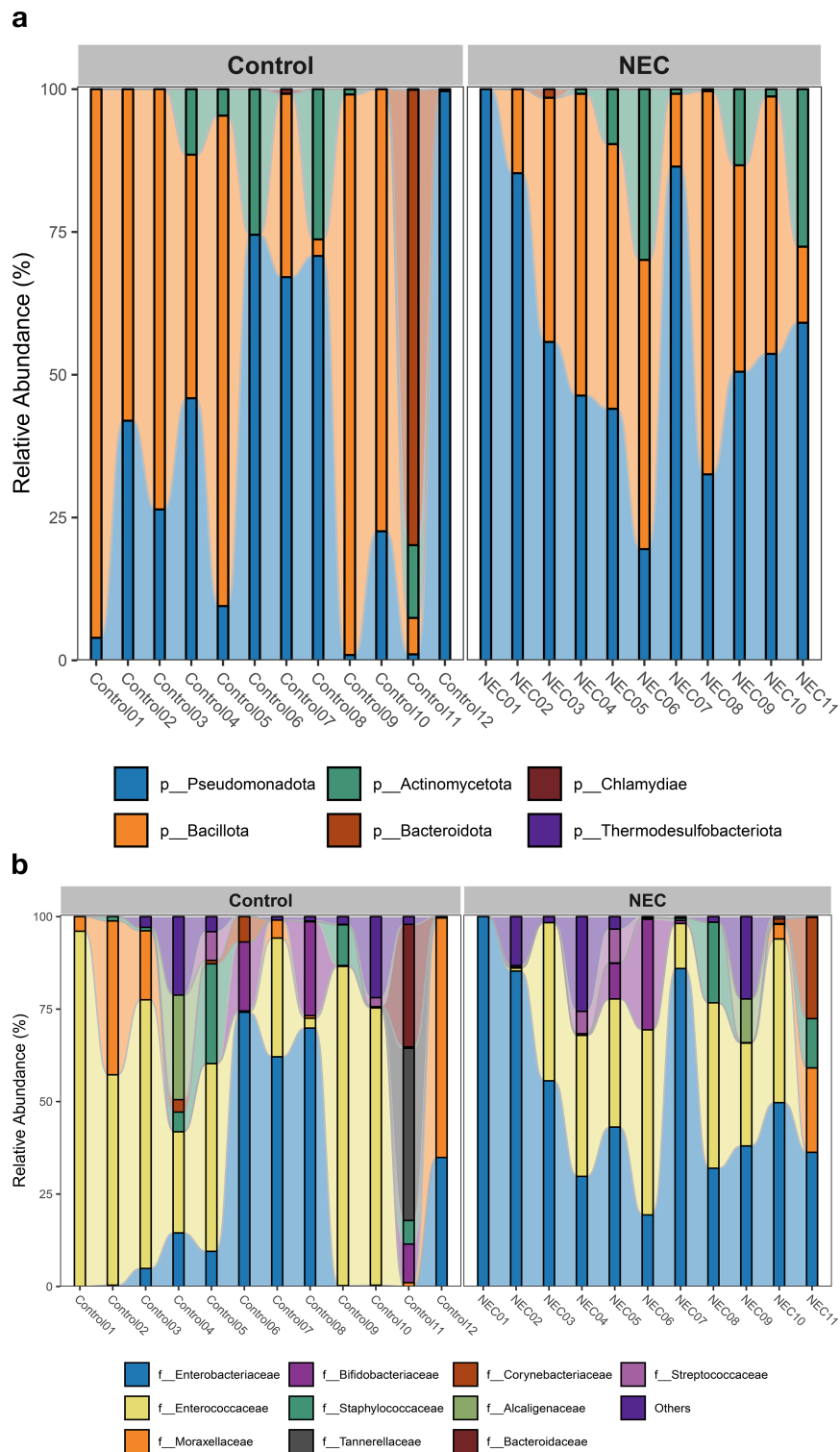


FIGURE 3
Bar plot represents taxonomic composition of both NEC and controls at (a) phylum and (b) family level.

