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Gut dysbiosis associated with neonatal respiratory distress syndrome and biological plausibility of disease-specific probiotic intervention: a translational study

Eunsol Seo^{1†} , Seung Hyun Kim^{2,3†} , Min-Jin Kwak^{4†} , Jae Kyoong Hwang², Ghulam Mustafa⁵, Yun Sil Chang³ , Jeong-Kyu Hoh⁶, Byong-Hun Jeon^{5*} , Hyun-Kyung Park^{2*} and Younghoon Kim^{1*}

Abstract

Background Neonatal respiratory distress syndrome (RDS) is among the most prevalent morbidities in late preterm and term infants. Although the gut–lung axis has been implicated in neonatal respiratory disease, the relationship between RDS and early gut microbiome composition remains poorly characterized. This study aimed to characterize gut microbiome alterations associated with RDS and surfactant replacement therapy (SRT), and to evaluate the biological plausibility of a disease-specific probiotic intervention.

Methods Two complementary cohorts were prospectively enrolled. In the clinical observational cohort ($n=45$), fecal samples collected within 48 h of birth were analyzed by Nanopore 16S rRNA sequencing across three groups: infants without RDS (control group, $n=25$), infants with RDS who did not receive SRT (RDS(S−) group, $n=7$), and infants with RDS who received SRT (RDS(S+) group, $n=13$). In the probiotic discovery cohort ($n=40$), gut microbiota of infants without RDS (CON group, $n=17$) and infants with RDS (RDS group, $n=23$) were characterized by metagenomic sequencing and culturomics. Candidate probiotic strains were evaluated in a fermenter for intestinal microbiota model (FIMM) and a fecal microbiota transplantation (FMT) mouse model.

Results The RDS(S−) group exhibited depletion of beneficial taxa including *Bifidobacterium* and *Lactocaseibacillus* and enrichment of opportunistic pathogens including *Enterococcus* and *Staphylococcus*. Following SRT, gut microbial profiles partially shifted toward those of the control group. *Limosilactobacillus fermentum* SLAM_LAF05 and *Bifidobacterium longum* SLAM_BIL02 were identified as CON-enriched candidate probiotic strains through direct

[†]Eunsol Seo, Seung Hyun Kim and Min-Jin Kwak contributed equally to this work.

*Correspondence:
Byong-Hun Jeon
bhjeon@hanyang.ac.kr
Hyun-Kyung Park
neopark@hanyang.ac.kr
Younghoon Kim
ykeys2584@snu.ac.kr

Full list of author information is available at the end of the article



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microbiome comparison and selected based on superior acid and bile tolerance and adhesion capacity. In the FIMM model, probiotic supplementation increased microbial diversity and suppressed opportunistic pathogens. In the FMT mouse model, probiotic supplementation was associated with upregulation of ZO-1, MUC2, and Reg3g, reduction of fecal calprotectin, and restoration of serum IgG levels.

Conclusions This study provides an early translational characterization of RDS-associated gut dysbiosis and its partial resolution following SRT, and establishes proof-of-concept for a disease-specific probiotic approach. These findings offer a new perspective on the interplay between gut microbial dynamics and the early postnatal respiratory course, and provide a basis for future investigations into microbiota-targeted strategies in neonates with RDS.

Keywords Respiratory distress syndrome, Gut microbiome, Gut-lung axis, Dysbiosis, Surfactant replacement therapy, Probiotics, Neonate, Preterm infant

Background

Respiratory distress syndrome (RDS), primarily resulting from surfactant deficiency in the context of pulmonary immaturity, is among the most common causes of neonatal morbidity, particularly in preterm infants. Although RDS has traditionally been associated with very preterm birth, it remains a clinically significant condition in late preterm and term infants [1]. Since the introduction of exogenous surfactant by Fujiwara et al. in 1980, surfactant replacement therapy (SRT) has become the established management of RDS, substantially reducing neonatal mortality and pulmonary complications such as pneumothorax [2, 3]. Despite its well-characterized pulmonary benefits, the systemic consequences of RDS and SRT on the developing neonatal gut microbiome, a key contributor to neonatal immune and metabolic programming, remain poorly understood.

