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Research Article

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Gut microbiota dynamics in preterm infants and the incidence of bronchopulmonary dysplasia: a prospective longitudinal cohort study

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Abstract

Background: Bronchopulmonary dysplasia (BPD) remains the most common chronic respiratory disease in preterm infants, and accumulating evidence implicates the gut–lung axis in its pathogenesis. However, prospective longitudinal data linking early gut microbiota dynamics to the onset of BPD are scarce. This study aimed to characterise gut microbiota dynamics within the first 28 days of life in preterm infants of ≤32 weeks' gestation and to evaluate their association with BPD and probiotic exposure.

Methods: Fifty preterm infants (≤32 weeks' gestation) were enrolled prospectively and allocated to four groups according to BPD status and probiotic use (n = 12–13 per group). Faecal samples were collected at days 0, 3, 14, and 28 after birth and at days 7, 14, and 21 after the start of probiotic administration (seven time points). The V3–V4 region of the 16S rRNA gene was sequenced, and amplicon sequence variants were generated with QIIME2/DADA2 and annotated against SILVA 138.1. α-Diversity (Chao1, Shannon and Simpson indices) and the relative abundance of Bifidobacterium were assessed, with Benjamini–Hochberg false discovery rate (FDR) correction applied to multiple comparisons.

Results: The Chao1 index dropped sharply from 375.7 ± 27.2 at day 0 to 15.5 ± 0.8 at day 3 (a decrease of 95.7%), then plateaued at a low level. From day 14 onwards, α-diversity was significantly higher in the non-BPD than in the BPD groups (Chao1: 15.18 ± 0.74 vs 14.06 ± 0.71, P = 0.001 at day 14), and this difference persisted until day 28. Probiotic intervention partially reduced but did not eliminate the between-group diversity deficit. After probiotic administration, the relative abundance of Bifidobacterium was higher in the non-BPD + probiotics group than in the BPD + probiotics group at day 14 (0.0241 ± 0.0136 vs 0.0151 ± 0.0058, P = 0.041) and day 21 (0.0400 ± 0.0128 vs 0.0285 ± 0.0111, P = 0.022).

Conclusions: The gut microbiota of preterm infants follows a three-stage pattern of high baseline, sharp decline, and subsequent differentiation. Infants who develop BPD show sustained reductions in diversity and delayed Bifidobacterium colonisation from day 14 onward. Probiotics partially improved diversity but did not fully correct BPD-associated dysbiosis. Day 14 may represent a critical window for microbiota-directed intervention.

Keywords: bronchopulmonary dysplasia; gut microbiota; preterm infants; 16S rRNA sequencing; Bifidobacterium; probiotics

1 Introduction

Bronchopulmonary dysplasia (BPD) is the most common chronic respiratory disease in preterm infants; in extremely preterm infants, the incidence of BPD can reach 68%[1]. Although the widespread use of antenatal glucocorticoids, pulmonary surfactant, and gentle ventilation strategies has significantly improved survival rates among preterm infants, the incidence of BPD has not decreased markedly[2]. The core pathological features of BPD are alveolar simplification and impaired microvascular development; its pathogenesis involves multiple factors, including intrauterine and postnatal infections, hyperoxia, mechanical ventilation-induced

injury, and an imbalance in the inflammatory response[3]. There is currently a lack of effective strategies for preventing BPD, and there is an urgent need to explore intervention targets from new mechanistic perspectives.

In recent years, the concept of the 'gut-lung axis' has provided a novel perspective for research into the pathogenesis of BPD[4]. The gut microbiota influences pulmonary immune homeostasis and inflammatory responses through mechanisms such as the production of short-chain fatty acids (SCFAs), the regulation of the Th17/Treg immune balance, and long-distance signalling via metabolites[5–6]. Animal studies have demonstrated that supplementation with Lactobacillus reuteri can alleviate hyperoxia-induced BPD-like lung injury in neonatal mice by activating the IL-22/STAT3 signalling pathway[7]. However, existing clinical studies have largely focused on microbiota differences at a single time point[8], with a lack of systematic tracking of the longitudinal evolution of the gut microbiota in preterm infants during the early postnatal period; in particular, there is a lack of prospective studies linking dynamic changes in the microbiota to the onset and progression of BPD.

Previous studies in adults and term infants have suggested that gut microbiota diversity typically increases gradually with age. However, it is worth noting that recent studies have reported a counterintuitive phenomenon: the microbial α-diversity in the meconium of preterm infants (D0) is significantly higher than at subsequent time points, exhibiting a 'high baseline–sharp decline' pattern[9–10]. The biological significance of this phenomenon and its role in the development of BPD remain unclear. Furthermore, there is a lack of longitudinal data to support whether probiotic intervention can correct BPD-associated dysbiosis, or to determine the optimal window for such intervention.

Accordingly, this study adopted a prospective cohort design to conduct longitudinal tracking of faecal microbiota at seven time points (days 0, 3, 14 and 28 after birth, plus days 7, 14 and 21 after the start of probiotic administration) in preterm infants with a gestational age ≤32 weeks, with the aim of: ① mapping the dynamic evolution of gut microbiota diversity in preterm infants; ② identifying the key time windows during which microbiota differences emerge between the BPD and non-BPD groups; ③ evaluate the modulatory effects of probiotic intervention on microbial diversity and Bifidobacterium colonisation. We hypothesise that preterm infants in the BPD group will exhibit reduced gut microbial diversity and delayed Bifidobacterium colonisation in the early postnatal period, and that probiotic intervention may partially correct this dysbiosis.

2 Methods

2.1 Study Design and Participants

This study was a prospective cohort study conducted in the Neonatal Department of the First Affiliated Hospital of Guilin Medical University from January 2022 to December 2024. Inclusion criteria: (1) gestational age ≤ 32 weeks; (2) admission within 24 hours of birth. Exclusion criteria: (1) concomitant congenital gastrointestinal malformations; (2) concomitant congenital heart disease (haemodynamically significant); (3) concomitant severe intrauterine infection (including intrauterine bacterial or viral infections leading to multi-organ dysfunction); (4) concomitant chromosomal abnormalities or inherited metabolic disorders; (5) refusal by the family to sign the informed consent form.

