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COMPARATIVE TRAJECTORIES OF GUT MICROBIOME COLONIZATION IN TWIN VERSUS SINGLETON NEONATES OVER SIX MONTHS POSTPARTUM: A LONGITUDINAL COHORT STUDY

Dr. Rimal Rashid[*1], Tooba Khanum[2], Danya Warsi[3], Haifa Arain[4] Komal Mushtaq[5], Dur e Shewar[6]

[*1]*MBBS, Master of Public Health, Dow University Hospital, Karachi, Pakistan*
[2]*M.Phil. Clinical Nutrition (Gold Medalist), Lecturer, Minhaj University Lahore, Pakistan*
[3]*PharmD student, Baqai Medical University, Karachi, Pakistan*
[4]*MBBS, Dow Medical College, Dow University of Health Sciences, Karachi, Pakistan*
[5]*MBBS, FCPS, MRCPCH, Senior Registrar Pediatric Medicine, Lady Willingdon Hospital, Lahore, Pakistan*
[6]*BS Microbiology, Federal Urdu University of Science and Technology, Karachi, Pakistan*

[1]https://orcid.org/0009-0005-5101-9486, [2]https://orcid.org/0009-0008-7697-7324, [4]https://orcid.org/0009-0009-5886-5238, [5]https://orcid.org/0000-0002-2995-871X

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gut microbiome, neonatal colonization, twins, singletons, longitudinal cohort, 16S rRNA sequencing, Pakistan, microbial diversity, infancy maturation.

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Corresponding Author: *
Dr. Rimal Rashid

Abstract

Objective:

We evaluated and compared the longitudinal patterns of gut microbiome colonization in twin and singleton neonates over the first six months postpartum in a tertiary care setting in Pakistan, aiming to delineate differences in microbial diversity, community composition, and temporal dynamics.

Methods:

A prospective longitudinal cohort of 120 neonates (60 twins – including 30 twin pairs – and 60 singletons) was enrolled at the neonatal unit of a tertiary hospital in Karachi, Pakistan, between January 2023 and December 2024. Fecal samples were collected at birth (meconium), 1 week, 1 month, 3 months, and 6 months. Microbial profiling was conducted via 16S rRNA gene sequencing, and taxa were annotated against SILVA and Greengenes databases. Alpha and beta diversity metrics were computed, and differential abundance analyses were performed. Statistical significance was assessed using mixed-effects models controlling for delivery mode, feeding practices, antibiotic exposure, and gestational age.

Results:

Both twins and singletons exhibited increasing microbial diversity over time. Singleton neonates had significantly higher alpha diversity (Shannon index) at week 1 and month 1 compared to twin neonates ($p < 0.05$). Twin pairs demonstrated delayed colonization by Bifidobacterium and Bacteroides species, and higher relative abundance of Proteobacteria at early time points compared to singletons. Beta diversity analyses revealed distinct community trajectories between twin and singleton groups from birth to six months (PERMANOVA, $p < 0.01$).

Conclusion:

Gut microbiome colonization followed distinct trajectories in twin versus singleton neonates within the first six months postpartum. These differences were influenced

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by host factors such as delivery mode and early feeding practices. Understanding divergent colonization patterns may inform targeted microbiome-based interventions in neonatal care to mitigate later-life health disparities.

INTRODUCTION

The establishment of the gut microbiome in early life represents a fundamental process that profoundly impacts health across the life course. The neonatal period marks a dynamic window in which microbial communities undergo rapid succession, thereby influencing immunological development, metabolic programming, and susceptibility to disease (Roager *et al.*, 2025). Unlike the relatively stable adult microbiome, the newborn gut microbiota is characterized by high plasticity and sensitivity to environmental and host-related factors, including mode of delivery, feeding practices, antibiotic exposure, and genetic background (Catassi *et al.*, 2024; Shao *et al.*, 2019).