Correlation of microbial community

The relationships between microbial communities at the genus level were evaluated using Pearson's correlation analysis. A strong

positive correlation was observed between *Finegoldia* and *Cutibacterium* as well as between *Paraburkholderia* and *Clostridioides*. Weak positive correlations were found between *Shigella* and *Klebsiella*, and between *Escherichia* and *Klebsiella*.

TABLE 3 Linear regression analysis at family levels between NEC and control groups.

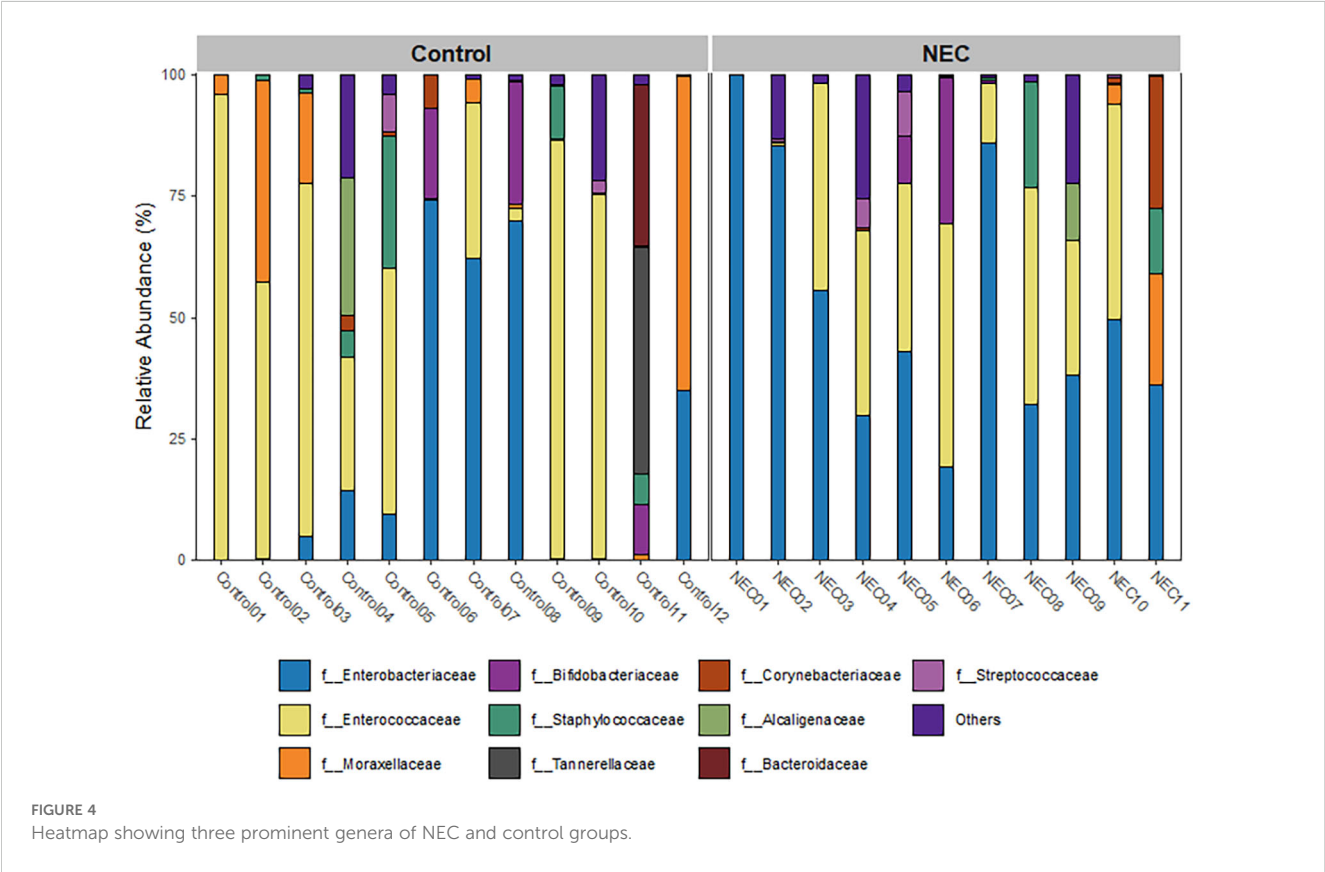
Variable	Coefficient	SE	P value	Adjusted p value	Abundance	Prevalence
<i>Bifidobacteriaceae</i>	-0.0182	0.9432	0.9847	0.9847	0.0417	0.2608
<i>Corynebacteriaceae</i>	0.2066	0.7670	0.7902	0.9659	0.01949	0.3043
<i>Enterobacteriaceae</i>	4.1053	1.2305	0.0031	0.0344	0.3660	0.9130
<i>Enterococcaceae</i>	0.07559	1.2171	0.9510	0.9847	0.3470	0.7826
<i>Listeriaceae</i>	0.1316	0.2551	0.6113	0.9453	0.0002	0.1304
<i>Moraxellaceae</i>	-3.0507	1.3559	0.0353	0.1294	0.0693	0.5217
<i>Staphylococcaceae</i>	-0.7351	1.2153	0.5517	0.9453	0.0364	0.6521
<i>Streptococcaceae</i>	0.9612	1.0025	0.3485	0.9453	0.0133	0.3478