This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Guilin Medical University (Approval No.: 2021KJJ40) and obtained written informed consent from the patients' guardians. The study protocol has been registered with the Guilin Municipal Bureau of Science and Technology (Registration No.: 20210227-7-7).

2.2 Grouping and Intervention

Neonates were divided into four groups based on the presence or absence of BPD and whether probiotics were administered clinically: the non-BPD + no probiotics group, the non-BPD + probiotics group, the BPD + no probiotics group, and the BPD + probiotics group. The diagnostic criteria for BPD were based on those of the National Institute of Child Health and Human Development (NICHD): the need for oxygen or respiratory support at 28 days postnatal age or 36 weeks' corrected gestational age[11]. Infants meeting this criterion at either time point were classified as having BPD.

In the probiotic groups, following the obtaining of informed consent from guardians, oral probiotics were administered when clinically indicated after the commencement of enteral feeding (Bifidobacterium Quadruple Live Bacteria Tablets, Hangzhou Yuanda Biopharmaceutical Co., Ltd., drug identification code: S20060010, containing Bifidobacterium infantis, Lactobacillus acidophilus, Enterococcus faecalis and Bacillus cereus; 1 tablet per dose, three times daily, with each tablet containing: Infant Bifidobacterium, Lactobacillus acidophilus and Enterococcus faecalis should not be less than $0.5x10^6$ CFU respectively, and Bacillus ceriumus should not be less than $0.5x10^5$ CFU live bacteria (CFU, per the manufacturer's specification)). The non-probiotic group did not receive any probiotic preparations. It should be noted that the use of probiotics was determined by the clinician based on an assessment of the child's clinical condition, rather than by random allocation.

2.3 Sample Collection and Processing

Fresh faecal samples were collected on day 0 (D0, first meconium), day 3 (D3), day 14 (D14) and day 28 (D28) after birth, and on day 7 (D7'), day 14 (D14') and day 21 (D21') after the start of probiotic administration. Note that the D0–D28 series reflects postnatal age, whereas the D7'–D21' series reflects the duration of probiotic exposure; the two series partially overlap in calendar time. For each collection, 0.5–1.0 g of faeces was placed in a sterile cryovial, immediately flash-frozen in liquid nitrogen, and then transferred to a freezer at –80 °C for storage. The D0 sample consisted of the first passage of meconium. DNA was extracted from all samples centrally within six months of collection.

2.4 DNA Extraction and 16S rRNA Gene Sequencing

Faecal genomic DNA was extracted using the TIANamp Stool DNA Kit (catalogue number: DP328, TIANGen Biochemical Technology Co., Ltd., Tai Bei, China) in accordance with the manufacturer's instructions. A negative control (sterile water substituted for the faecal sample) was included in each extraction run for subsequent contamination control analysis. The V3–V4 variable regions of the 16S rRNA gene were amplified using the primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Following purification, the PCR products were sequenced using paired-end sequencing (2 × 250 bp) at Novogene.

2.5 Bioinformatics Analysis

Raw sequencing data were processed using the QIIME2 workflow (version 2023.x). Quality control and noise reduction were performed using the DADA2 algorithm, and amplicon sequence variants (ASVs) were generated after chimera removal. Species annotation was based on the SILVA 138.1 database. α-diversity indices (Chao1, Shannon, Simpson) were calculated based on the rarefied ASV table. The R package decontam (frequency method, threshold = 0.5) was used to remove contaminant ASVs detected in the negative control.

2.6 Statistical Analysis

Continuous variables are expressed as mean ± standard deviation. Comparisons between groups were performed using one-way analysis of variance (ANOVA), with post-hoc tests conducted using Tukey's HSD test. Longitudinal trends in diversity indices were analysed using repeated measures ANOVA; where the time × group interaction effect was significant, separate analyses were conducted for each time point. Categorical variables are expressed as frequencies (percentages); comparisons between groups were performed using the chi-squared test or Fisher's exact test. Prior to one-way ANOVA, normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test) were assessed; for repeated measures ANOVA, normality and sphericity (Mauchly's test) were assessed, and Greenhouse-Geisser correction was applied if the assumptions were not met. Effect sizes were reported as Cohen's d with 95% confidence intervals (CIs) for pairwise between-group comparisons of diversity indices. To control for multiple comparisons across groups, time points, and diversity indices, Benjamini–Hochberg false discovery rate (FDR) correction was applied to all post-hoc pairwise comparisons, and adjusted P values are reported. Handling of missing data: For missing data resulting from failed faecal sample collection at individual time points, a linear mixed model was fitted using repeated-measures ANOVA to make full use of all available data. All statistical tests were two-sided; a P-value < 0.05 was considered statistically significant.

2.7 Sample Size Estimation

Based on the between-group difference in the Chao1 index at time point D14 observed in the pilot study (effect size f = 0.45), with α = 0.05 and β = 0.20, G*Power 3.1 was used to calculate the minimum sample size required for a four-group comparison as 11 participants per group. Taking into account a 20% dropout rate, it was planned to enrol 13–15 participants per group, resulting in a total sample size of approximately 50 participants.

3 Results

3.1 Baseline Characteristics of Study Participants

This study included a total of 50 preterm infants with a gestational age of ≤32 weeks, comprising 25 in the non-BPD group and 25 in the BPD group. There were no statistically significant differences between the four groups in baseline indicators such as gestational age, birth weight, sex, mode of delivery, 1-minute and 5-minute Apgar scores, rate of antenatal steroid use, and duration of antibiotic treatment (P > 0.05) (Table 1). The duration of mechanical ventilation and the length of hospital stay were significantly longer in the BPD group than in the non-BPD group (P < 0.05). There were no statistically significant differences in the distribution of feeding methods (breast milk, formula milk, or mixed feeding) across the four groups (P > 0.05).