While numerous studies have delineated the general course of microbial colonization in infancy, comparative data between twin and singleton neonates remains sparse. Twins represent a unique model system to disentangle genetic versus environmental contributions to microbial assembly. A longitudinal investigation from the Wuhan Twin Birth Cohort demonstrated that gut microbiota richness and diversity increased with age and identified specific genera that followed distinct developmental trajectories over time (Mei *et al.*, 2022). However, most research in this domain has been conducted outside South Asia, and there is a paucity of data from low- and middle-income countries where nutritional and environmental exposures differ markedly from high-resource settings.

The neonatal gut microbiome undergoes rapid maturation in the early postnatal period. The meconium, once considered sterile, has been found to host low biomass microbial communities, although the "sterile womb" hypothesis remains debated (Perez-Munoz *et al.*, 2017). Initial colonizers predominantly derive from the maternal vaginal, skin, and gastrointestinal microbiota during childbirth and early contact (Dominguez-Bello *et al.*, 2010). Delivery mode exerts a profound influence: vaginally delivered neonates generally acquire microbiota resembling maternal vaginal and fecal communities, enriched in Lactobacillus and Bifidobacterium, while cesarean section births show delayed acquisition of beneficial taxa and enrichment of opportunistic Proteobacteria taxa (Ma *et al.*, 2024).

Furthermore, the role of host genetics and shared environment in shaping early-life microbiomes has been increasingly recognized. Twin studies are particularly informative, as monozygotic twins share identical genetics whereas dizygotic twins share only ~50 %, enabling partitioning of heritable versus environmental influences on microbial colonization (Goodrich *et al.*, 2014). However, the existing longitudinal research predominantly focuses on later childhood and adult twins rather than neonatal cohorts. Moreover, preterm twins in neonatal intensive care settings exhibit distinct microbial patterns influenced by hospital environment and antibiotic exposure (Cong *et al.*, 2021).

In resource-limited settings like Pakistan, early-life exposures such as maternal nutrition, enteric infections, and environmental enteric dysfunction may further modulate gut microbiome development. Longitudinal work among Pakistani children has identified age-related increases in alpha diversity and shifts in taxonomic composition over the first two years, underscoring the importance of age and local environmental factors (Balaji *et al.*, 2023). Nonetheless, comparative trajectories of twins versus singletons in South Asian contexts have not been comprehensively described.

The maturation of neonatal microbial communities also correlates with profound immunological and metabolic outcomes. Disrupted or suboptimal colonization has been linked to atopy, obesity, and neurodevelopmental

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impairment in later life (Arrieta *et al.*, 2015; Carlson *et al.*, 2018). Consequently, understanding how early colonization differs across birth types — and how such differences persist or converge over time — holds clinical relevance.

In this study, we implemented a longitudinal design to chart gut microbiome trajectories in twin and singleton neonates through six months of age at a tertiary hospital in Karachi, Pakistan. We hypothesized that microbial colonization patterns would diverge between the two groups, potentially due to differences in intrauterine environment, initial microbial seeding, and postnatal exposures.

We aimed to:

- **Characterize temporal changes in microbial diversity and community structure** from birth to six months in both twins and singletons.
- **Identify specific taxa and community features** that followed distinct colonization trajectories in twin versus singleton neonates.
- **Assess the influence of perinatal and environmental factors**, including delivery mode, feeding practices, and antibiotic exposure, on gut microbiome development.

By providing a detailed comparison of early gut microbiome maturation in twin and singleton neonates, we sought to advance understanding of early microbial ecology in a South Asian clinical cohort. Our findings are expected to inform tailored neonatal care strategies and future interventional studies aimed at optimizing microbiome-linked health trajectories.

MATERIALS AND METHODS

Study Design and Setting

This study was conducted as a prospective longitudinal cohort investigation at the neonatal and pediatric units of a tertiary care teaching hospital in Karachi, Pakistan. The study period extended from January 2023 to December 2024. The hospital served as a major referral center for both urban and peri-urban populations, providing obstetric and neonatal services to a diverse patient population. The longitudinal design was selected to capture dynamic changes in gut microbiome composition from birth through six months of postnatal life.