Additionally, weak negative correlations were noted between *Bifidobacterium* and *Enterococcus* and between *Enterobacter* and *Acinetobacter* (Supplementary Figure 1).

Functional pathway analysis

Functional analysis using SEED classification revealed that pathways associated with type IV secretion systems, conjugative

transfer, Enterobacterial common antigen (LPS O-antigen), quorum sensing in *Yersinia*, the L-rhamnose pathway, and iron transport systems—including ABC transporters and the Shikimate kinase SK3 cluster—were significantly enriched in infants with NEC compared to controls (Figures 7, 8). These pathways were significant in both linear regression analysis and Welch’s t-test using the STAMP tool (Supplementary Figure 2). Additionally, genes involved in folate biosynthesis and lactose utilization were reduced in the NEC group compared to the control group.



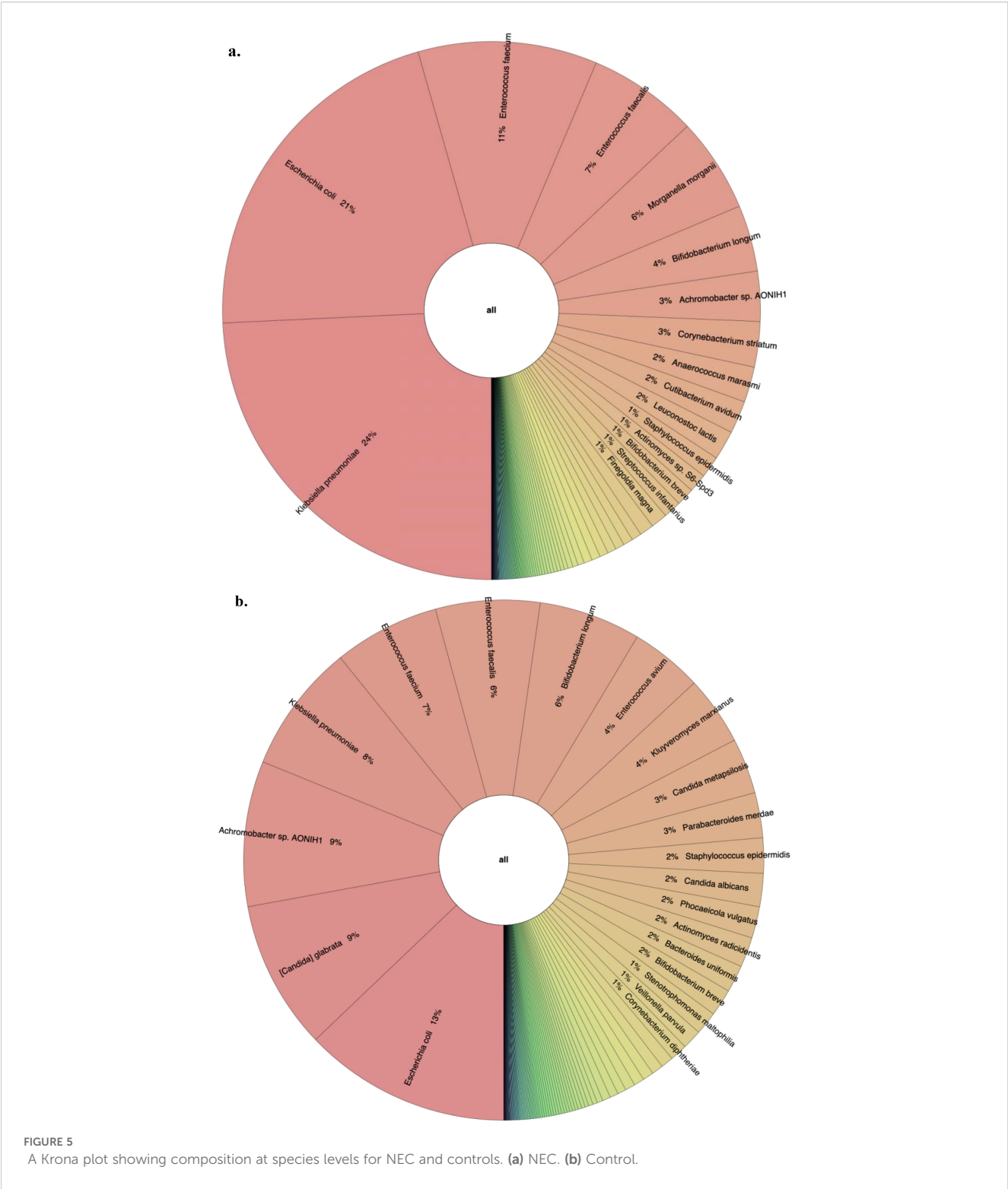
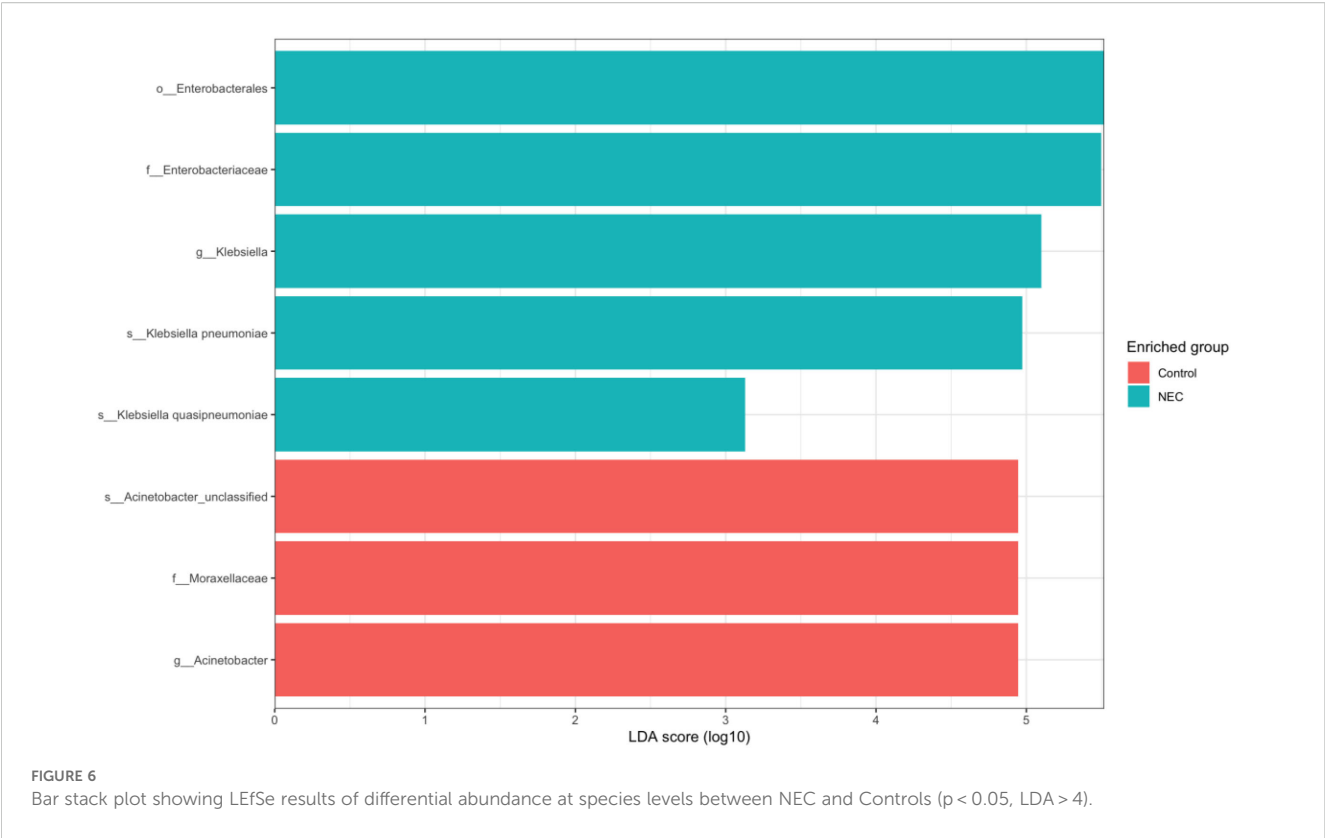