Table 1. Comparison of baseline clinical characteristics among the four groups of preterm infants

Parameter	Non-BPD + No Probiotics (n=12)	Non-BPD + Probiotics (n=13)	BPD + no probiotics (n=12)	BPD + probiotics (n=13)	P-value
Gestational age (weeks)	30.1 ± 1.5	29.8±1.7	29.5 ± 1.8	29.3 ± 1.6	0.612
Birth weight (g)	1325±215	1285±238	1255±256	1,238 ± 242	0.783
Male/Female	7/5	8/5	7/5	8/5	0.985
Caesarean section	8 (66.7%)	9 (69.2%)	8 (66.7%)	9 (69.2%)	0.991
Prenatal hormones	10 (83.3%)	11 (84.6%)	10 (83.3%)	11 (84.6%)	0.999
Feeding method (breast milk/formula/mixed)	5/3/4	6/3/4	4/4/4	5/3/5	0.962
Duration of antibiotic treatment	4.2 ± 1.8	4.5 ± 2.0	5.1 ± 2.3	5.3 ± 2.1	0.342
Number of days on mechanical ventilation	2.1 ± 1.5	2.3 ± 1.7	6.5 ± 3.2*	6.8 ± 3.5*	<0.001
Length of hospital stay	28.5 ± 7.2	29.1±6.8	52.3±12.5*	53.1 ± 11.8*	<0.001

*Indicates a comparison with the corresponding non-BPD group, P < 0.05. Continuous variables are presented as mean ± SD; categorical variables are presented as n (%).

3.2 Three-stage dynamic evolution of gut microbiota α-diversity

A longitudinal analysis of α-diversity indices at the four postnatal time points (D0–D28) in the four groups of preterm infants revealed a distinct three-stage evolutionary pattern (Figure 1).

Stage 1: High diversity at baseline (D0)

At D0 (meconium), the mean Chao-1 index was 375.73 ± 27.24, the Shannon index was 4.71 ± 0.78, and the Simpson index was 0.73 ± 0.12. There were no statistically significant differences in any of the indices between the four groups at D0 (P > 0.05), suggesting that BPD-related differences in the microbiota had not yet emerged at birth.

Stage 2: Sharp decline in diversity at D3

From D0 to D3, the Chao-1 index plummeted from 375.73 to 15.50, a decrease of 95.7%; the Shannon index fell from 4.71 to 1.57, a decrease of 66.67%; the Simpson index fell from 0.73 to 0.60, a decrease of 17.81%. This ‘diversity collapse’ occurred simultaneously across all four groups, with no statistically significant

differences between groups (P > 0.05), suggesting that this sharp decline was primarily driven by the postnatal environmental transition (from the uterus to the NICU) rather than by BPD-specific factors.

Stage 3: Differentiation between groups after D14

From D14 onwards, the diversity indices across the four groups showed significant divergence, which persisted until D28. The Chao1, Shannon and Simpson indices in the non-BPD group (regardless of probiotic use) were significantly higher than those in the BPD group (P < 0.05), whereas differences between groups at D0 and D3 were not statistically significant. The emergence of this time point occurs significantly earlier than the clinical diagnostic window for BPD (typically D28–D36), suggesting that reduced microbial diversity may be an early concomitant event in the development of BPD rather than a late consequence.

Table 2. Comparison of α-diversity indices (Chao1 index) across the four groups of preterm infants at various time points (mean ± SD)

Time point	Non-BPD+ No probiotics	Non-BPD+ Probiotics	BPD+ No probiotics	BPD+ Probiotics	F	P-value
D0	375.73 ± 27.24	376.29 ± 17.25	374.25 ± 27.76	374.65 ± 34.64	0.015	0.997
D3	15.50 ± 0.79	15.61 ± 1.51	15.33 ± 0.36	15.43 ± 0.63	0.195	0.899
D14	15.18 ± 0.74	15.25 ± 0.82	14.06 ± 0.71*#	14.36 ± 0.69*#	7.881	<0.001
D28	15.25 ± 0.64	15.42±0.86	14.11 ± 0.58*#	14.58 ± 0.58*#	9.974	<0.001

Note: Data are expressed as mean ± standard deviation. * indicates a comparison with the non-BPD+ group without probiotics at the same time point, P < 0.05. # indicates a comparison with the non-BPD+ group with probiotics at the same time point, P < 0.05. There were no statistically significant differences between the four groups at time points D0 and D3.

Table 3. Comparison of α-diversity indices (Shannon index) among the four groups of preterm infants at various time points (mean ± SD)

Time point	Non-BPD+ No probiotics	Non-BPD+ Probiotics	BPD+ No probiotics	BPD+ Probiotics	F	P-value
D0	4.71 ± 0.78	4.88 ± 0.54	4.61 ± 0.54	4.69 ± 0.44	0.449	0.719
D3	1.57 ± 0.08	1.58 ± 0.10	1.54 ± 0.08	1.56 ± 0.13	0.359	0.783
D14	1.52±0.09	1.54 ± 0.08	1.37 ± 0.06*#	1.41 ± 0.05*#	17.252	<0.001
D28	1.56 ± 0.06	1.59 ± 0.09	1.44 ± 0.07*#	1.49 ± 0.05*#	10.394	<0.001

Note: Data are expressed as mean ± standard deviation. * indicates a comparison with the non-BPD+ group without probiotics at the same time point, P < 0.05. # indicates a comparison with the non-BPD+ group with probiotics at the same time point, P < 0.05. Differences between the four groups at time points D0 and D3 were not statistically significant.

Table 4. Comparison of α-diversity indices (Simpson's index) among the four groups of preterm infants at various time points (mean ± SD)

Time point	Non-BPD+ No probiotics	Non-BPD+ Probiotics	BPD+ No probiotics	BPD+ Probiotics	F	P-value
D0	0.73 ± 0.12	0.74 ± 0.07	0.73 ± 0.12	0.73 ± 0.07	0.015	0.998
D3	0.60 ± 0.12	0.61 ± 0.08	0.57 ± 0.06	0.59 ± 0.06	0.434	0.730
D14	0.58±0.07	0.58 ± 0.08	0.50 ± 0.06*#	0.52 ± 0.04*#	5.231	0.003
D28	0.59±0.05	0.60±0.07	0.52±0.04*#	0.53±0.06*#	6.920	<0.001

Note: Data are expressed as mean ± standard deviation. * indicates a comparison with the non-BPD+ group without probiotics at the same time point, P < 0.05. # indicates a comparison with the non-BPD+ group with probiotics at the same time point, P < 0.05. There were no statistically significant differences between the four groups at time points D0 and D3.

