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Original article

Impact of early probiotic supplementation on the gut microbiota and urinary and fecal metabolome of formula-fed infants delivered via elective cesarean section

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Abstract

Early postnatal life is a critical period for gut microbiota establishment and metabolic programming, particularly in infants born by cesarean section and fed with infant formula, two factors known to disrupt physiological microbial colonization. This exploratory pilot study aimed to provide a longitudinal characterization of microbiota and metabolome dynamics in infants born by elective cesarean section and formula-fed receiving Lactobif® (Buona S.p.a. Società Benefit, Sesto Fiorentino, Italy), a commercial probiotic formulation containing *Lacticaseibacillus rhamnosus*, LGG® (DSM33156) and *Bifidobacterium longum subsp. infantis*, Bifin02™ (DSM33361). A multi-omics approach was applied, integrating 16S rRNA gene-based microbiota profiles with ^{1}H NMR-based metabolomics of fecal and urinary samples collected before and during supplementation. Longitudinal analyses revealed dynamic shifts in gut microbial composition, notably a post-treatment increase in *Bifidobacteriaceae* and a reduction in *Enterobacteriaceae*, accompanied by changes in fecal metabolites associated with fermentation, including lactate, acetate, and propionate. An expansion of *Veillonellaceae* was also observed,

suggesting the progressive emergence of a more functionally interconnected microbial ecosystem. Urinary metabolomic profiles showed time-dependent modulation of metabolites involved in energy metabolism, amino acid turnover, and microbial co-metabolism, reflecting systemic metabolic adaptation during early infancy. The longitudinal, multi-compartment design offers descriptive evidence of temporal positive changes in microbial composition and metabolic outputs, revealing both shared patterns and marked interindividual variability. The integrated analysis of microbiota and metabolomics data highlights the value of multi-biofluid, multi-omics strategies for capturing functional maturation of the gut ecosystem and supports the need for personalized approaches to early-life microbiome modulation.

Keywords

Metabolomics, NMR, microbiome, gut microbiota, urinary metabolome, fecal metabolome, formula-fed infants, elective cesarean section, probiotics, early probiotic supplementation.

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Introduction

The first months of life represent a critical window for the establishment of the gut microbiota, metabolic programming, and immune system maturation, all of which profoundly influence infant health and long-term disease susceptibility [1, 2]. During this early-life period, the intestinal microbiota acts not merely as a passive colonizer but as a dynamic and essential partner in host development, shaping digestion, nutrient assimilation, intestinal barrier integrity, and immune homeostasis. This sensitive colonization window can be perturbed by several perinatal factors, among which cesarean section and formula feeding have emerged as key disruptors of the physiological succession of gut microbiota [2, 3]. Infants born by cesarean section are deprived of exposure to maternal vaginal and intestinal microbes and instead acquire microbes primarily from the skin and hospital environment, resulting in reduced microbial diversity, delayed colonization by *Bifidobacterium*, and a higher prevalence of facultative anaerobes such as *Enterobacteriaceae* [4]. Similarly, formula feeding fails to provide the bioactive components found in human milk, including human milk oligosaccharides, secretory immunoglobulins, and beneficial microbes, that promote the expansion of symbiotic taxa and support mucosal immune development [5-8]. In contrast, infants who are breastfed and delivered vaginally typically develop a microbiota dominated by *Bifidobacterium* and other beneficial taxa, which support efficient nutrient utilization, metabolic homeostasis, and colonization resistance against pathogens [9].

Disruptions in early microbial colonization have been increasingly linked to dysbiosis, suboptimal immune maturation, altered metabolic programming, and a greater susceptibility to non-communicable diseases later in life, including obesity, allergic disorders, and chronic inflammatory conditions [2]. These observations highlight the critical need for early interventions that support the establishment of a resilient and balanced gut ecosystem. Within this framework, probiotic supplementation during infancy has gained attention as a potential strategy to beneficially shape the gut microbiota and promote a microbial profile resembling that of breastfed infants in formula-fed neonates [10-13]. In particular, probiotic strains such as *Lacticaseibacillus rhamnosus*, LGG® (DSM33156) (hereafter, LGG®) and *Bifidobacterium longum subsp. infantis*, Bifin02™ (DSM33361) (hereafter, *B. infantis* Bifin02™) have demonstrated encouraging effects in enhancing microbial balance, strengthening epithelial barrier integrity, and reducing the risk of intestinal inflammation and necrotizing enterocolitis in preterm infants [12].

Alongside microbial profiling, metabolomic analysis has become a powerful tool to investigate the functional consequences of early-life interventions. By focusing on low-molecular-weight (< 1.5 kDa) metabolites in biofluids such as urine and feces, metabolomics enables insight into the biochemical output of both host and microbiota. In particular, urinary metabolomics offers a non-invasive approach to track systemic changes [14], while fecal metabolomics provides a localized

snapshot of gut microbial activity, allowing for a more comprehensive interpretation of microbe-host interactions [15, 16].

A recent systematic review has highlighted the growing relevance of combining metabolomic and microbiome data to better capture the functional impact of microbiome-modulating interventions in early life [17]. Although several studies have reported alterations in fecal short-chain fatty acids (SCFAs), bile acids, and aromatic amino acid metabolites following probiotic or synbiotic administration [11, 15, 18-20], the current evidence remains heterogeneous and strongly influenced by differences in study design, analytical methodologies, and population characteristics. As emphasized [17, 21], moving beyond taxonomic profiling to explore microbial functionality is essential to unravel host-microbe interactions and to inform precision-based interventions in early life. In this context, the integration of metabolomics with microbiome analysis represents a key strategy to capture the functional maturation of the gut ecosystem and its systemic metabolic impact.

Building on this background, the present study investigated the effects of Lactobif® (Buona S.p.a. Società Benefit, Sesto Fiorentino, Italy), a commercially available dual-strain probiotic supplement (containing LGG® and *B. infantis* Bifin02™), in healthy full-term neonates born via elective cesarean section and fed with infant formula. Using an integrated microbiome-metabolome approach, we evaluated whether this supplementation could modulate intestinal microbial colonization and metabolic profiles towards a healthier trajectory, mimicking features typical of breastfed infants. Our findings aim to inform future strategies for individualized neonatal nutrition and microbial modulation, with the goal of optimizing infant health from the earliest stages of life.

Material and methods

Study population

This study was designed as a prospective, exploratory pilot investigation aimed at characterizing metabolic and microbiota-related changes in neonates receiving probiotic supplementation. A total of 12 neonates were initially enrolled. Due to drop-out during follow-up, complete longitudinal data across all scheduled time points were available for 9 subjects, who were included in the final analyses. Given the exploratory design, no formal *a priori* sample size calculation was performed, and the resulting cohort was considered suitable for a pilot investigation.

To ensure a homogeneous cohort, all infants met the following general criteria: born at term (≥ 37 weeks of gestation); delivered via elective cesarean section; healthy and appropriate for gestational age (AGA); exclusively or predominantly formula-fed (> 80%) with type-1 infant formula during the first 6 months of life; no major congenital anomalies or perinatal complications, including absence of birth asphyxia. All participants were recruited as outpatient cases and enrolled under full anonymization.

Furthermore, to minimize confounding variables and guarantee a clinically and demographically homogeneous maternal population, the following inclusion criteria were applied: Italian nationality; age between 18 and 40 years; singleton pregnancy; spontaneous conception (no assisted reproductive technologies); absence of relevant maternal diseases, including diabetes, thyroid disorders, and hypertension.