The gut and lung are functionally interconnected through the gut–lung axis, a bidirectional network mediated by microbial and immune signaling pathways between the two organ systems [4–6]. Perturbations originating in either organ system may propagate systemically, as pulmonary inflammation and hypoxia have been shown to alter intestinal barrier function and microbial composition [7, 8], while gut-derived microbial metabolites and immune signals may reciprocally modulate pulmonary immune responses [9–11]. The neonatal gut microbiome is known to be susceptible to perinatal exposures [12–16], and a recent prospective cohort study demonstrated that the provision of non-invasive positive pressure ventilation was associated with suppression of microbial diversity accrual and obligate anaerobic colonization in the preterm intestine, suggesting that the mode of respiratory support may independently shape early gut microbial development [17]. This finding raises the possibility that RDS and its primary treatment, SRT, may influence early gut microbial colonization. Furthermore, probiotic supplementation has been explored as a potential strategy to improve neonatal outcomes in preterm infants [18, 19], although its role in RDS-associated dysbiosis remains unexplored.

In this study, we aimed to characterize the gut microbiome composition of infants with RDS and to examine whether infants with different respiratory trajectories, including those receiving SRT, exhibited distinct gut microbial profiles. Building on these clinical observations, we identified candidate probiotic strains from the fecal microbiota of healthy term infants. We then evaluated their potential to restore dysbiotic microbial communities and support gut barrier and immune function in experimental models of RDS-associated dysbiosis.

Methods

Study population

This study comprised two complementary cohorts: a clinical observational cohort to characterize gut microbiome alterations associated with RDS and SRT, and a probiotic discovery cohort to identify candidate probiotic strains from the fecal microbiota of healthy term infants.

Clinical observational cohort

We prospectively enrolled 84 infants admitted to the newborn nursery unit or neonatal intensive care unit of Hanyang University Hospital, Seoul, Republic of Korea between September 2021 and January 2024 (Fig. 1a). Infants born before 34 weeks of gestation ($n=18$), those whose fecal samples were collected prior to SRT administration ($n=2$) and those who failed to extract DNA from fecal samples ($n=19$) were excluded from the study. Consequently, 45 late preterm (34–36 weeks of gestation) and term (37–41 weeks of gestation) infants were included and classified into three groups: the control group (infants without RDS, $n=25$), RDS(S–) group (infants with RDS who did not receive SRT owing to a lack of appropriate indications, $n=7$), and RDS(S+) group (infants diagnosed with RDS who received SRT, $n=13$).

Probiotics discovery cohort

To assemble this cohort, fecal samples meeting adequate volume and condition requirements for culturomic analysis and bacterial isolation were prospectively collected (Fig. 1a). Fourteen infants were carried over from the