Study Population

Neonates were recruited consecutively from the labor and delivery units within 48 hours of birth. The cohort consisted of two groups: twin neonates and singleton neonates. The twin group included both monozygotic and dizygotic twins, identified through obstetric records and placental examination. The singleton group comprised neonates born from uncomplicated singleton pregnancies.

Inclusion Criteria

- Live-born neonates delivered at the study hospital
- Gestational age ≥34 weeks
- Birth weight ≥1,800 grams
- Availability for follow-up until six months of age
- Written informed consent obtained from parents or legal guardians

Exclusion Criteria

- Major congenital anomalies
- Known chromosomal abnormalities
- Neonates requiring prolonged mechanical ventilation (>7 days)
- Maternal history of chronic inflammatory or autoimmune diseases
- Maternal antibiotic use within two weeks prior to delivery (except intrapartum prophylaxis)

A total of 132 neonates were initially enrolled. Twelve participants were excluded during follow-up due to relocation or incomplete sample collection, resulting in a final analytical cohort of 120 neonates (60 twins and 60 singletons).

Clinical Data Collection

Baseline maternal and neonatal data were collected using structured case record forms. Maternal variables included age, parity, antenatal antibiotic exposure, gestational complications, and mode of delivery. Neonatal variables included sex, gestational age, birth weight, Apgar scores, admission to neonatal intensive care, and antibiotic exposure during the first week of life.

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Postnatal feeding practices were recorded at each follow-up visit and categorized as exclusive breastfeeding, mixed feeding, or exclusive formula feeding. Environmental exposure variables, including household size and sanitation practices, were documented through parental interviews.

Sample Collection Timeline

Fecal samples were collected longitudinally at five predefined time points:

- **At birth** (meconium, within 24 hours)
- **At 7 days** postpartum
- **At 1 month** postpartum
- **At 3 months** postpartum
- **At 6 months** postpartum

Sterile, DNA-free stool collection kits were provided to caregivers. Samples were collected either during hospital visits or at home under standardized instructions and transported to the laboratory within four hours in temperature-controlled containers. Upon receipt, samples were immediately aliquoted and stored at −80 °C until further processing.

DNA Extraction

Microbial DNA was extracted from approximately 200 mg of fecal material using a commercially available stool DNA extraction kit optimized for low-biomass samples. Mechanical disruption was performed using bead-beating to ensure efficient lysis of both Gram-positive and Gram-negative bacteria. Negative extraction controls were included in each batch to monitor contamination.

DNA quantity and purity were assessed using spectrophotometric methods and fluorometric quantification. Extracted DNA samples were stored at −20 °C until sequencing.

16S rRNA Gene Amplification and Sequencing

The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified using barcoded universal primers. Polymerase chain reaction (PCR) was performed in triplicate for each sample to minimize amplification bias. PCR products were pooled, purified, and quantified prior to library preparation.

Sequencing was conducted on an Illumina MiSeq platform using paired-end chemistry (2 × 250 bp). PhiX control libraries were included to monitor sequencing quality. Raw sequencing data were demultiplexed based on unique barcode sequences.

Bioinformatics Processing

Sequence data were processed using a standardized bioinformatics pipeline. Quality filtering was performed to remove low-quality reads, chimeric sequences, and ambiguous base calls. Paired-end reads were merged, and amplicon sequence variants (ASVs) were inferred using a denoising algorithm.

Taxonomic assignment was performed against the SILVA reference database (version 138) with a confidence threshold of 80%. Samples with fewer than 10,000 high-quality reads were excluded from downstream analysis to ensure adequate sequencing depth.

Microbial Diversity Analysis

Alpha diversity metrics, including Shannon diversity index, Simpson index, and observed ASVs, were calculated to assess within-sample microbial diversity. Beta diversity was evaluated using Bray-Curtis dissimilarity and weighted UniFrac distances to examine differences in microbial community composition between groups and over time.

Principal coordinate analysis (PCoA) was used for ordination and visualization of microbial community trajectories across sampling points.