FIGURE 5
A Krona plot showing composition at species levels for NEC and controls. (a) NEC. (b) Control.

Discussion

The present study identified that *Enterobacteriaceae* were significantly more abundant in the stool samples of infants with NEC. Functional analysis revealed an increased abundance of genes involved in the biosynthesis of lipopolysaccharide (LPS) O-antigen, the type IV secretion system (T4SS), the L-rhamnose pathway and

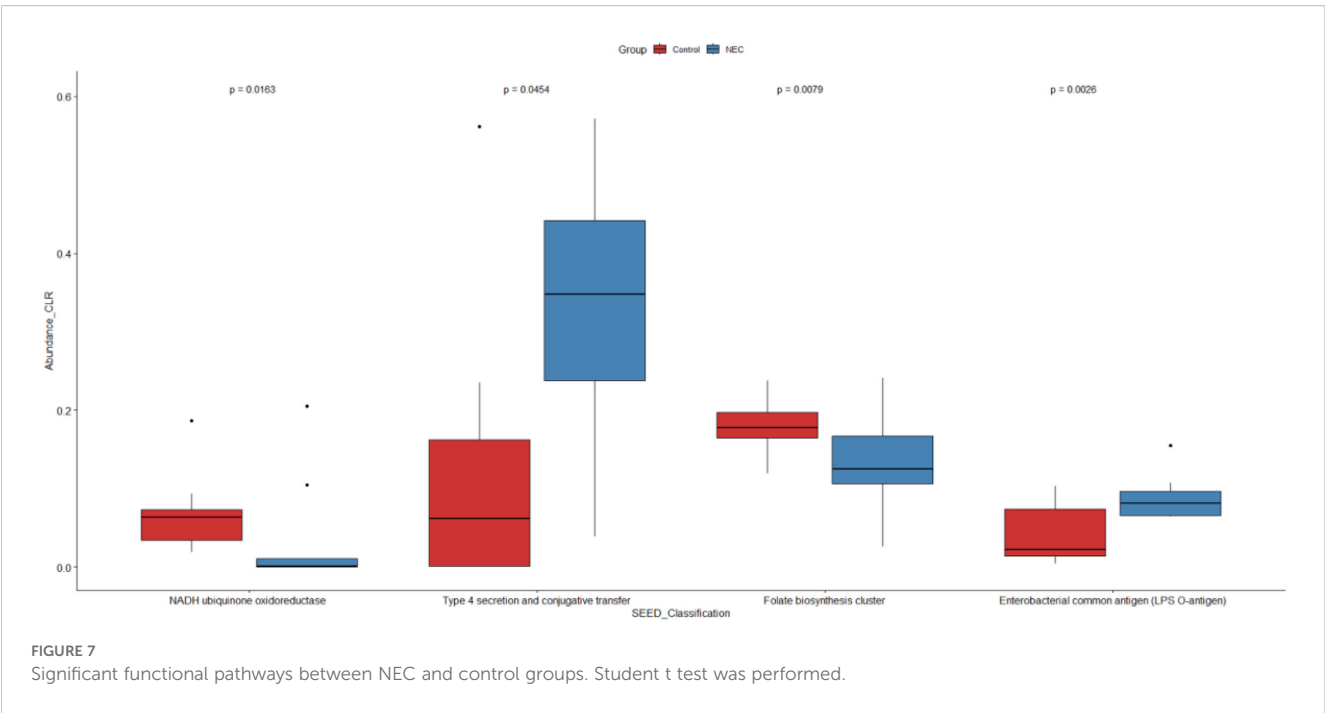
quorum sensing in the stool samples of infants with NEC. To the best of our knowledge, this is the first study to examine gut microbial communities using whole-genome shotgun sequencing in Indian preterm infants with NEC. PERMANOVA analysis demonstrated that the mode of delivery influenced the beta diversity of the gut microbiome. However, the differential bacterial abundance identified through linear regression