The study was conducted in accordance with the principles of the Declaration of Helsinki and complied with all applicable national and institutional regulations. The study protocol was reviewed and approved by the Ethics Committee Liguria Region (approval number: 413/2022). Written informed consent was obtained from both parents of enrolled neonates prior to participation.

Study design and probiotic administration

Families participating in the study received, free of charge and on a *pro bono* basis, a commercial probiotic supplement containing LGG® and *B. infantis* Bifin02™ suspended in sunflower oil. The product, commercially available in Italy under the trade name Lactobif® (Ministry of Health registration no. 147755; label code: R00.0821.30840100AV), was distributed through family pediatricians. Caregivers were instructed to store the supplement in a dry place at room temperature (≤ 25°C), avoiding exposure to heat, sunlight, and moisture. The recommended dosage was 6 drops once daily, providing approximately 1×10^9 colony-forming units (CFU) of LGG® and 1×10^8 CFU of *B. infantis* Bifin02™.

The study followed a longitudinal observational design with predefined sampling time points. Baseline sampling (T0) was performed before the initiation of probiotic supplementation, when infants were approximately 1 month old. Probiotic administration was then started and continued throughout the entire study period. Follow-up samples were collected 1

month after supplementation onset (T1) and 3 months after initiation (T2), while supplementation was still ongoing.

Urine samples were collected at all 3 time points (T0, T1, and T2) and analysed by ^{1}H NMR-based metabolomics. Missing samples were mainly due to insufficient urine volume or technical issues during acquisition. Fecal samples were collected at baseline (T0) and at the end of the supplementation period (T2) and used for both microbiota profiling (16S rRNA gene sequencing) and ^{1}H NMR-based metabolomic analysis.

Sample collection

Urine and fecal samples from each infant were collected at home by the parents. For urine collection, a sterile cotton pad was placed inside the infant's diaper. Once soaked, the pad was manually squeezed into sterile 15 mL Eppendorf™ conical tubes with screw caps and stored at –20°C in the participants' home freezers. Fecal samples were collected directly from diapers using sterile containers (Coprotainer® Feces, FL Medical, Italy) and stored at 4°C until pick-up. All biological samples were retrieved within 24 hours of collection and stored at –80°C at the University of Cagliari until processing. Samples were labelled using a unique code combining the infant ID and the sampling timepoint.

Microbiome analysis

Fecal bacterial composition was determined by 16S rRNA gene sequencing. For DNA extraction, about 0.5 g of feces were combined with 2 mL of sodium citrate (2%, pH 7.5) and homogenized by vigorously vertexing for 2 min in a 2 mL tube. A 1.5 mL aliquot was centrifuged at 10,000 × g for 30 min at 4°C and the pellet was combined with 1,000 µL lysis buffer (200 mM Tris–HCl, pH 8.0, 250 mM NaCl, 25 mM EDTA, SDS 0.5% w/v) and the tube was vigorously vortexed for 1 min. 200 µL of samples were then transferred in another tube and 50 µL of SDS (10% w/v) and 18 µL of proteinase K solution (5 mg/mL, Sigma-Aldrich) were added. The sample was vigorously vortexed and incubated for 60 min at 65°C before addition of 100 µL 2.5 M potassium acetate (Sigma-Aldrich). The tubes were slowly inverted, placed at –20°C for 2 hours and then centrifuged at 11,000 x g for 30 min at 4°C. 300 µL of the supernatant were transferred to a new tube and an equal volume of 2-propanol (Sigma-Aldrich) was added to precipitate nucleic acids. The tubes were left to stay at room temperature for 5 min and then were centrifuged at 11,000 x g for 15 min at 4°C. The resulting DNA pellet was washed with 500 µL 70% ethanol and the tubes were centrifuged again at 11,000 x g for 15 min. Afterwards, the DNA pellet was air-dried and resuspended in a 120 µL TE buffer (10 mM Tris–Cl, pH 8.0, 1 mM EDTA). DNA concentration and purity were checked by optical density at 260 nm and ratio O.D. 260 nm/280 nm were determined using the microplate spectrophotometer SPECTRO-star® Nano equipped with LVIS Plate (BMG LABTECH, Germany). DNA samples were submitted to BMR Genomics (Padua, Italy) for the sequencing of the V3–V4 region of the bacterial 16S rRNA gene on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) using 2 × 300 bp paired-end reads. Bioinformatics analysis of bacterial 16S rRNA amplicon sequencing data was conducted using the Quantitative Insights Into Microbial Ecology (QIIME) software. Sequencing reads were processed using the DADA2 pipeline. Reads were trimmed and filtered, paired reads were merged, and chimeric sequences were removed. The SILVA ribosomal RNA reference database and GreenGenes2 database were used for taxonomic assignment of the amplicon sequence variants.

Sample preparation for NMR analysis

On the day of NMR analysis, urine and fecal samples were thawed on ice and prepared for NMR spectral acquisition. For urine samples, 1 mL of sample was mixed with 10 µL of 10% sodium azide (NaN_3, ≥ 99.5%, Sigma-Aldrich) to prevent bacterial growth and centrifuged at 12,000 × g for 10 min at 4°C. Subsequently, a 900 µL aliquot of the supernatant was combined with 100 µL of phosphate buffer in D_2O (1.5 M, pH 7.4) containing 1 mM trimethylsilyl propionate sodium salt (TSP) as an internal chemical shift reference. The mixture was vortexed and the pH was adjusted to 7.00 ± 0.05 using NaOD (40 wt% in D_2O, 99.5 atom% D) or DCl (35 wt% in D_2O, ≥ 99 atom% D), both from Sigma-Aldrich. A final volume of 650 µL was transferred to a standard 5 mm NMR tube for analysis. When less than 1 mL of urine was available, the same protocol was followed by proportionally scaling down the reagent volumes.

Fecal samples were processed by suspending approximately 1 g of fresh stool in 5 mL of Milli-Q water (Millipore, Billerica, MA, USA), followed by vortexing to ensure homogenization and centrifugation at 4,000 × g for 15 minutes at 4°C. Then, 1.5 mL of fecal water was separated by the solid phase and subjected to a second centrifugation at

18,000 × g for 30 minutes at 4°C to further clarify the extract. Then, 500 µL of the supernatant was filtered through a 3 kDa molecular weight cut-off centrifugal filter (Amicon Ultra) by centrifugation at 13,000 × g for 30 minutes at 4°C. Prior to use, the filters were thoroughly washed with Milli-Q water to remove residual glycerol, as recommended by the manufacturer. The filtrate (300 µL) was mixed with 400 µL of phosphate buffer (0.1 M, pH 7.4) in D_2O containing 0.1 mM TSP. A total volume of 650 µL was transferred into a 5 mm NMR tube for spectral acquisition.

^{1}H NMR data acquisition and processing

^{1}H NMR spectra were acquired at 300 K on a Varian Unity Inova 500 spectrometer (Agilent Technologies, Santa Clara, CA, USA) using a standard one-dimensional NOESY pulse sequence (noesypresat), which includes presaturation during the relaxation delay and mixing time for effective water signal suppression. Acquisition parameters were as follows: spectral width of 6,000 Hz, acquisition time of 1.5 seconds, relaxation delay of 3.5 seconds, and a mixing time of 1 ms. Each spectrum was obtained by accumulating 216 scans for urine samples and 512 scans for fecal samples.