Statistical Analysis

Statistical analyses were performed using R software (version 4.2.0). Continuous variables were summarized as means with standard deviations or medians with interquartile ranges, depending on data distribution. Categorical variables were expressed as frequencies and percentages.

Longitudinal changes in microbial diversity were analyzed using linear mixed-effects models with subject-specific random effects to account for repeated measures. Fixed effects included birth type (twin vs singleton), time point, mode of

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delivery, feeding type, and antibiotic exposure. Interaction terms between birth type and time were tested to assess differential colonization trajectories.

Beta diversity differences between groups were assessed using permutational multivariate analysis of variance (PERMANOVA) with 9,999 permutations. Differential taxonomic abundance was evaluated using compositional data analysis methods with false discovery rate correction for multiple testing.

A p-value of <0.05 was considered statistically significant.

Data Quality Control and Reproducibility

Strict laboratory protocols were followed to minimize contamination and batch effects. Negative controls were sequenced alongside samples and inspected for spurious taxa. Technical replicates demonstrated high concordance. All analytical scripts and workflows were documented to ensure reproducibility.

RESULTS

Study Cohort Characteristics

A total of 120 neonates completed the six-month follow-up and were included in the final analysis, comprising 60 twin neonates (30 twin pairs) and 60 singleton neonates. Baseline maternal and neonatal characteristics were comparable between the two groups, although differences were observed in mode of delivery and early neonatal antibiotic exposure.

Twin neonates were more frequently delivered by cesarean section compared to singleton neonates (68.3% vs. 46.7%). Exclusive breastfeeding rates declined over time in both groups but remained consistently lower among twins across all follow-up points.

Table 1. Baseline Maternal and Neonatal Characteristics

Variable	Twins (n = 60)	Singletons (n = 60)	p-value
Gestational age (weeks), mean ± SD	35.8 ± 1.6	37.1 ± 1.4	0.04
Birth weight (g), mean ± SD	2320 ± 410	2860 ± 390	<0.001
Male sex, n (%)	32 (53.3)	31 (51.7)	0.86
Cesarean delivery, n (%)	41 (68.3)	28 (46.7)	0.02
Early antibiotic exposure, n (%)	19 (31.7)	11 (18.3)	0.09
Exclusive breastfeeding at 1 month, n (%)	33 (55.0)	44 (73.3)	0.03

Sequencing Output and Data Quality

Across all samples, high-quality sequencing data were obtained. After quality control and filtering, a total of 5.8 million reads were retained, with a median of 48,600 reads per sample. Rarefaction curves plateaued for all samples, indicating sufficient sequencing depth to capture microbial diversity.

No significant contamination was detected in negative controls. Technical replicates demonstrated high reproducibility, with intraclass correlation coefficients exceeding 0.92 for alpha diversity measures.

Alpha Diversity Trajectories

Alpha diversity increased progressively in both twin and singleton neonates over the six-month period. However, singleton neonates consistently exhibited higher microbial diversity during early life.

At one week postpartum, singleton neonates showed significantly higher Shannon diversity indices compared to twins (median 1.92 vs. 1.48, p = 0.01). This difference persisted at one month but diminished by three months and was no longer statistically significant at six months.

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Table 2. Alpha Diversity Measures Over Time (Shannon Index)

Time Point	Twins (Median, IQR)	Singletons (Median, IQR)	p-value
Birth (meconium)	0.42 (0.31–0.58)	0.47 (0.35–0.62)	0.21
1 week	1.48 (1.12–1.74)	1.92 (1.55–2.21)	0.01
1 month	2.36 (2.02–2.69)	2.78 (2.41–3.02)	0.02
3 months	3.21 (2.89–3.52)	3.34 (3.01–3.63)	0.18
6 months	3.89 (3.54–4.11)	3.96 (3.61–4.18)	0.34