analysis between NEC and control groups was not affected by the mode of delivery. This is likely because the number of infants delivered vaginally and by C-section was equal in both the NEC and control groups.

The median gestational age of infants with NEC in our cohort was 30.5 ± 2.17 weeks, comparable to that reported in Western

studies evaluating the gut microbiome in these infants, which showed a median gestational age of 30.1 ± 2.4 weeks (Pammi et al., 2017). No significant differences were observed between the NEC and control groups in alpha diversity indices or beta diversity measures. This finding is consistent with previous meta-analyses on the subject (Pammi et al., 2017).



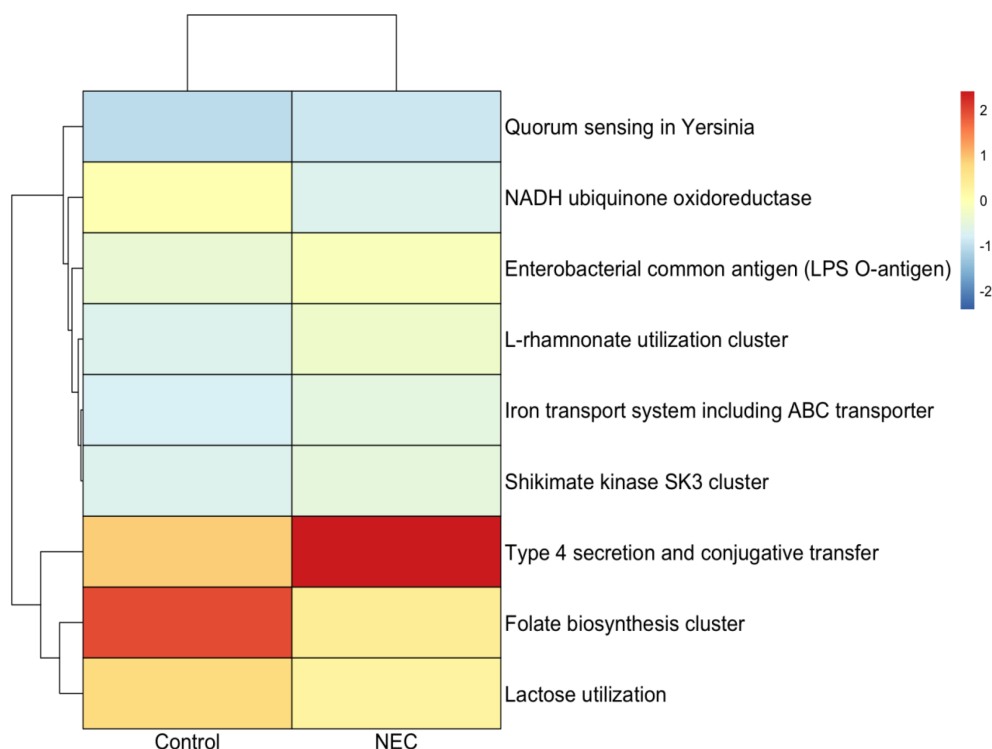


FIGURE 8
Heatmap showing differential functional pathway analysis between NEC and control groups.