Spectral processing was performed using MestReNova software (version 14.0, Mestrelab Research SL, Valencia, Spain). After Fourier transformation with an exponential line broadening of 0.3 Hz and zero-filling to 64K data points, spectra were manually corrected for phase and baseline distortions. Chemical shifts were referenced to the internal standard TSP at $\delta = 0.00$ ppm. To reduce potential artifacts, spectral regions corresponding to TSP (–0.5 to 0.5 ppm) and residual water (4.6 to 5.2 ppm) were excluded from further analysis. All spectra were manually aligned, segmented into equidistant bins of 0.002 ppm, and PQN-normalized [22].

Metabolite identification and quantification

Metabolite annotation was performed by matching experimental chemical shifts and multiplicity patterns with reference spectra in the Human Metabolome Database (HMDB) [23] and the Chenomx NMR Suite library (v8.5, evaluation version, Edmonton, Canada). Only metabolites confidently identified across all samples were retained for downstream analysis. Their relative abundances were determiners by integration of the selected corresponding spectral peaks.

Statistical analysis

Given the exploratory nature of the study, statistical analyses were primarily aimed at describing longitudinal trends and identifying metabolomic and microbiota-related changes over time. Differences in the relative abundances of microbial taxa at the phylum, class, order, family, and genus levels were assessed using the one-tailed Wilcoxon signed-rank paired test. Univariate comparisons of urinary metabolite concentrations were conducted using the Mann-Whitney U test to evaluate differences between T1 and T0, and between T2 and T0. Statistical significance was assessed based on p-values, with $p < 0.05$ considered statistically significant. All univariate analyses were performed using GraphPad Prism (version 10.0).

Multivariate statistical analysis of the NMR spectra was conducted using SIMCA software (version 17.0.1, Sartorius, Göttingen, Germany). Principal component analysis (PCA) was applied to explore the overall structure of the metabolomic dataset across timepoints, following Pareto scaling. Outlier detection was based on Hotelling's T^2 and distance-to-model in X-space (DModX), using both 95% and 99% confidence intervals.

Results

Study population

The study included 9 neonates (7 males and 2 females), enrolled approximately 1 month of age. At birth, the infants had a mean weight of 3.09 ± 0.25 kg, a mean length of 49.7 ± 1.9 cm, and an average head circumference of 33.8 ± 0.8 cm. Anthropometric measurements collected longitudinally confirmed normal postnatal growth trajectories. Mean body weight increased to 4.19 ± 0.59 kg at T0, 5.54 ± 0.76 kg at T1, and 7.0 ± 0.45 kg at T2. Body length increased to 54.6 ± 2.7 cm at T0, 59.1 ± 1.4 cm at T1, and 64 ± 0.37 cm at T2. Head circumference progressively increased, measuring 36.8 ± 1.7 cm at T0, 38.6 ± 1.2 cm at T1, and 41.5 ± 2.1 cm at T2.

Gut microbiome

The analysis of fecal microbiota before (T0) and after 3 months (T2) of probiotic supplementation in 9 infants revealed marked interindividual variability (**Figures 1-4**).

At the phylum level (**Fig. 1** and **Fig. 4A**), the predominant phyla at T0 were *Proteobacteria*,

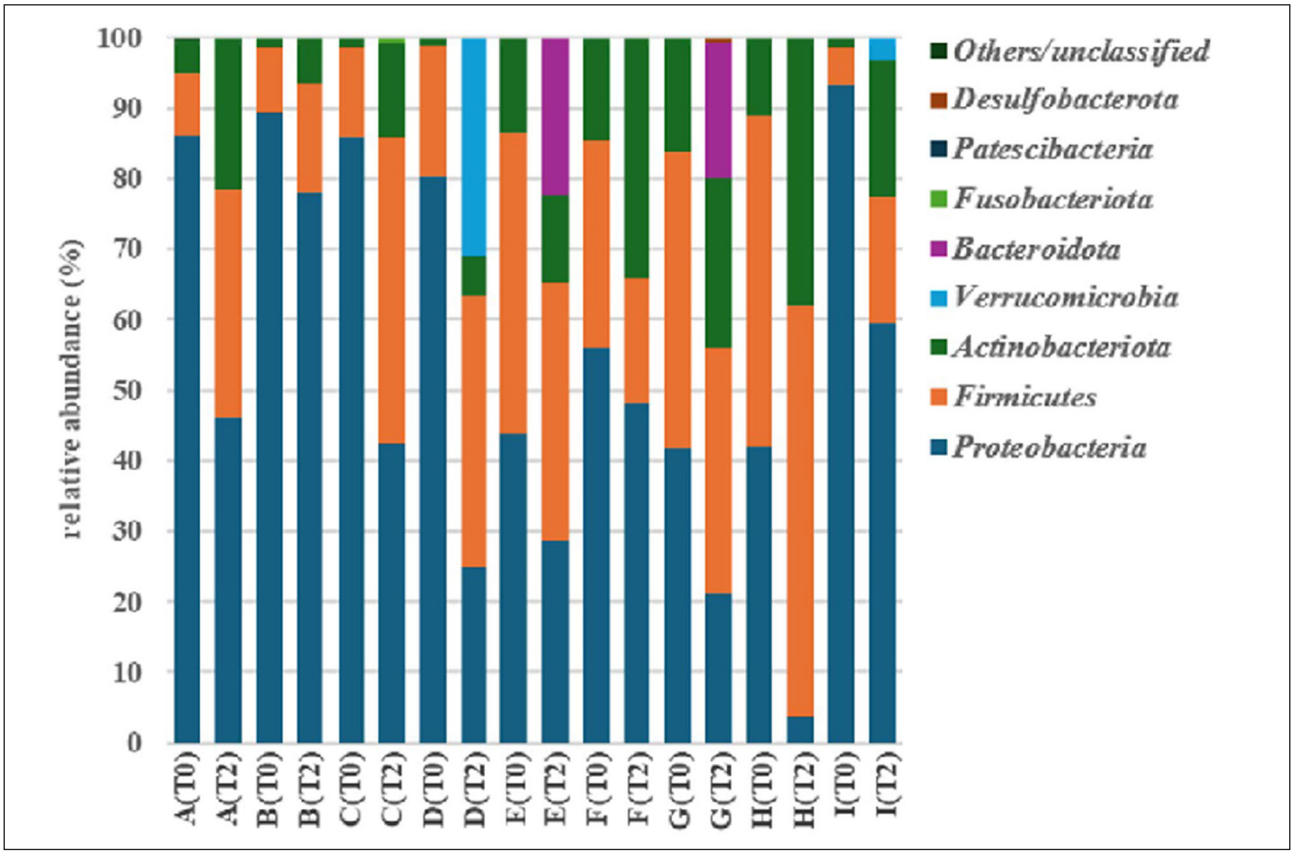

Figure 1. Stacked bar plots showing the relative fecal microbiota composition at the phylum level in the 9 infants at baseline (T0, 1 month of age) and after 3 months of probiotic administration (T2). Each bar represents a fecal sample from a single subject, with taxa color-coded according to the legend.