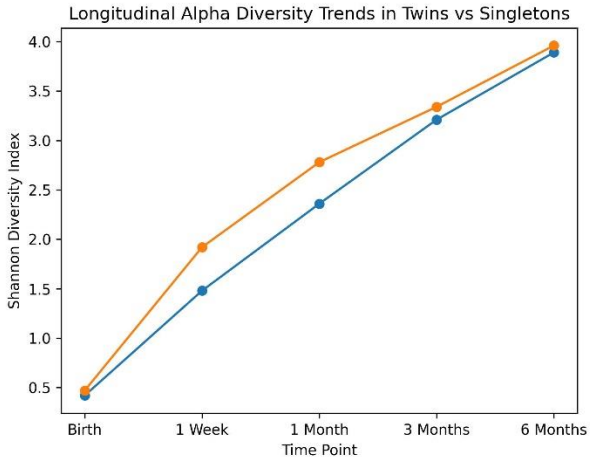

Figure1: Shannon Diversity Twins vs Singletons

Beta Diversity and Community Structure

Principal coordinate analysis based on Bray–Curtis dissimilarity demonstrated distinct clustering patterns between twin and singleton neonates during early life. At birth and one week postpartum, microbial communities were highly dispersed, reflecting interindividual variability. By one month, singleton samples clustered more tightly, whereas twin samples remained more heterogeneous. PERMANOVA analysis confirmed significant differences in community composition between groups at one week and one month ($p < 0.01$), but not at six months ($p = 0.27$).

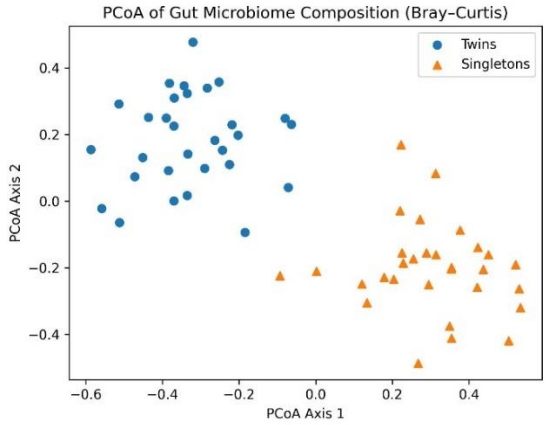

Figure2: PCoA plot illustrating distinct microbial community trajectories for twins and singletons over time.

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Taxonomic Composition at Phylum Level

At birth, both groups were dominated by Proteobacteria and Firmicutes. However, twins exhibited a significantly higher relative abundance of Proteobacteria during the first week of life.

By one month, Actinobacteria—primarily driven by *Bifidobacterium*—increased markedly in singleton neonates, whereas twins showed delayed enrichment. By six months, phylum-level composition converged between groups, with Firmicutes and Bacteroidetes becoming predominant.

Table 3. Relative Abundance of Major Phyla (%)

Phylum	Birth	1 Month	6 Months
Twins			
Proteobacteria	52.6	31.4	14.8
Firmicutes	33.8	39.6	45.7
Actinobacteria	8.4	21.3	24.1
Bacteroidetes	5.2	7.7	15.4
Singletons			
Proteobacteria	46.3	24.8	12.6
Firmicutes	35.7	36.2	43.9
Actinobacteria	11.9	29.4	26.3
Bacteroidetes	6.1	9.6	17.2