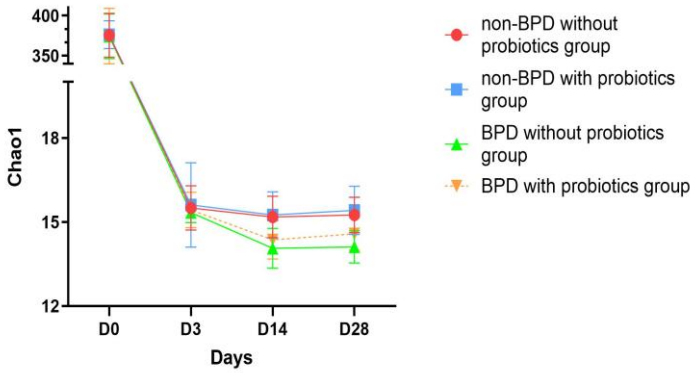


Fig 1 (b)

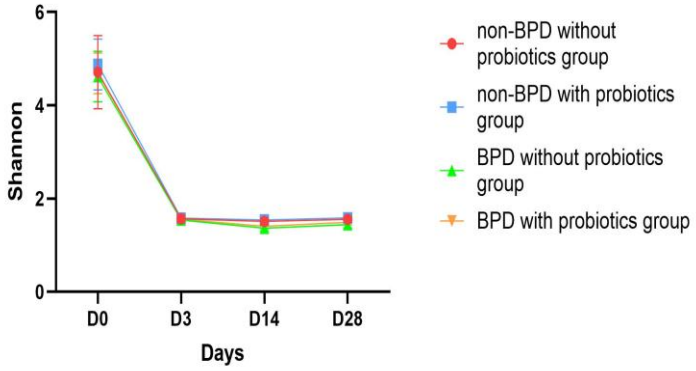


Fig 1 (c)

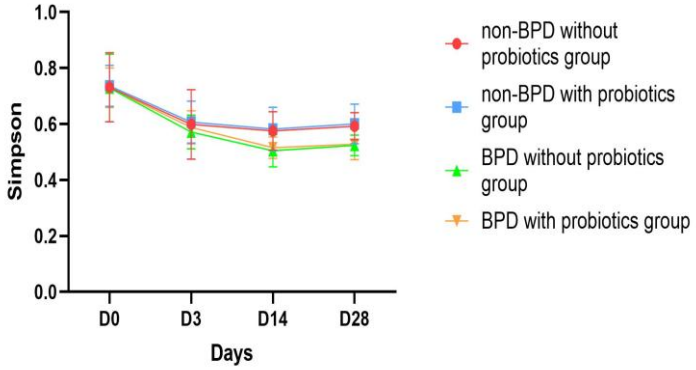


Figure 1. Longitudinal changes in α-diversity over the first 28 days of life: (a) Chao1 index; (b) Shannon index; (c) Simpson index. Lines represent group means; error bars indicate SD.

3.3 Key time windows for diversity differences between the BPD and non-BPD groups

To determine when BPD-associated microbiota differences emerge, we compared the diversity indices across the four groups at each time point. Under conditions without probiotic supplementation, a comparison between the non-BPD group without probiotics and the BPD group without probiotics revealed that the differences in the Chao1, Shannon and Simpson indices were not statistically significant at D0 and D3 ($P > 0.05$); D14: All indices in the non-BPD group were significantly higher than those in the BPD group (Chao1: 15.18±0.74 vs 14.06±0.71, P=0.001; Shannon: 1.52±0.09 vs 1.37±0.06, P<0.001; Simpson: 0.58±0.07 vs 0.50±0.06, P=0.011); D28: All indices in the non-BPD group were significantly higher than those in the BPD group (Chao1: 15.25 ± 0.64 vs 14.11 ± 0.58, $P < 0.001$; Shannon: 1.56 ± 0.06 vs 1.44 ± 0.07, $P < 0.001$; Simpson: 0.59 ± 0.05 vs 0.52 ± 0.04, $P < 0.001$); this difference persisted at D14 and D28 and showed a trend towards widening (Tables 2–4).

Comparisons between subgroups receiving probiotics revealed a similar pattern: statistically significant differences in the Chao1, Shannon, and Simpson indices were observed between the non-BPD + probiotics group and the BPD + probiotics group at D14 and D28 ($P < 0.05$), although the magnitude of these differences was smaller than that observed in the non-probiotics subgroup. This suggests that probiotic intervention partially improved the reduced microbial diversity in the BPD group but failed to fully restore it to non-BPD levels.

Fig 2 (a)

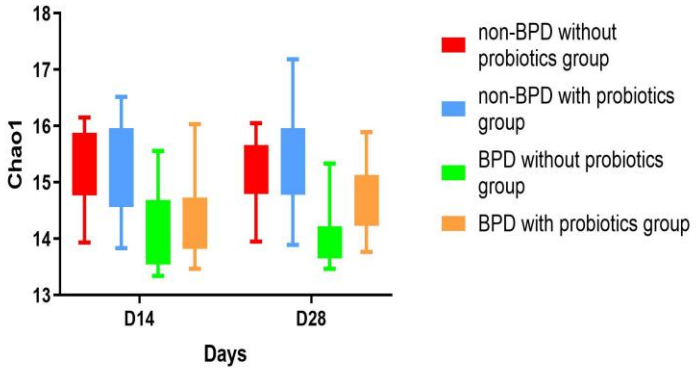


Fig 2 (b)