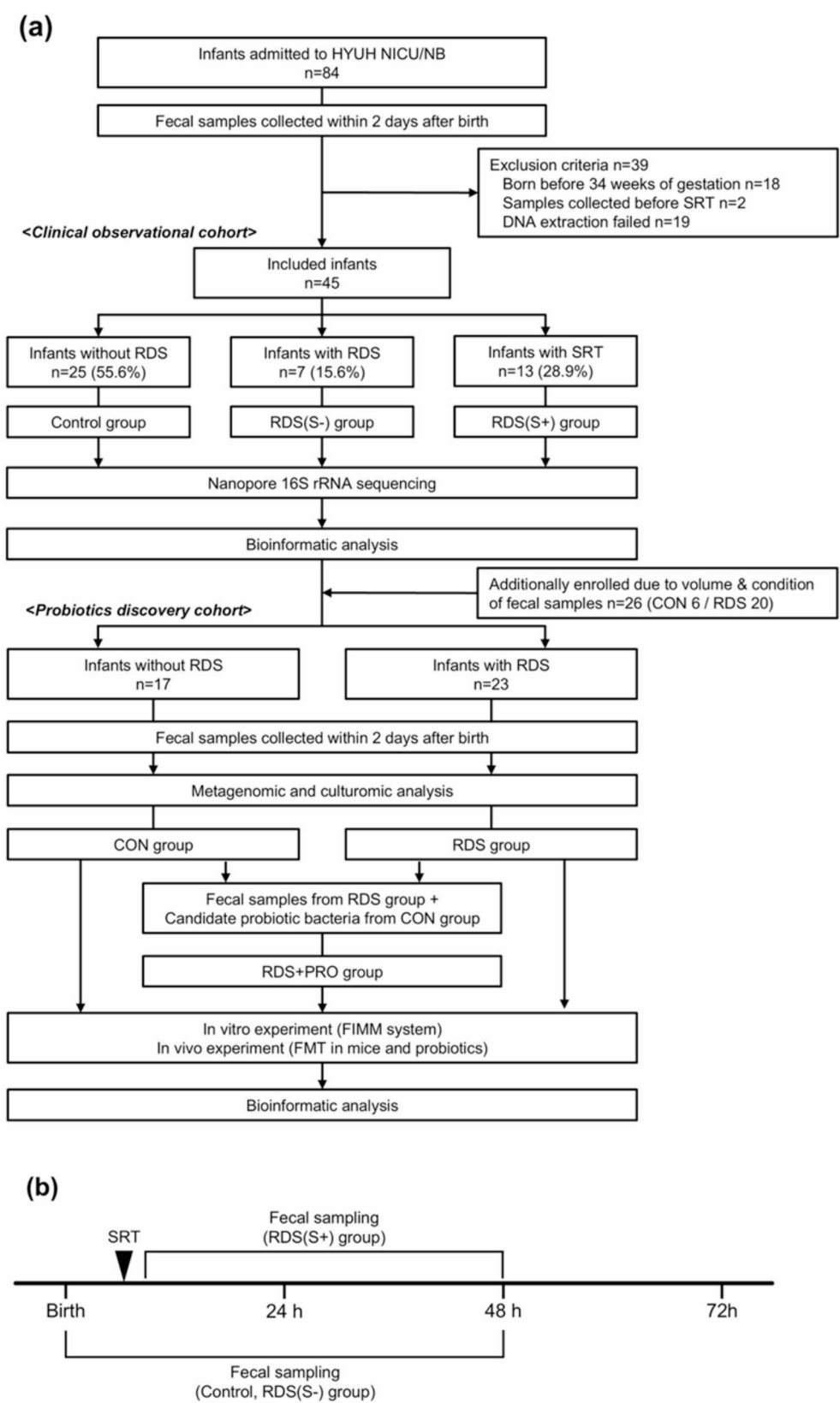


Fig. 1 (See legend on next page.)

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Fig. 1 Participant enrollment and study design. **(a)** Flow diagram illustrating the enrollment and allocation of participants across the clinical observational cohort and probiotic discovery cohort. **(b)** Timeline of fecal sample collection relative to birth. For the control and RDS(S−) groups, samples were collected between birth and 48 h. For the RDS(S+) group, samples were collected between SRT administration and 48 h after birth. NICU, neonatal intensive care unit; NB, newborn nursery; RDS, respiratory distress syndrome; SRT, surfactant replacement therapy; FIMM, fermenter for intestinal microbiota model; FMT, fecal microbiota transplantation

clinical observational cohort, and an additional 26 late preterm and term infants were enrolled between February and April 2024. A total of 40 infants were classified into two groups: the CON group (infants without RDS, $n = 17$) and the RDS group (infants with RDS, $n = 23$).

Sample collection

Fecal samples were collected at the time of first defecation within 48 h of birth across both cohorts, reflecting the typical timing of initial defecation in neonates (Fig. 1b). In the RDS(S+) group, samples were collected within 24 h after SRT administration, which is consistent with the 48-hour collection window given that SRT is typically administered within hours of birth. All samples were immediately frozen and stored at -80°C until further analysis.