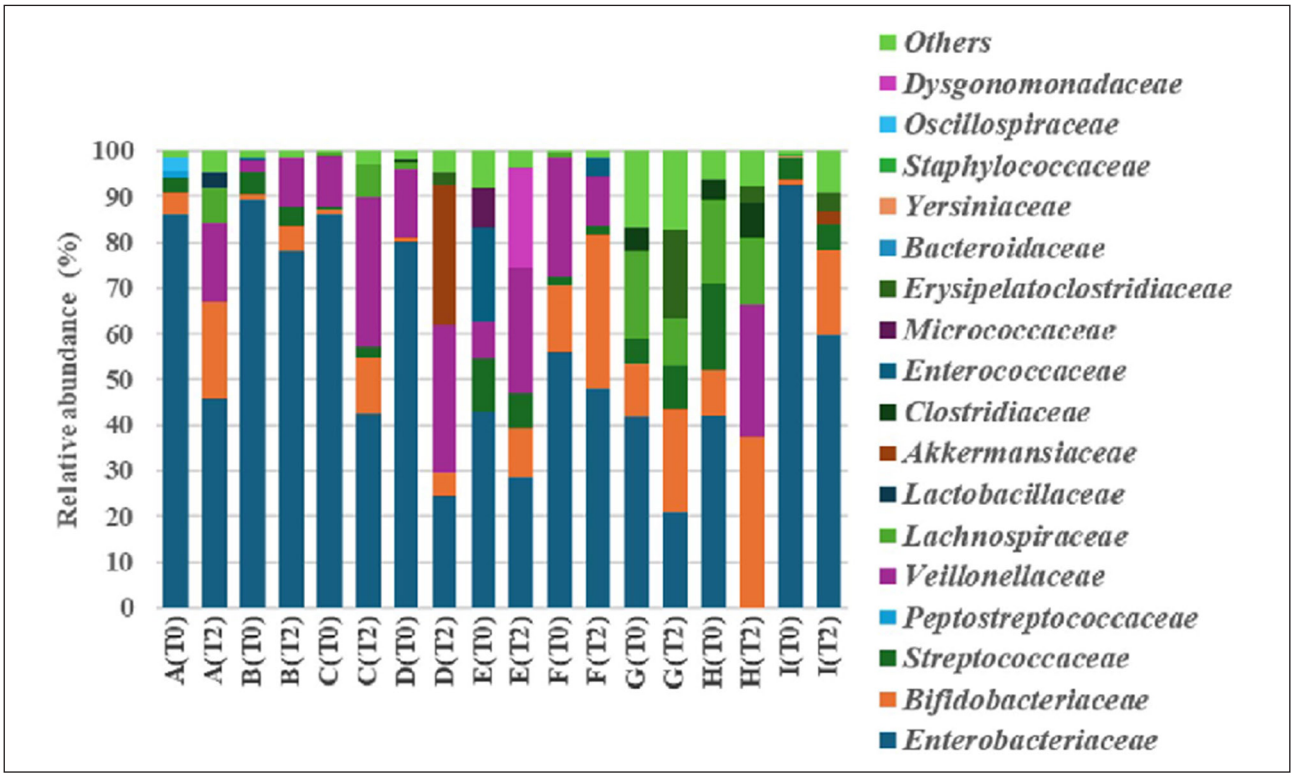

Figure 2. Stacked bar plots showing the relative fecal microbiota composition at the family level in the 9 infants at baseline (T0, 1 month of age) and after 3 months of probiotic administration (T2). Each bar represents a fecal sample from a single subject, with taxa color-coded according to the legend.

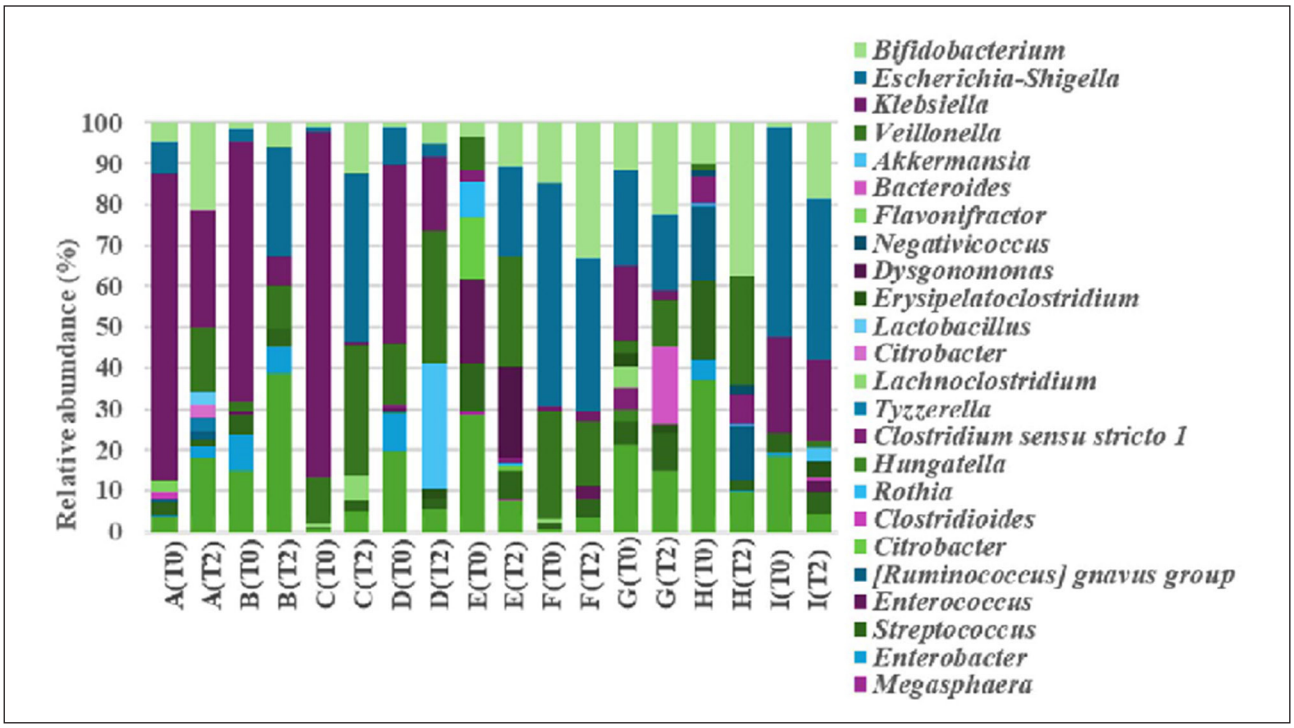

Figure 3. Stacked bar plots showing the relative fecal microbiota composition at the genus level in the 9 infants at baseline (T0, 1 month of age) and after 3 months of probiotic administration (T2). Each bar represents a fecal sample from a single subject, with taxa color-coded according to the legend.

followed by *Firmicutes* and *Actinobacteriota*. A significant ($p < 0.05$) reduction in *Proteobacteria* and a relative significant increase in *Actinobacteriota* were observed after treatment.

At the family level (**Fig. 2** and **Fig. 4B**), *Enterobacteriaceae* were dominant in all infants at T0, indicating a typical early-life microbiota composition [5, 6]. *Bifidobacteriaceae* showed a statistically significant increase following probiotic supplementation ($p < 0.01$), indicating effective colonization by the administered strain. However, the magnitude of the increase in *Bifidobacteriaceae* abundance varied among the subjects, with some showing a pronounced rise after probiotic administration, while others exhibited only minimal changes. This variability may reflect heterogeneous gut colonization, potentially influenced by individual factors such as baseline microbiota composition, intestinal environment, or feeding regimen [17, 21]. Differently, the *Lactobacillaceae* family was detected in only 1 infant after probiotic supplementation. The limited detection of *Lactobacillaceae* and the genus *Lactobacillus* in most fecal samples may be partially explained by technical factors, such as the DNA isolation method and potential amplification bias resulting from the limited affinity of commonly used universal primers for targeting specific rDNA regions in *Lactobacilli* [24]. These limitations could lead to underestimation or apparent absence of these taxa in DNA-based fecal analyses.