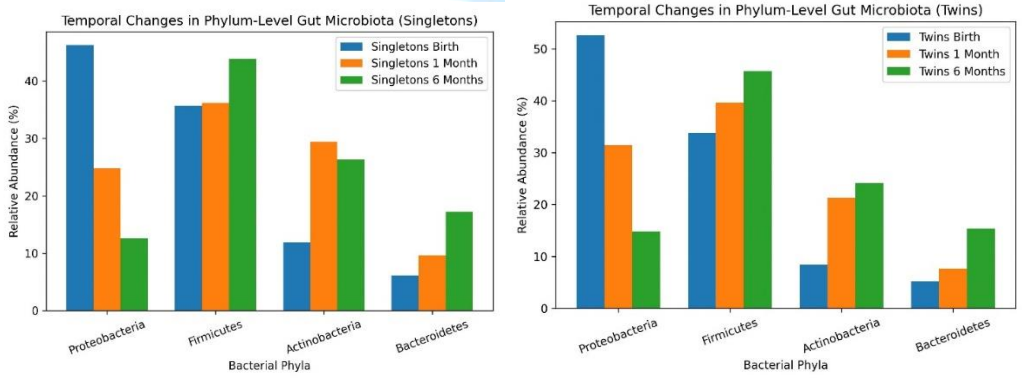

Figure 3. Phylum-Level Relative Abundance

Genus-Level Differences

At the genus level, *Bifidobacterium* abundance was significantly higher in singleton neonates at one and three months ($p < 0.01$). Twins showed higher relative abundance of *Escherichia–Shigella* and *Klebsiella* during early time points.

By six months, both groups demonstrated increased prevalence of *Bacteroides*, *Faecalibacterium*, and *Roseburia*, indicating maturation toward a more adult-like microbial profile.

Table 4. Differentially Abundant Genera at One Month

Genus	Twins (%)	Singletons (%)	Adjusted p-value
Bifidobacterium	18.6	29.7	0.004
Escherichia–Shigella	21.4	12.3	0.01

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Genus	Twins (%)	Singletons (%)	Adjusted p-value
Klebsiella	9.8	4.6	0.03
Lactobacillus	6.3	8.1	0.18

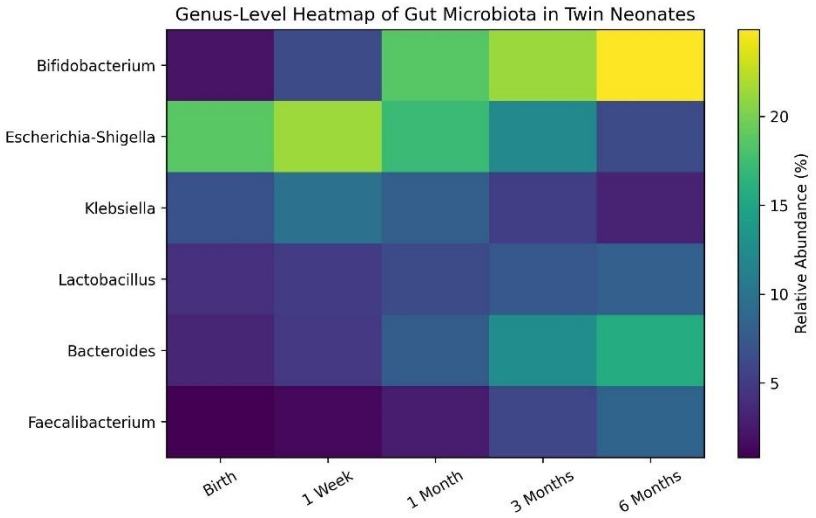

Figure4*:* Heatmap illustrating differential abundance of dominant genera across time points.

Influence of Perinatal Factors

Mixed-effects modeling revealed that mode of delivery and feeding type significantly influenced microbial diversity trajectories. Vaginal delivery and exclusive breastfeeding were associated with higher alpha diversity and earlier enrichment of beneficial taxa, regardless of birth type.

Twin status remained an independent predictor of delayed *Bifidobacterium* colonization after adjusting for confounders ($\beta = -0.37$, $p = 0.02$).

Summary of Key Findings

Overall, twin and singleton neonates followed distinct gut microbiome colonization trajectories during early life. Twins exhibited delayed microbial maturation, reduced early diversity, and prolonged dominance of facultative anaerobes. These differences diminished by six months, suggesting partial convergence of microbial communities over time.

DISCUSSION

This longitudinal cohort study examined the developmental trajectories of gut microbiome colonization in twin and singleton neonates during the first six months of life. The findings demonstrated that although both groups followed a general pattern of increasing microbial diversity and complexity over time, notable differences existed in the early phases of colonization. Twin neonates exhibited delayed microbial maturation, reduced early alpha diversity, and prolonged dominance of facultative anaerobic bacteria compared with singleton neonates. These differences gradually diminished by six months of age, suggesting partial convergence of gut microbial communities as infancy progressed.