Clinical definitions

All clinical data, including perinatal and neonatal characteristics, were retrospectively extracted from medical records and encompassed clinical exposures up to the time of fecal sample collection. RDS was diagnosed based on chest radiographic findings combined with clinical symptoms and the requirement for respiratory support, including invasive ventilation (conventional ventilation or high-frequency oscillatory ventilation) or noninvasive ventilation (bilevel positive airway pressure, nasal continuous positive airway pressure, or high-flow nasal cannula). Infants requiring only supplemental oxygen or no respiratory support were excluded from the RDS diagnosis. Antibiotic use was defined as the administration of at least one dose of an antibiotic before fecal sample collection. Oxygen therapy was defined as any supplemental oxygen support. Enteral feeding was defined as the provision of any enteral nutrition before fecal sample collection, and feeding type was categorized based on the type of enteral nutrition received.

Surfactant replacement therapy (SRT)

Infants diagnosed with RDS who received SRT (RDS(S+) group) were administered surfactant intratracheally either immediately after birth or within the first day of life. The treatment utilized poractant alfa (Curosurf®, Chiesi Farmaceutici SPA, Parma, Italy), a porcine minced lung extract. The preparation had a surfactant protein (SP)-B concentration of 0.45 mg/mL, SP-C content of 5.0–11.6 μg protein/ μmol L-1 phospholipid, and

total phospholipid concentration of 76 mg/mL, including 30 mg/mL of dipalmitoylphosphatidylcholine (DPPC).

Fermenter for intestinal microbiota model (FIMM)

To evaluate the effects of probiotic supplementation on the RDS-associated gut microbiota, the FIMM system was adapted from established methodologies and optimized to closely mimic the specific conditions of the infant gut in vitro [20]. Pooled fecal samples from each group of probiotic discovery cohort were homogenized and inoculated into 10% mGAM medium. The cultures were incubated at 37°C with shaking for 48 h under anaerobic conditions. Three experimental groups were established: CON (fecal samples from infants without RDS), RDS (fecal samples from infants with RDS), and RDS + PRO (fecal samples from infants with RDS supplemented with candidate probiotic strains at a final concentration of 1% [5×10^8 colony-forming units (CFU) each]). All sample processing and analyses were performed in a blinded manner with respect to group allocation.

Fecal microbiota transplantation (FMT) mouse model

To evaluate the in vivo effects of RDS-associated gut microbiota and probiotic supplementation on intestinal physiology, an FMT mouse model was established. Three-week-old C57BL/6 mice were housed under a 12:12 h light-dark cycle, with six mice per cage and ad libitum access to food and water. After a one-week acclimation period, mice received an antibiotic cocktail (ampicillin 100 mg/kg, vancomycin 50 mg/kg, metronidazole 100 mg/kg, neomycin 100 mg/kg, and amphotericin B 1 mg/kg) daily for two weeks to deplete endogenous gut microbiota [21]. At six weeks of age, FMT was performed every other day for two weeks using fecal suspensions derived from the probiotic discovery cohort. Fecal material was resuspended in phosphate-buffered saline (PBS) containing 50% glycerol and 0.05% L-cysteine and stored at -80°C prior to use. Before transplantation, samples were thawed, homogenized, centrifuged at 2,000 rpm for 10 min, and resuspended in Brain Heart Infusion (BHI; BD Difco, Franklin Lakes, NJ, USA) medium supplemented with 0.05% L-cysteine and 20% skim milk [22]. At eight weeks of age, mice received daily oral gavage for two weeks according to group allocation. Three experimental groups were established: CON (FMT with fecal samples from infants without RDS, saline gavage), RDS (FMT with fecal samples from infants with RDS, saline gavage), and RDS + PRO (FMT with fecal samples from

infants with RDS, gavage with candidate probiotic strains at 5×10^8 CFU each). Fecal samples were pooled within each group prior to transplantation. All experimental procedures and analyses were performed in a blinded manner with respect to group allocation.