The significant decrease in *Enterobacteriaceae* after treatment ($p < 0.01$) was evident across multiple individuals and reflects the typical ecological transition from a *Proteobacteria*-dominated microbiota to one enriched in *Actinobacteria*. These changes suggest a maturation of the gut microbiota over time and a shift toward a more balanced microbial community, with decreased abundance of early-dominant, potentially opportunistic taxa such as *Enterobacteriaceae*. Additionally, families such as *Streptococcaceae* and the lactate-utilizing, hydrogen-producing *Veillonellaceae* were detected in most subjects, with the latter showing a significantly higher abundance after treatment (**Fig. 4B**). The emergence or expansion of *Veillonellaceae* is generally associated with microbiota maturation and was particularly notable in infants who also showed increased *Bifidobacteria*. Notably, *Veillonellaceae* were already present in some infants at baseline (e.g., subject C), indicating the potential for spontaneous microbial maturation even in formula-fed conditions.

At the genus level (**Fig. 3** and **Fig. 4C**), the relative abundance of *Bifidobacterium* increased significantly ($p < 0.01$) at T2, consistent with

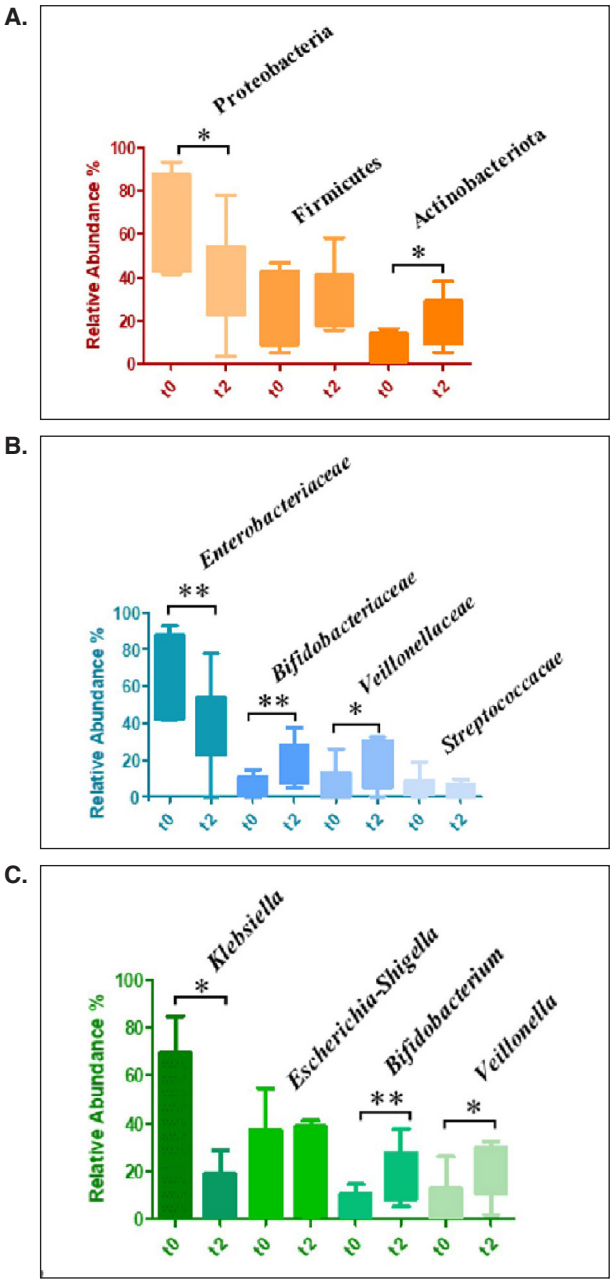

Figure 4. Box plots summarizing temporal changes in selected taxa at the phylum (**A**), family (**B**) and genus (**C**) levels.

Significant differences are indicated by asterisks: *p < 0.05, **p < 0.01.

the family-level findings. In contrast, the genus *Klebsiella*, a member of *Enterobacteriaceae*, showed a significant ($p < 0.05$) decline, further supporting the transition toward a more favourable gut environment.

Overall, these findings describe a partial reconfiguration of the fecal microbiota over time toward a profile consistent with functional maturation under probiotic supplementation. These patterns are in line with dynamic changes in microbial composition during early life, with the extent and direction of these shifts varying markedly among individuals [17, 21].

Urinary metabolomics

The ^{1}H NMR-based metabolomic analysis of infant urine samples provided valuable insights into the temporal evolution of systemic metabolism during the early postnatal period captured in our sampling window. PCA revealed a clear separation of samples by timepoint (**Fig. 5A**), indicating a progressive metabolic shift as the infants aged, in agreement with known developmental processes occurring in the neonatal period [25-27].

The PCA loadings plot (**Fig. 5B**) identifies the spectral features most responsible for the separation of urine samples across principal components. Along PC1, which accounts for 21.9% of the total variance, creatinine and creatine showed the strongest positive loadings. These metabolites, linked to muscle turnover and renal function, are predominantly associated with later timepoints (T2), evidencing an age-related increase consistent with physiological maturation [25-31]. Another noteworthy observation concerns the urinary excretion of dimethylamine (DMA), which showed temporal variation across the study period. DMA mainly originates from the microbial metabolism of dietary precursors, including choline and carnitine. In particular, members of the *Enterobacteriaceae* family are known to contribute significantly to DMA production [32]. Its detection in urine reflects not only early microbial colonization by taxa capable of degrading trimethylated compounds, but also the host's capacity to absorb, process, and excrete microbially derived metabolites. As such, DMA represents a systemic marker of microbiota-host crosstalk during early life.

Conversely, propylene glycol, glycerol, and glycine display negative loadings along PC1, indicating higher abundance in earlier timepoints (T0). Glycerol may originate from lipid metabolism or dietary intake, whereas propylene glycol is frequently used as a solvent and excipient in pediatric formulations. Elevated levels of propylene glycol at T0 could result from perinatal exposure or early supplementation, while its decline over time may indicate reduced exogenous intake or improved renal clearance [26]. Glycine, a multifunctional amino acid involved in protein synthesis, one-carbon metabolism, and detoxification, also contributes to muscle development [14]. It is considered a conditionally essential amino acid in mammals to