Differential abundance analysis at the family level revealed elevated levels of *Enterobacteriaceae* and *Pseudomonadaceae* in the fecal samples of infants with NEC. However, the increase in *Pseudomonadaceae* did not reach statistical significance after p-value adjustment. The overrepresentation of *Enterobacteriaceae* in fecal samples from infants with NEC has been documented in studies using both culture-based and molecular techniques (Millar et al., 1992; Marolda and Valvano, 1995; Brower-Sinning et al., 2014). *Pseudomonadaceae* has also been associated with NEC and sepsis in preterm infants (Morrow et al., 2013; Wang et al., 2024). A previous study demonstrated that colonization with uropathogenic *E. coli* (UPEC) is associated with NEC-related mortality (76%) (Ward et al., 2016). In contrast, the current study found that only 21% of the infants were colonized with *E. coli*.

Klebsiella pneumoniae and *Klebsiella quasipneumoniae* were the dominant species identified in infants with NEC in our study. A previous study found that *K. pneumoniae* and *Klebsiella oxytoca* are associated with NEC (Paveglio et al., 2000; Thänert et al., 2024). *K. quasipneumoniae* is a recently classified subspecies of *K. pneumoniae*. Numerous studies have reported that *K. quasipneumoniae* acquires antimicrobial resistance genes (ARGs) and is associated with hospital-acquired infections (Mathers et al., 2019). *K. quasipneumoniae* has been detected in the rectal and fecal samples of preterm infants admitted to the neonatal intensive care unit (NICU) (Crellen et al., 2019; Chen et al., 2020). Previous studies showed that *Clostridium neonatale* and *Clostridium perfringens* were associated with NEC onset (Tarracchini et al.,

2021). However, we did not find any association between *Clostridium* and NEC in our cohort.

Functional pathway analysis revealed that pathways associated with type IV secretion systems (T4SS), LPS O-antigen, quorum sensing in *Yersinia*, the L-rhamnose pathway, and iron transport systems including ABC transporters and the shikimate kinase SK3 cluster, were significantly increased in NEC. T4SS facilitates horizontal gene transfer among bacteria, promoting the dissemination of antibiotic resistance genes and the delivery of virulence factors. The LPS O-antigen is a conserved surface antigen found in the *Enterobacteriaceae* family. The increased abundance of *Enterobacteriaceae* in NEC samples may result in elevated levels of LPS antigens. LPS antigen-induced activation of Toll-like receptor 4 (TLR4) pathway is a well-characterized mechanism contributing to intestinal inflammation and necrosis in animal models of NEC as well as patients (Shaw et al., 2021). The L-rhamnose pathway is involved in the synthesis of LPS O-antigen in Gram-negative bacteria, including *Enterobacteriaceae* (Marolda and Valvano, 1995). The shikimate kinase SK3 pathway is crucial for the synthesis of aromatic amino acids, such as tyrosine and tryptophan, which are essential for bacterial growth and survival, including in *E. coli* (Ely and Pittard, 1979). Moreover, shikimate kinase is a target for the therapeutic inhibition of multi-resistant strains, including *K. pneumoniae* (Li et al., 2025). Overall, the functional pathways identified in this study highlight that *Enterobacteriaceae* and their metabolic functions contribute to the pathogenesis of NEC.

The limitations of this study include its single-center design and small sample size. However, to our knowledge, this is the first study in India to use a shotgun metagenomic approach to investigate the association of gut microbiota in fecal samples of infants with NEC.

Conclusion

Enterobacteriaceae were more abundant in stool samples from infants with NEC than infants without NEC. Differential abundance analysis using Linear Discriminant Analysis Effect Size (LEfSe) identified *Klebsiella pneumoniae* and *Escherichia coli* as enriched in the gut microbiota of preterm infants with NEC. Functional analysis revealed increased expression of genes associated with the LPS O-antigen, the Type IV secretion system, the L-rhamnose pathway, quorum sensing, and iron transporters, including ABC transporters, in the NEC samples.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repository and accession number(s) can be found below: NCBI-PRJNA1304237.

Ethics statement

The studies involving humans were approved by Institutional Ethics Committee, PGIMER. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

PD: Formal analysis, Methodology, Investigation, Conceptualization, Funding acquisition, Writing – original draft. SA: Conceptualization, Writing – review & editing, Supervision, Project administration. JKu: Methodology, Resources, Writing – review & editing. SD: Writing – review & editing, Conceptualization. JKa: Writing – review & editing, Project administration, Methodology, Formal analysis, Supervision, Visualization, Conceptualization.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2025.1649384/full#supplementary-material>

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