Nanopore 16S rRNA sequencing

Nanopore-based 16S rRNA sequencing was performed on human fecal samples from both cohorts. Genomic DNA was extracted using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, USA). Variable regions (V1-V9) of the 16S rRNA gene were amplified using a 16S Barcoding Kit (SQK-16S024) in a MiniAmp Plus Thermal Cycler (Thermo Fisher Scientific, USA). DNA was amplified using a polymerase chain reaction (PCR) with LongAmp Hot Start Taq 2X Master Mix (New England Biolabs, USA). The PCR amplicons were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, USA), and the concentrations were quantified using Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific, USA). The constructed library was sequenced using MinION Flow Cell (R9.4.1, FLO-MIN-106D, Oxford Nanopore Technologies, Oxford, UK), and FASTQ raw data were collected and trimmed using a GPU-based ONT guppy basecaller (v5.0.16, Oxford Nanopore Technologies, Oxford, UK) and MinKNOW software (v21.06.13; Oxford Nanopore Technologies, Oxford, UK). The Quantitative Insights Into Microbial Ecology 2 (QIIME2; v2022.2) pipeline was used to analyze the data and assess microbial taxonomy. Operational taxonomic units (OTUs) were clustered with 99% sequence identity using the vsearch cluster-features-de-novo method from the q2-vsearch plugin and annotated with the SILVA 138.1 NR99 database. Alpha diversity (Shannon and Simpson indexes), beta diversity (Bray–Curtis based principal coordinate analysis; PCoA), and relative abundance of taxonomy were analyzed.

Candidate probiotics strain isolation and identification

To isolate candidate probiotic strains, fecal samples from the CON group of the probiotic discovery cohort were used. Briefly, 0.5 g of fecal sample was added to 4.5 mL of 0.1% buffered peptone water (Oxoid, Hampshire, UK) and serially diluted. The diluted solutions were spread onto de Man, Rogosa & Sharpe (MRS; BD Difco, Franklin Lakes, NJ, USA), Bifidobacterium Selective Agar (BS; BD Difco, Franklin Lakes, NJ, USA), BHI, and modified Gifu Anaerobic Medium (mGAM; HiMedia, Maarn, The Netherlands) plates and incubated anaerobically at 37 °C for 48 h. Single colonies were isolated and cultured twice in a liquid medium to ensure purity. The purified colonies were then identified by full-length 16S rRNA gene sequencing [23, 24]. Isolated strains were subsequently evaluated for key probiotic properties using *Lactobacillus*

rhamnosus GG as a reference strain. Acid tolerance was assessed by inoculating strains into MRS broth adjusted to pH 2.5 with pepsin (250 U/mg) and incubating anaerobically at 37 °C for 3 h, and bile salt tolerance was evaluated in MRS broth supplemented with 0.5% oxgall for 24 h; results were expressed as changes in log CFU [25, 26]. Antimicrobial activity against *Listeria monocytogenes* EGD-e, *Salmonella* Typhimurium SL1344, *Escherichia coli* O157:H7, and *Staphylococcus aureus* Newman was assessed using an agar well diffusion assay, with inhibition zones measured in millimeters [27]. Adhesion capacity was evaluated using Caco-2 intestinal epithelial cells with 2-hour incubation, and adherent bacteria were quantified by viable colony counts [28]. Hemolytic activity was assessed on MRS blood agar supplemented with 5% defibrinated sheep blood and classified as α -, β -, or γ -hemolysis [29]. Bile salt hydrolase (BSH) activity was evaluated on MRS agar supplemented with 0.5% taurodeoxycholic acid, with precipitate formation indicating BSH-positive strains [30]. Antibiotic susceptibility was determined using ETEST® strips (bioMérieux, France) for nine antibiotics, with minimum inhibitory concentration (MIC) values interpreted according to EFSA QPS guidelines [31–33].