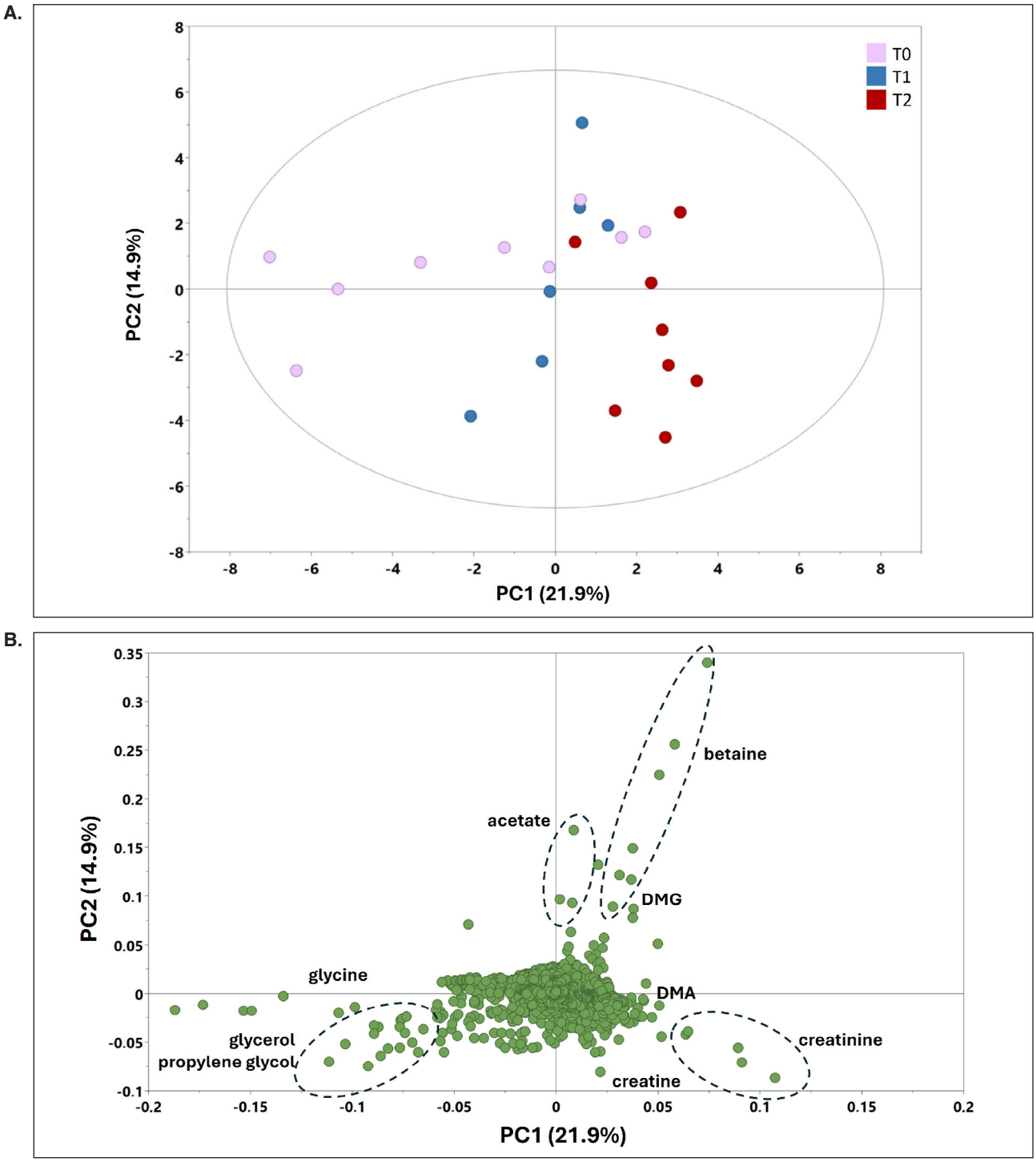

Figure 5. PCA scores (**A**) and loadings (**B**) plot of the PCA model built with the urinary ^{1}H NMR spectra of neonate samples collected at 3 timepoints: baseline (T0, pink); 1 month after initiation of probiotic supplementation (T1, blue); 3 months after initiation of supplementation (T2, red).
DMA: dimethylamine; DMG: dimethylglycine.

support optimal growth, particularly during early life stages [33]. The observed decline in urinary glycine is consistent with previous studies showing dynamic remodelling of amino acid metabolism during infancy [25].

Scores clustering in the upper right quadrant of the PCA loadings plot (**Fig. 2A**) were primarily associated with elevated urinary levels of betaine, acetate, and dimethylglycine (DMG). During early infancy, increased betaine excretion likely reflects a combination of renal immaturity, limited enzymatic utilization, and potentially enhanced endogenous production through choline metabolism [34]. Both betaine and DMG serve as key methyl donors within

one-carbon metabolism, contributing to methionine synthesis and homocysteine regulation. Acetate, in turn, represents a major microbial fermentation product and is widely recognized as a marker of bifidobacterial activity in the gut [35]. Notably, the clustering of high betaine, DMG, and acetate levels in this quadrant was not restricted to T2 samples, despite continued probiotic administration up to that timepoint. This observation suggests that these metabolic patterns are unlikely to be solely driven by supplementation or infant age. Rather, they may reflect interindividual variability in systemic metabolic processing, renal excretion capacity, and host-microbiota co-metabolism, operating independently or in parallel with probiotic exposure.

In addition to the metabolic changes highlighted by the PCA model, we further explored potential temporal variations in other metabolites that did not strongly contribute to the main components of the loading plot. To investigate these trends at the cohort level, we applied the Mann-Whitney U test to compare metabolite levels across timepoints (T1 vs. T0 and T2 vs. T0). The most pronounced differences emerged between T2 and T0 (**Fig. 6**). In addition to the aforementioned changes, we observed a progressive increase in urinary trimethylamine (TMA), a microbial-derived metabolite resulting from the fermentation of dietary choline and other methylated compounds [32]. Additionally, lactate levels declined significantly at T2. This trend aligns with previous evidence of urinary lactate decline during the first months of life [26], reflecting key metabolic transitions during neonatal development. Lastly, alanine exhibited a moderate decline, reflecting changes in nitrogen metabolism or amino acid turnover associated with postnatal development [14].

Fecal metabolomics

PCA analysis of the ^{1}H NMR fecal profiles offered a relevant snapshot of gut microbial activity, capturing metabolic shifts occurring during probiotic supplementation. As illustrated in the scores plot in **Fig. 7A**, a time-dependent shift in metabolic profiles is discernible, albeit less pronounced than in urine. To facilitate subject-level interpretation, each score is labeled with the corresponding sample code, allowing the visual tracking of individual metabolic trajectories. As with the microbiota composition, the plot revealed substantial interindividual variability in both the direction and magnitude of temporal changes. While some infants displayed marked metabolic shifts following supplementation,