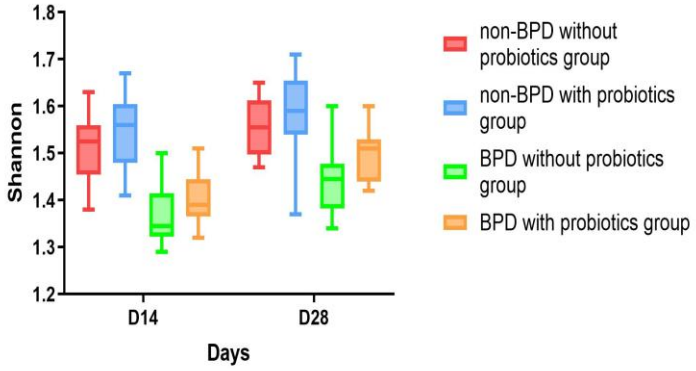


Fig 2 (c)

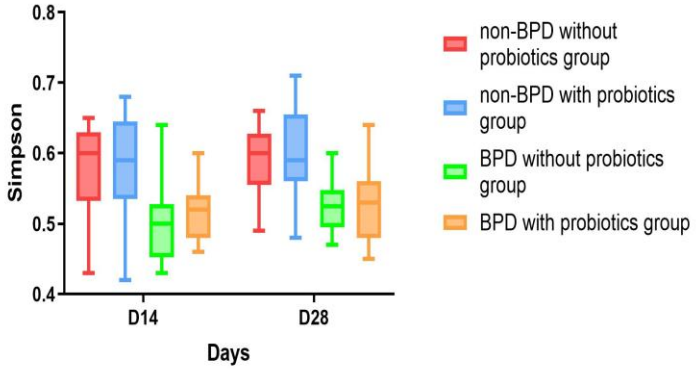


Figure 2. Between-group comparisons of α-diversity at D14 and D28: (a) Chao1 index; (b) Shannon index; (c) Simpson index. Boxes show medians with interquartile ranges.

3.4 Modulatory effects of probiotic intervention on microbial diversity

Within the non-BPD group, differences in diversity indices between the probiotic subgroup and the non-probiotic subgroup at all time points were not statistically significant ($P > 0.05$), suggesting that in preterm infants without BPD who had a better baseline gut microbiota status, the effect of probiotics on enhancing overall diversity was limited.

Within the BPD group, the Chao1 and Shannon indices in the probiotic subgroup showed a trend towards higher values than those in the non-probiotic subgroup; however, these differences did not reach statistical significance, which may be attributed to the limited sample size. However, when comparing the effects of probiotics between the BPD subgroups (i.e. non-BPD + probiotics vs BPD + probiotics), the differences in diversity indices at D14' and D21' following probiotic administration were statistically significant ($P < 0.05$), indicating that even under probiotic intervention, the BPD group still exhibited significant diversity deficits.

The above results suggest that BPD-associated dysbiosis may involve 'colonisation resistance'—that is, the gut microenvironment in the BPD group resists the colonisation and proliferation of probiotics, making it difficult for exogenous probiotics to effectively establish themselves in the gut of the BPD group and restore microbial diversity. This phenomenon is similar to the 'microbial colonisation resistance' observed in adult inflammatory bowel disease[12].

Table 5. Comparison of α-diversity indices (Chao1 index) for probiotic subgroups at various time points following probiotic administration (mean ± SD)

Time point	Non-BPD+ Probiotics	BPD+ Probiotics	t	P
D7'	15.38±0.72	15.13 ± 0.81	0.83	0.414
D14'	15.16 ± 0.45	14.52 ± 0.77	2.56	0.017
D21'	15.50 ± 1.00	14.78 ± 0.51	2.31	0.030

Note: Data are expressed as mean ± standard deviation.

Table 6. Comparison of α-diversity indices (Shannon index) across probiotic subgroups at various time points following probiotic administration (mean ± SD)

Time point	Non-BPD+ Probiotics	BPD+ Probiotics	t	P
D7'	1.52 ± 0.90	1.50 ± 0.06	0.76	0.451
D14'	1.54 ± 0.12	1.46±0.04	2.20	0.044
D21'	1.59 ± 0.13	1.50 ± 0.06	2.24	0.038

Note: Data are presented as mean ± standard deviation.

Table 7. Comparison of α-diversity indices (Simpson's index) across probiotic subgroups at various time points following probiotic administration (mean ± SD)

Time point	Non-BPD+ Probiotics	BPD+ Probiotics	t	P
D7'	0.53±0.04	0.50 ± 0.04	1.98	0.059
D14'	0.53±0.04	0.45±0.03	5.17	<0.001
D21'	0.60 ± 0.09	0.50 ± 0.03	3.30	0.004

Note: Data are expressed as mean ± standard deviation.

Fig 3 (a)

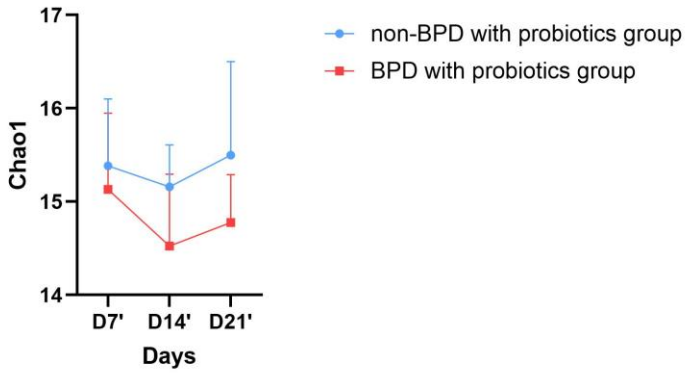


Fig 3 (b)

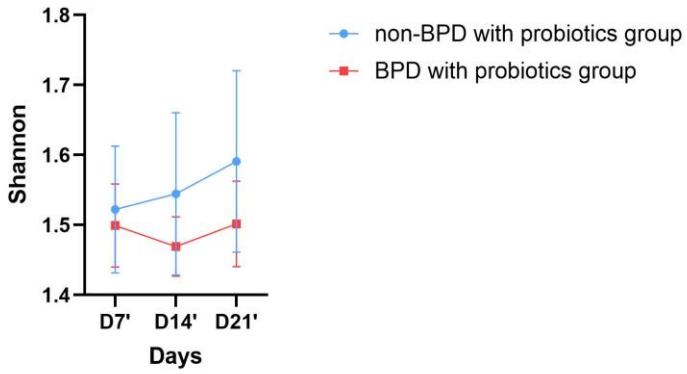


Fig 3 (c)