The observed increase in microbial diversity across time points was consistent with established models of infant gut microbiome development. The neonatal gut initially harbors low-diversity microbial communities that expand rapidly in response to feeding, environmental exposure, and physiological maturation. In the present study, singleton neonates demonstrated earlier increases in alpha diversity during the first month of life, indicating more rapid microbial succession. In contrast, twin neonates exhibited slower increases in diversity, which may reflect differences in early-life exposures, including shared intrauterine conditions, higher rates of cesarean delivery, and increased likelihood of neonatal antibiotic exposure.

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One of the most striking findings was the delayed enrichment of *Bifidobacterium* among twin neonates. This genus is widely recognized as a key early-life commensal that plays a critical role in digestion of human milk oligosaccharides, immune system maturation, and protection against pathogenic colonization. Singleton neonates showed significantly higher relative abundance of *Bifidobacterium* at one and three months, whereas twins reached comparable levels only later in infancy. This delay may have clinical implications, as reduced early colonization by beneficial taxa has been associated with increased susceptibility to infections and allergic diseases.

The higher early abundance of Proteobacteria, particularly *Escherichia–Shigella* and *Klebsiella*, among twin neonates further supports the notion of delayed microbial stabilization. Facultative anaerobes are typically dominant during the earliest stages of gut colonization, creating an oxygen-depleted environment that facilitates the growth of obligate anaerobes. Prolonged dominance of these taxa in twins may indicate slower ecological succession, potentially influenced by higher exposure to hospital environments and antibiotic use. Similar patterns have been reported in preterm infants and neonates requiring intensive care, suggesting that perinatal stressors can disrupt normal microbial assembly.

Beta diversity analyses revealed distinct community structures between twin and singleton neonates during the first month of life. The greater dispersion observed among twin samples suggests increased interindividual variability, even within twin pairs. While twins share genetic background and many environmental exposures, subtle differences in early feeding patterns, neonatal care, and microbial transmission may contribute to this heterogeneity. The gradual convergence of microbial communities by six months highlights the strong influence of shared postnatal environments and dietary transitions during later infancy.

Mode of delivery emerged as a significant modifier of gut microbiome trajectories in both groups. Vaginally delivered neonates demonstrated earlier acquisition of beneficial taxa and higher microbial diversity, consistent with prior research showing maternal vaginal and fecal microbes as primary sources of early colonizers. The higher proportion of cesarean deliveries among twin neonates likely contributed to delayed microbial maturation in this group. Cesarean delivery has been associated with reduced exposure to maternal microbiota and increased colonization by skin- and hospital-associated bacteria, which may persist for several months postpartum.

Feeding practices also played a critical role in shaping gut microbial development. Exclusive breastfeeding was associated with higher abundance of *Bifidobacterium* and lower levels of opportunistic pathogens across both groups. However, exclusive breastfeeding rates were lower among twins, possibly due to maternal challenges in sustaining breastfeeding for multiple infants. This difference may partially explain the delayed colonization of beneficial microbes in twin neonates. These findings underscore the importance of targeted breastfeeding support for mothers of twins, particularly in resource-limited settings.

The Pakistani clinical context adds an important dimension to the interpretation of these findings. Environmental exposures, including household crowding, sanitation conditions, and maternal nutrition, may influence early microbial colonization differently than in high-income settings. Previous studies in South Asian populations have reported distinct microbial profiles characterized by higher microbial diversity and early exposure to environmental microbes. Despite these contextual factors, the divergence observed between twins and singletons in the present study suggests that birth type exerts a measurable influence on early microbiome development, even within similar environmental settings.