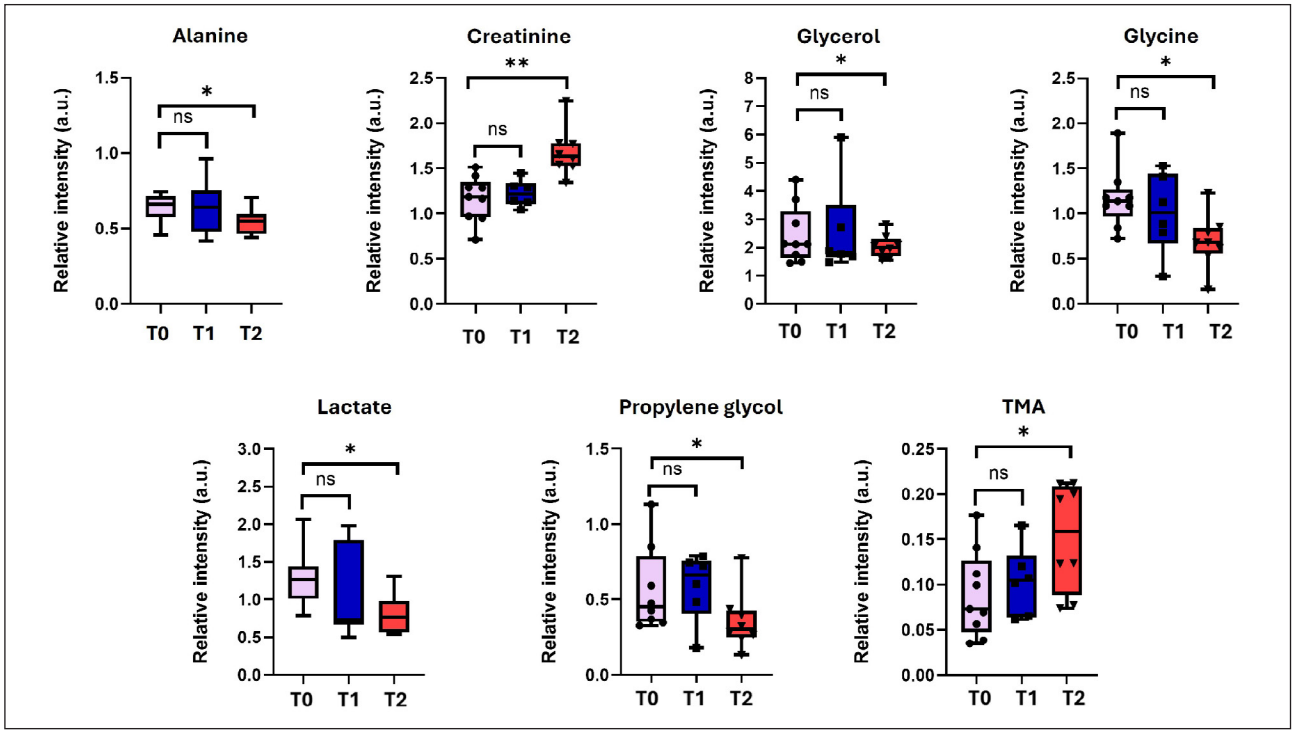

Figure 6. Box plots representing the relative intensities of selected urinary metabolites at baseline (T0), after 1 month (T1), and 3 months (T2) of probiotic supplementation, as assessed by ^{1}H NMR spectroscopy.
Statistical comparisons between T1 vs. T0 and T2 vs. T0 are indicated (*p < 0.05, **p < 0.001; ns = not significant).
TMA: trimethylamine.

others exhibited minimal change or even partial reversals over time. This divergence closely mirrors the interindividual variability seen in microbiota development, highlighting the complex and non-uniform nature of early-life gut maturation, and suggests differential responsiveness to the probiotic intervention, an effect further magnified by the small sample size. Rather than hindering interpretation, such heterogeneity highlights the value of subject-specific resolution in capturing personalized trajectories of metabolic adaptation.

Inspection of the PCA loadings plot provided insight into the metabolites contributing to the observed sample variability (**Fig. 7B**). Samples located in the lower right quadrant (positive PC1 and negative PC2), including post-treatment samples C, E, and F, were characterized by higher levels of lactate. It is worth noting that the increase in fecal lactate occurred in parallel with a decline in urinary lactate levels over time. This apparent divergence is not contradictory, as fecal lactate primarily reflects the activity of early-life gut microbiota, particularly

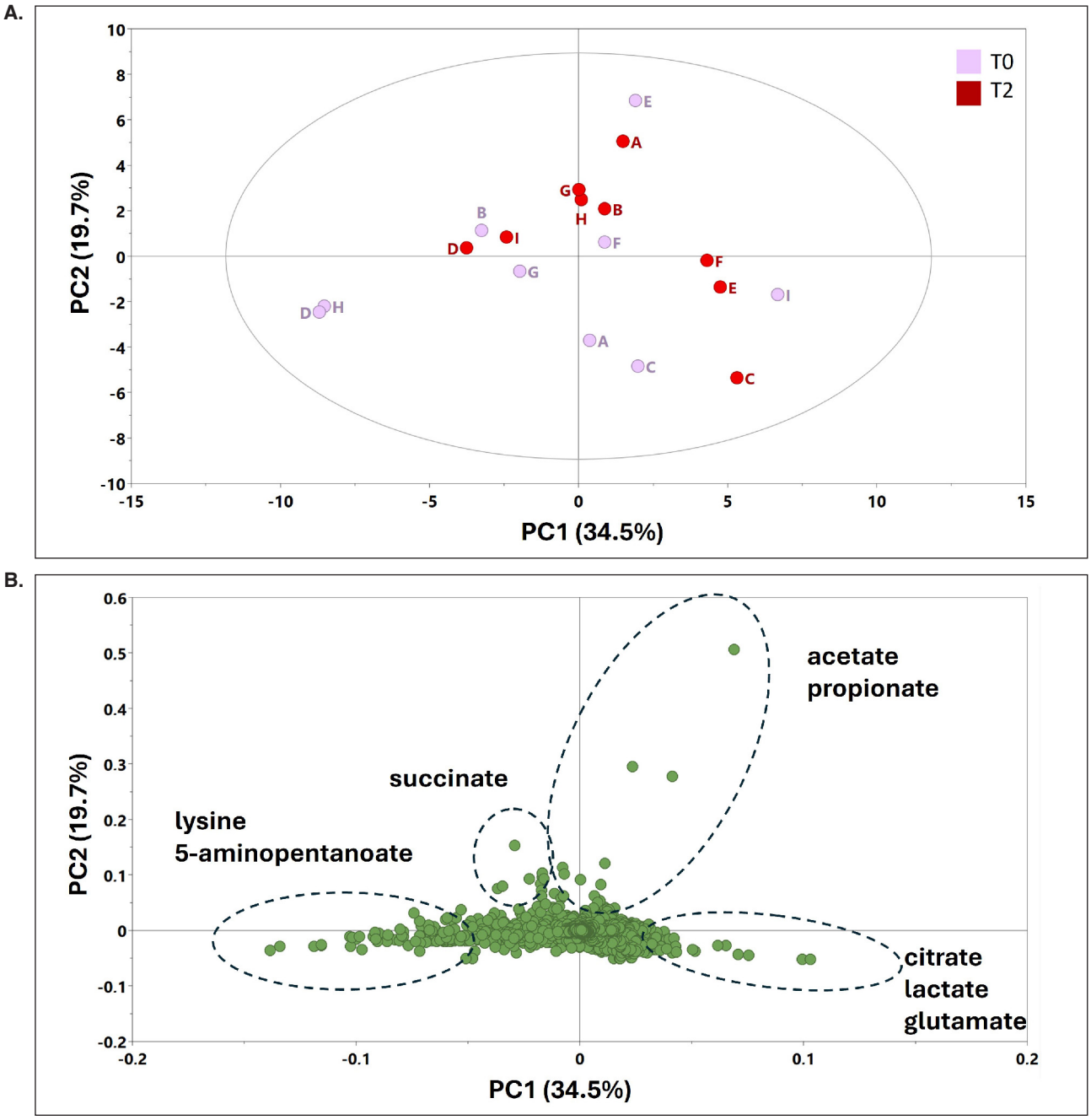

Figure 7. PCA scores (**A**) and loadings (**B**) plot of the PCA model built with the fecal ^{1}H NMR spectra of neonate samples collected at baseline (T0, pink) and after 3 months of probiotic supplementation (T2, red).
Letters denote individual infant identifiers.

Bifidobacterium and *Lactobacillus* [13, 36], whereas urinary lactate mirrors systemic host metabolism and a maturation of mitochondrial oxidative capacity, as previously reported [26]. Citrate and glutamate also contributed to this sample clustering, suggesting changes in metabolites related to energy and nitrogen metabolism. Conversely, the negative side of PC1 was predominantly driven by lysine and its degradation product 5-aminopentanoate, which showed the highest negative loadings on this axis. Lysine has been previously reported in association with amino acid-related fermentation pathways and detected at higher levels in the gut of formula-fed infants compared to breast-fed newborns [37]. Both metabolites were particularly abundant in pre-treatment samples D and H.