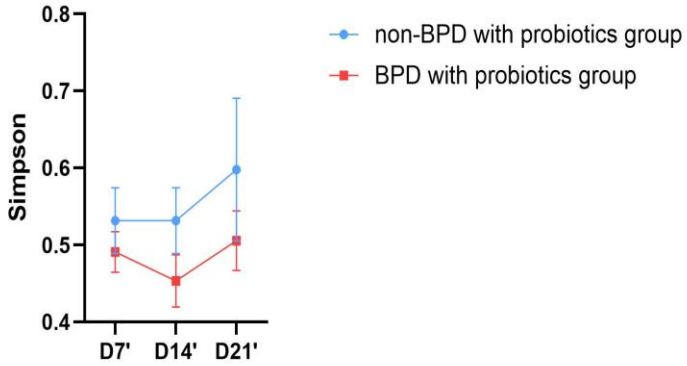


Figure 3. α-Diversity following probiotic administration (D7'–D21'): (a) Chao1 index; (b) Shannon index; (c) Simpson index.

3.5 Bifidobacterium colonisation kinetics

Given that Bifidobacterium is a key beneficial bacterium in the gut of preterm infants and the primary component of the probiotic intervention in this study, we further analysed the longitudinal changes in the relative abundance of Bifidobacterium following probiotic administration (Table 8, Figure 4). Within the probiotic subgroups (non-BPD + probiotics vs BPD + probiotics), the non-BPD + probiotics group exhibited higher relative abundance of Bifidobacterium at D14' following probiotic administration (0.0241 ± 0.0136 vs 0.0151 ± 0.0058, P = 0.041) and D21' (0.0400 ± 0.0128 vs 0.0285 ± 0.0111, P = 0.022). At D7', the non-BPD group was higher than the BPD group, but the difference was not statistically significant (P = 0.815).

Longitudinal observations showed that the relative abundance of Bifidobacterium in both groups increased with the duration of probiotic administration; however, the upward trend in the BPD+probiotic group lagged behind that of the non-BPD+probiotic group, suggesting delayed colonisation of Bifidobacterium in the BPD group.

Table 8. Relative abundance of Bifidobacterium at various time points following probiotic administration (mean ± SD)

Time point	Non-BPD + probiotic group	BPD + probiotics group	t	P
D7'	0.0051 ± 0.0015	0.0048 ± 0.0041	0.237	0.815
D14'	0.0241 ± 0.0136	0.0151 ± 0.0058	2.188	0.044
D21'	0.0400 ± 0.0128	0.0285 ± 0.0111	2.459	0.022

Note: Data are expressed as mean ± standard deviation.

Fig 4

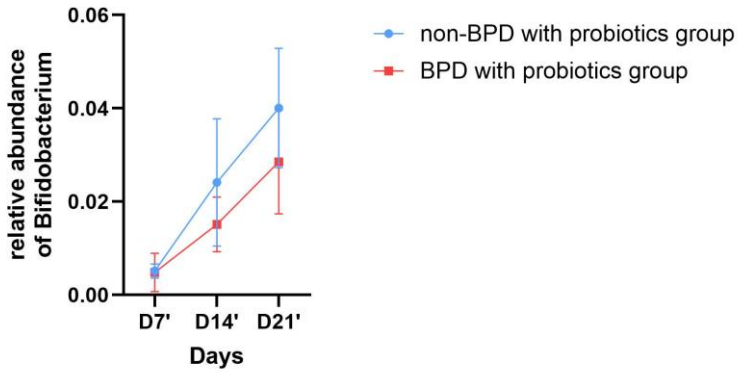


Figure 4. Relative abundance of Bifidobacterium following probiotic administration (D7'–D21').

4 Discussion

This study identified three key findings through longitudinal tracking of the faecal microbiota at seven time points in 50 preterm infants with a gestational age of ≤32 weeks. Firstly, the α-diversity of the gut microbiota in preterm infants exhibited a three-stage evolutionary pattern characterised by a 'high baseline–sharp decline–differentiation' pattern: at D0 (meconium), diversity was extremely high (Chao1 = 375.73); by D3, it had plummeted to an extremely low level (Chao1 = 15.50, a decrease of 95.7%); thereafter, whilst maintaining a low plateau, the BPD and non-BPD groups began to diverge significantly from D14 onwards. Secondly, the BPD group consistently exhibited reduced α-diversity and delayed Bifidobacterium colonisation between D14 and D28, whereas this difference was not yet apparent at birth (D0), suggesting that microbiota dysbiosis is an early acquired event in the development of BPD. Thirdly, probiotic intervention partially improved microbial diversity but failed to fully correct the BPD-associated dysbiosis, suggesting the presence of 'colonic colonisation resistance' in the BPD group.

4.1 Peak diversity in meconium at D0: biological significance and controversy

The high diversity observed in meconium at D0 (Chao1 = 375.73 ± 27.24) in this study is consistent with several recent studies. Kim et al. [9] found in a South Korean cohort of preterm infants that meconium α-diversity was highest at all time points, declined significantly during NICU admission, and rebounded after discharge. Dong et al.[10] further found in a Chinese multicentre study that the diversity of meconium in extremely preterm infants (GA < 28 weeks) was anomalously higher than in other gestational age groups, suggesting that this phenomenon is more pronounced in the more immature preterm infants. However, the biological explanation for this phenomenon remains controversial. The 'in utero microbial colonisation hypothesis' posits that foetal faeces reflect the in utero microbial environment[13]; however, a study by Kennedy et al.[14] published in *Nature*, employing rigorous contamination control analyses, cast doubt on this, suggesting that the microbial signals detected in foetal faeces likely stemmed from contamination during sampling and experimental procedures, and that the gut of healthy foetuses may be sterile.