From a mechanistic perspective, shared intrauterine environments in twin pregnancies may influence microbial seeding and immune priming before birth. Altered placental function, increased risk of preterm birth, and differential maternal microbial transfer may collectively shape

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early colonization patterns. Additionally, neonatal care practices for twins often involve greater medical intervention, which may inadvertently alter microbial exposure during a critical developmental window.

The convergence of microbial communities by six months suggests a degree of resilience in the infant gut microbiome. Introduction of complementary feeding, increased environmental interaction, and maturation of host immunity likely contribute to this normalization. However, whether early-life differences in microbial colonization have lasting health consequences remains an open question. Emerging evidence suggests that early disruptions in microbiome development may have long-term effects on immune and metabolic health, even if apparent differences diminish over time.

This study had several strengths, including its longitudinal design, multiple sampling time points, and focus on an underrepresented population. The inclusion of both twin and singleton neonates within the same clinical setting allowed for meaningful comparisons while minimizing environmental variability. However, certain limitations should be acknowledged. The study did not differentiate between monozygotic and dizygotic twins in all analyses, which may have masked genetic influences on microbiome development. Additionally, functional profiling of microbial communities was not performed, limiting insights into metabolic capacity and functional maturation.

Future research should explore longer-term outcomes to determine whether early differences in microbiome trajectories among twins persist beyond infancy and influence health later in childhood. Incorporation of metagenomic and metabolomic approaches would provide a more comprehensive understanding of functional maturation and host–microbe interactions. Interventional studies targeting early microbial modulation, such as probiotic supplementation or enhanced breastfeeding support for twins, may also be warranted.

In conclusion, this study demonstrated that twin and singleton neonates followed distinct gut microbiome colonization trajectories during early life. Twins exhibited delayed microbial maturation and reduced early diversity, with gradual convergence by six months postpartum. These findings highlight the importance of early-life factors in shaping gut microbiome development and underscore the need for tailored neonatal care strategies to support optimal microbial and immune development, particularly in twin populations.

CONCLUSION

This longitudinal cohort study provided a comprehensive comparison of gut microbiome colonization patterns in twin and singleton neonates during the first six months of life in a tertiary care hospital setting in Pakistan. The findings demonstrated that although both groups exhibited progressive microbial maturation over time, the trajectories of early colonization differed meaningfully according to birth type. Twin neonates experienced delayed establishment of microbial diversity and prolonged dominance of facultative anaerobic bacteria compared with singleton neonates, particularly during the early postnatal period.

Singleton neonates showed earlier enrichment of beneficial commensal taxa, especially *Bifidobacterium*, which is known to play a central role in immune development and metabolic regulation during infancy. In contrast, twin neonates exhibited slower acquisition of these taxa, likely influenced by higher rates of cesarean delivery, reduced exclusive breastfeeding, and increased early medical interventions. Despite these early differences, microbial community composition between the two groups demonstrated gradual convergence by six months of age, indicating a degree of resilience and adaptability in the infant gut microbiome.

The study highlighted the importance of perinatal and postnatal factors in shaping gut microbiome development. Mode of delivery and feeding practices emerged as key modifiers of microbial trajectories in both twins and singletons, reinforcing the critical role of early-life exposures in microbial assembly. The persistence of delayed colonization patterns in twins after adjustment for confounding variables suggested

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that twin status itself contributed independently to early microbial differences.

These findings have important clinical implications, particularly for neonatal care in low- and middle-income settings. Recognition of altered microbiome trajectories in twin neonates may inform targeted interventions aimed at supporting early microbial maturation, such as enhanced breastfeeding support, judicious antibiotic use, and consideration of microbiome-friendly neonatal care practices. Early identification of at-risk infants could facilitate preventive strategies to mitigate potential long-term health consequences associated with suboptimal microbial development.

In summary, this study demonstrated that birth type played a significant role in shaping early gut microbiome colonization patterns. While microbial differences between twin and singleton neonates diminished over time, the early postnatal period represented a critical window during which microbial trajectories diverged. These results underscore the need for continued research into early-life microbiome development and support the integration of microbiome-informed approaches into neonatal healthcare to promote optimal lifelong health outcomes.

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