Scores distribution along positive PC2 was mainly driven by acetate and propionate that were particularly abundant in post-treatment samples A, B, G, and H. These SCFAs represent major end-products of microbial carbohydrate fermentation and are commonly associated with *Bifidobacterium*, *Bacteroides*, and *Veillonella* [38]. Succinate was more abundant in samples B and G at T0. Although classically considered an intermediate of the tri-carboxylic acid cycle, it is intermediate metabolite or end-product of many intestinal microorganisms in infants [39]. Elevated succinate levels have previously been reported in preterm infants and correlated with unclassified *Enterobacteriaceae* [16].

Discussion

The neonatal period constitutes a highly sensitive window for the establishment of the gut microbiota and the functional maturation of host-microbe interactions [1]. Perturbations during this early developmental phase may exert long-term consequences on immune competence, metabolic regulation, and disease susceptibility [2]. In this exploratory pilot study, we investigated the impact of a commercial combined probiotic supplementation (Lactobif®: *B. infantis* Bifin02™ and LGG®), on the fecal microbiota composition and metabolic output in a cohort of 9 full-term, formula-fed infants. Despite the limited sample size, the within-subject longitudinal design enabled the identification of consistent trends, revealing both common and individualized patterns of response. This interindividual variability aligns with accumulating evidence that early-life gut colonization is highly personalized and shaped by a complex interplay of host, environmental, and nutritional factors [1, 2, 17].

The integration of microbiota and metabolomics datasets revealed coherent and biologically meaningful shifts in both fecal microbial composition and metabolic output over the 3-month observation period. Taxonomic analyses indicated a significant increase in *Bifidobacteria*, a key taxon consistently associated with physiological maturation of the infant gut ecosystem and typically enriched in breastfed infants compared with formula-fed counterparts [11, 15, 18, 21, 40]. These microbial changes were accompanied by a concomitant decline in *Enterobacteriaceae* such as *Escherichia*, *Shigella* and *Klebsiella*, genera frequently linked to dysbiosis and opportunistic pathogenicity in early life. In particular, *Klebsiella* has been widely associated with healthcare-related infections and inflammatory conditions, particularly in vulnerable pediatric populations [41]. In line with previous reports [15, 18], the overall microbial trajectory appeared to reflect a shift toward a microbial composition more characteristic of breastfed infants, a pattern consistent with the expected impact of supplementation with *B. infantis* Bifin02™ and LGG®. Of particular interest was the detection of *Veillonellaceae*, not only after supplementation but also in some infants prior to treatment. *Veillonella* species are lactate-utilizing bacteria that are typically more abundant in breastfed than in formula-fed infants [42]. Their early presence may therefore suggest spontaneous microbial succession driven by host-specific or environmental factors, potentially operating in parallel with probiotic-driven modulation. This observation underscores the complexity of microbial colonization dynamics in early life and suggests that probiotic effects may interact with endogenous or ecological processes already in motion.

The fecal metabolome changes observed by PCA reflect a broad metabolic reprogramming associated with microbiota restructuring. Among the detected SCFAs, acetate emerged as one of the major contributors of the temporal change of the fecal metabolome of infants A, B, G, and H. This trend mirrors the compositional shift in the gut microbiota, particularly the post-intervention rise in *Bifidobacterium* abundance, a genus well known for its capacity to produce acetate and lactate [35]. Similar metabolic signatures have been reported in preterm and term infants receiving bifidobacterial supplementation [18] and in formula-fed infants receiving pre-/postbiotic-enriched formulas [15]. Beyond its microbial origin, acetate plays a crucial role in maintaining intestinal barrier integrity, host energy metabolism, and immune modulation.

Increased acetate production are associated with protection against pathobiont expansion, including *Enterobacteriaceae* which declined in our cohort following supplementation. This functional coupling between microbial composition and metabolic output supports a shift toward a more symbiotic gut ecology.

Similarly, fecal propionate dynamics appeared closely linked to microbiota composition. Propionate is typically associated with genera such as *Bacteroides* and *Veillonella* [38]. In our cohort, the increase in propionate levels, particular evident in post-treatment samples A, B, G, and H, closely paralleled the expansion of *Veillonella*. One possible explanation for this pattern is that increased lactate availability in the gut, produced by lactic acid-producing bacteria such as *Bifidobacterium*, may have favored the growth of lactate-utilizing taxa, including *Veillonella*, thereby enhancing propionate production. This metabolic profile resembles those previously reported in vaginally delivered infants [40] and may reflect a transition toward a more physiologically mature gut ecosystem.

The above-mentioned metabolic shifts were accompanied by a temporal decrease in lysine and 5-aminopentanoate which were particularly abundant at baseline (T0) in infants D and H, and to a lesser extent in B and G. In early life, lysine availability in the gut is largely determined by formula composition [37]. However, the reduction in fecal lysine observed over time is more likely to reflect increased microbial utilization rather than changes in dietary intake. Indeed, previous metagenomic studies have shown that the microbiota of formula-fed infants is enriched in lysine biosynthesis pathways, mainly associated with genera such as *Bacteroides* and *Firmicutes*, indicating an active microbial turnover of this amino acid [44]. Notably, lysine has been described as a metabolic substrate for SCFA linking amino acid metabolism to microbial fermentative activity. In this context, the concurrent decrease in fecal lysine and its degradation product, 5-aminopentanoate, observed in our cohort may reflect enhanced microbial catabolism and incorporation into downstream metabolic pathways, potentially associated with a progressive functional maturation of the gut ecosystem. This interpretation aligns with the concept that the early-life microbiota dynamically adapts to nutrient availability, transitioning from simple fermentative processes toward more complex metabolic networks involving amino acid turnover.

Finally, analysis of the urinary metabolome provided complementary insights into systemic metabolic changes occurring alongside the observed fecal and microbial dynamics. Several metabolites, among which creatinine, glycine, lactate, exhibited clear temporal trends that are consistent with maturation-related processes, while changes in microbiota-derived metabolites such as TMA and DMA suggest an increasing contribution of gut microbial activity to systemic metabolism.

Overall, the observed associations between specific microbial taxa and metabolomics profiles are consistent with shifts toward more functionally mature gut ecosystems in early life [10-13, 15, 18]. It is worth reminding that in our cohort these patterns were not uniform across all infants. While some subjects exhibited coordinated microbiota-metabolome trajectories, others showed distinct or divergent responses, reflecting substantial interindividual variability. This heterogeneity mirrors observations from larger longitudinal cohorts and highlights the dynamic, personalized nature of gut ecosystem development during infancy. This variability cautions against over-generalization and reinforces the need for personalized frameworks when evaluating probiotic interventions in infancy.

Conclusions

This study integrates fecal microbiota profiling with fecal and urinary metabolomics to provide an exploratory characterization of longitudinal microbiota and metabolome dynamics in healthy, full-term, cesarean-born, formula-fed infants receiving a commercial dual-strain probiotic formulation (LGG® and *B. infantis* Bifin02™) over a 3-month period. Although the small cohort size and lack of an untreated comparator limit causal inference and generalizability, the longitudinal, multi-compartment design offers descriptive evidence of temporal positive changes in microbial composition and metabolic outputs, revealing both shared patterns and marked interindividual variability. These findings support the utility of integrative multi-omics approaches for exploring early-life host-microbiota interactions and for informing future efforts toward personalized microbiome modulation strategies in infancy.

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Declaration of interest

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