The data from this study provide indirect but significant corroborating evidence for this debate. Firstly, the high diversity observed on Day0 was highly consistent across the four groups (Chao1: 374.25–376.29), with no systematic bias between groups; this does not support the hypothesis of purely random contamination—if contamination were the cause, greater inter-group variability would be expected, and this would be correlated with the batch of sample processing. Secondly, the 'diversity collapse' observed from D0 to D3 exhibits a temporal pattern (occurring simultaneously across all four groups), which is more consistent with the biological explanation of a 'transition from the intrauterine to the postnatal environment' rather than random fluctuations in contamination. Although this study incorporated negative controls during DNA extraction and utilised the decontam package for contamination control, the extremely low biomass of the D0 (meconium) samples limited the detection performance of the decontam frequency method, and the contribution of low-biomass

contamination cannot be entirely ruled out. Future studies should combine absolute bacterial quantification (qPCR) with metagenomics to validate the authenticity of the meconium microbiota.

4.2 Three-stage evolutionary pattern and the 'NICU environmental bottleneck effect' hypothesis

This study describes, for the first time in a BPD cohort, a three-stage evolutionary pattern characterised by 'high baseline–sharp decline–differentiation'; based on this, we propose the 'NICU environmental bottleneck effect' hypothesis. The first stage (high diversity at D0) likely reflects initial colonisation following exposure to intrauterine or intrapartum microbiota; the second stage (sharp decline at D3) is driven by postnatal environmental transition—factors such as the sterilised environment of the NICU, the use of broad-spectrum antibiotics, and formula feeding constitute a 'bottleneck', screening the initially highly diverse microbiota down to a low-diversity state dominated by a small number of opportunistic pathogens; the third stage (differentiation after D14) reflects differences in the recovery trajectories of the microbiota across different host backgrounds—whilst the non-BPD group was able to gradually re-establish diversity, the BPD group was unable to recover effectively due to factors such as persistent inflammation, oxygen exposure and an immature intestinal barrier.

This hypothesis is consistent with the findings of Beck et al.[15] and Thänert et al.[16]—namely, that the period of NICU admission represents a critical window of disruption in the development of the microbiota in preterm infants, the intensity of which may exceed the influence of genetic background. The novelty of this study lies in linking this evolutionary process to the development of BPD and identifying the onset of intergroup differentiation (D14), thereby providing direct evidence for selecting the timing of intervention.

4.3 Gut-lung axis mechanisms underlying BPD-associated dysbiosis

The persistent reduction in diversity and delayed colonisation by Bifidobacterium observed in the BPD group from D14 onwards may contribute to the pathogenesis of BPD via the gut-lung axis. As a key beneficial bacterium in the gut of preterm infants, Bifidobacterium's metabolic activity promotes the production of butyrate in the gut[17]. Studies have confirmed that butyrate can induce the differentiation of regulatory T cells in the colon by inhibiting the activity of histone deacetylases (HDACs)[18]. In an animal study, the colonisation of the lungs of germ-free mice with Lactobacillus resulted in changes in the number, size and mucus content of their alveoli[19]. This study found that Bifidobacterium colonisation in the BPD group was delayed by approximately one week compared with the non-BPD group; this time lag coincides precisely with the critical window for the pathological development of BPD (the first 1–2 weeks after birth, which is the key period for alveolar sac–alveolar development), suggesting that delayed Bifidobacterium colonisation may weaken the protective immunoregulation of the gut–lung axis by reducing the supply of SCFAs.

Furthermore, reduced α-diversity is typically accompanied by dysregulation of microbial metabolic functions, leading to inflammatory responses and cytokine production, and is associated with the regulation of cytokines such as IL-1β, IL-4, IL-6, IL-8, IL-10 and TNF-α[20]. This study provides preliminary microbiota phenotypic data supporting this mechanistic hypothesis; further validation is required in future studies combining quantitative analysis of faecal SCFAs with serum inflammatory cytokine assays.

It is worth noting that at D14, the difference in the Chao1 index between the BPD group and the non-BPD group was approximately 1 (15.18 vs 14.06); although this difference was statistically significant ($P < 0.001$), its biological significance during the low-diversity plateau phase should be interpreted with caution. We believe that at an extremely low Chao-1 level of approximately 15, even a difference of a single ASV may reflect the successful colonisation of a specific bacterial species (such as Bifidobacterium), and its impact on the metabolic function of the gut-lung axis may extend beyond the scope reflected by the diversity index itself. Future studies should comprehensively evaluate the biological significance of intergroup differences by combining effect sizes (such as Cohen's d) with functional indicators.

4.4 Effects of Probiotic Intervention and 'Microbiota Colonisation Resistance'

The third key finding of this study is the 'partial compensation' pattern observed with probiotic intervention—whilst probiotics improved the diversity trend in the BPD group, they failed to correct it to the level seen in the

non-BPD group. This phenomenon suggests that the gut microenvironment in the BPD group exhibits 'colonisation resistance' to exogenous probiotics. The concept of colonisation resistance originates from research on the adult gut microbiota and refers to the established microbial ecosystem resisting the colonisation of exogenous bacteria through mechanisms such as nutritional competition, bacteriocin production and niche occupation[12]. In preterm infants with BPD, we hypothesise that the mechanisms underlying colonisation resistance may differ: opportunistic pathogens already dominant in the gut of the BPD group (such as facultative anaerobes including Klebsiella and Enterobacter) may inhibit the colonisation of strict anaerobes, such as Bifidobacterium, through rapid nutrient depletion and alterations in the microenvironment's pH. Furthermore, the BPD group often experiences prolonged antibiotic exposure and intestinal barrier dysfunction, which may also limit the survival and proliferation of probiotics[12].

A meta-analysis by Villamor-Martínez et al.[21] (incorporating 15 RCTs involving 4,782 preterm infants) showed that whilst probiotics prevented NEC and late-onset sepsis, they did not significantly reduce the risk of BPD. This finding corroborates the 'colony resistance' phenomenon observed in the present study—suggesting that probiotic supplementation alone may be insufficient to overcome BPD-associated dysbiosis. Future research should explore strategies such as combining probiotics with faecal microbiota transplantation, personalised probiotic formulations, or intestinal barrier repair agents.

4.5 Clinical Implications: Day 14 as a Critical Window for Microbiota Intervention

The most important clinical implication of this study is that D14 may represent a critical time window for gut microbiota intervention to prevent BPD. Firstly, differences in microbiota diversity between the BPD and non-BPD groups became significantly apparent from D14 onwards and persisted until D28, suggesting that D14 is a 'point of no return' for the divergence of microbiota trajectories—after which, even with probiotic supplementation, differences between the groups persisted. Secondly, whilst the clinical diagnosis of BPD is typically established at D28 or 36 weeks' corrected gestational age, microbiota dysbiosis had already emerged two weeks earlier (D14), providing an objective temporal basis for early microbiota intervention in BPD. Thirdly, in a recent study of preterm infants with BPD, cytokines associated with the inflammatory response were markedly elevated between D14 and D28, suggesting that the development of BPD is linked to an imbalance in gut-derived inflammatory factors[20]. Based on these findings, we recommend that for preterm infants with high-risk factors for BPD (such as very low birth weight, prolonged mechanical ventilation, and exposure to intrauterine inflammation), microbiota intervention may be initiated before day 14, depending on the infant's condition (e.g., establishment of enteral nutrition), rather than waiting until BPD has been clinically diagnosed. This concept of an 'early window' is consistent with the recent trend in BPD prevention strategies towards 'immediate postnatal intervention'[22].

4.6 Limitations

This study has the following limitations: Firstly, the sample size was limited (12–13 cases per group); whilst this was sufficient for statistical power regarding the primary outcome, it may have failed to detect intergroup differences in some secondary endpoints (e.g., $P = 0.054–0.975$ for comparisons within the BPD group's probiotic subgroups), which requires validation in a larger, multicentre study. Secondly, as this was a single-centre cohort study, the generalisability of the NICU antimicrobial strategy and feeding regimens requires validation in external cohorts. Thirdly, although negative controls were included in the DNA extraction batches for D0 (meconium) samples, the extremely low biomass of meconium limited the effectiveness of the decontam frequency method in identifying contamination, making it difficult to completely rule out the contribution of contamination in a low-biomass context; future validation using absolute bacterial quantification (qPCR) is required. Fourthly, this study analysed only 16S rRNA gene sequencing data and lacked quantitative faecal SCFA measurements, metagenomic functional annotation, and serum immunological markers, thereby limiting an in-depth elucidation of the gut-lung axis mechanism. Fifth, the study did not collect samples from the mother's birth canal, skin, or the NICU environment, making it impossible to determine the source of the microbiota (vertical transmission versus environmental acquisition). Sixth, the use of probiotics was based on clinical decision-making rather than random allocation, which may introduce 'confounding by indication': children eligible for probiotic treatment may have better intestinal function and a lower inherent risk of BPD. Although differences in baseline characteristics (including feeding method) across the four groups were not

statistically significant, residual confounding cannot be entirely ruled out. Seventh, the diagnosis of BPD was made at D28 or 36 weeks' corrected gestational age; differences in the microbiota at D28 may partly reflect the consequences of BPD rather than its causes; data from D14 are more reliable for causal inference. Moreover, the 2024 NICHD revision of the BPD diagnostic criteria may reclassify some infants; future studies should report both definitions. Eighth, although the duration of antibiotic treatment did not differ significantly between groups (P = 0.342), infants who later developed BPD received antibiotics for approximately one day longer on average; given the high sensitivity of the developing microbiota to antibiotic exposure, residual confounding by antibiotics cannot be excluded and may partly underlie the diversity differences observed from D14 onwards.

4.7 Conclusions

The gut microbiota of preterm infants with a gestational age of ≤32 weeks exhibits a three-stage evolution pattern characterised by a 'high baseline–sharp decline–differentiation'. The BPD group showed a sustained reduction in α-diversity and delayed Bifidobacterium colonisation from D14 onwards. Probiotic intervention partially improved but failed to fully correct BPD-associated dysbiosis, suggesting the presence of 'colonisation resistance'. Day 14 postnatal may represent a critical window for gut microbiota intervention to prevent BPD. Future studies are needed to validate the predictive value of D14 microbiota characteristics for BPD in multicentre prospective cohorts and to explore combined intervention strategies that go beyond single-strain probiotic supplementation.

Declarations

Abbreiations

ANOVA, analysis of variance; ASVs, amplicon sequence variants;

BPD, bronchopulmonary dysplasia; CFU, colony-forming units;

CI, confidence interval; DNA, deoxyribonucleic acid;

FDR, false discovery rate; GA, gestational age;

HDAC, histone deacetylase; HSD, honestly significant difference;

IL, interleukin; NEC, necrotising enterocolitis;

NICHD, National Institute of Child Health and Human Development;

NICU, neonatal intensive care unit; PCR, polymerase chain reaction;

qPCR, quantitative polymerase chain reaction; RCT, randomised controlled trial;

rRNA, ribosomal ribonucleic acid; SCFAs, short-chain fatty acids;

TNF-α, tumour necrosis factor-alpha.

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Guilin Medical University (Approval No.: 2021KJJ40), and written informed consent was obtained from the guardians of all participating infants. All experiments were performed in accordance with relevant guidelines and regulations, including the Declaration of Helsinki. The study protocol was registered with the Guilin Municipal Bureau of Science and Technology (Registration No.: 20210227-7-7).

Consent for publication

Not applicable. This manuscript contains no individual persons' data in any form.

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available due to ethical and privacy restrictions involving clinical data from preterm infants, but are available from the corresponding author on reasonable request. The raw 16S rRNA sequencing data can be made available by the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Man Liu: Conceptualization, Investigation, Data curation, Writing – original draft. Xinyi Liang: Methodology, Data curation, Formal analysis, Writing – original draft. Sifeng Yue: Investigation, Sample collection, Formal analysis. Tianli Liang: Data curation, Visualization. Zi'ang Gao: Investigation, Visualization. Syed Kamran Bukhari: Writing – review & editing, Language polishing. Yuan Tan: Conceptualization, Supervision, Funding acquisition, Project administration, Writing – review & editing. All authors read and approved the final manuscript.

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Man Liu and Xinyi Liang contributed equally to this work as co-first authors.

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