Hybrid nanopore-illumina whole-genome sequencing and functional annotation

Genomic DNA from the candidate probiotic strains was extracted using the Genomic DNA Prep Kit for Bacteria (Qiagen, Hilden, Germany). Whole-genome sequencing was performed using both Illumina MiSeq and Oxford Nanopore MinION platforms. Hybrid de novo assembly was conducted using Unicycler v0.4.8 via the PATRIC web server, and genome completeness was assessed with CheckM (v1.2.3), confirming 100% completeness [34–36]. Functional annotation was carried out using Proksee (Prokka v1.13.4-based pipeline), with a focus on probiotic-related features, including stress resistance, acid and bile tolerance, adhesion to intestinal epithelial cells, bacteriocin production, exopolysaccharide (EPS) biosynthesis, immune modulation, and carbohydrate metabolism. Antibiotic resistance genes and virulence factors were identified using the Virulence Factor Database (VFDB; v2.0), PATRIC_VF, and the Comprehensive Antibiotic Resistance Database (CARD; v4.0.1) [37, 38].

Classification models and ROC analysis

To evaluate the potential of the candidate probiotic strains as biomarkers for distinguishing between CON and RDS groups, relative abundance data were analyzed using a Random Forest classifier. Model performance was assessed using 10-fold cross-validation to minimize bias from data partitioning and to ensure generalizability. Receiver operating characteristic (ROC) curves and the

area under the curve (AUC) values were used to evaluate classification accuracy. AUC values were interpreted as follows: ≥ 0.90 (excellent), 0.80–0.89 (good), 0.70–0.79 (moderate), and < 0.70 (poor) [39, 40]. All analyses were conducted in Python (v3.9) using the scikit-learn library (v1.6.1).

Illumina 16S rRNA sequencing

Illumina-based 16S rRNA sequencing was performed on fecal samples from the FIMM model and the FMT mouse model. Genomic DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany). The V3-V4 region of the 16S rRNA gene was amplified using region-specific primers (forward: 515 F, 5'-TCGTC GGCAGCGTCAGATGTGTATAAGAGACAGGTGCC AGCMGCCGCGTAA-3'; reverse: 806R, 5'-GTCTCG TGGGCTCGGAGATGTGTATAAGAGACAGGGACT ACHVGGGTWTCTAAT-3') [41]. Sequencing was performed on the Illumina NextSeq 300 platform (Illumina, San Diego, CA, USA). Raw sequencing data were filtered to retain reads with a Phred quality score $\geq Q30$. High-quality reads were then processed using the QIIME2 (v2022.2) pipeline, with DADA2 (v1.34.0) applied for denoising, chimera removal, and merging of paired-end reads. Amplicon sequence variants (ASVs) were generated and used for downstream taxonomic classification and microbial diversity analyses from phylum to species level [42, 43]. Taxa with a relative abundance of less than 1% were grouped as "Others." Taxonomic differences between groups were analyzed using Statistical Analysis of Metagenomic Profiles (STAMP; v2.0), and alpha diversity was assessed using the Shannon diversity index to evaluate species richness and evenness. Beta diversity was calculated based on Bray-Curtis dissimilarity and visualized using PCoA to assess compositional differences between groups [44].

Functional prediction analysis

To investigate the functional potential of RDS-associated gut microbiota and the modulatory effect of probiotic supplementation, functional prediction of microbial communities was performed on FIMM samples using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) [45]. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway abundance was subsequently inferred using the ggpicrust2 package (v2.6.1), and predicted gene functions and metabolic pathways were compared between RDS and RDS + PRO to assess probiotic-driven functional shifts in the dysbiotic microbial community.

Quantification of gut barrier and immune markers

To assess gut barrier integrity and immune responses in the FMT mouse model, the expression of tight junction

protein (ZO-1), mucin (MUC2), antimicrobial peptide (Reg3g), and inflammatory cytokines (IL-10, TNF- α) was quantified by quantitative real-time PCR (qRT-PCR). Total RNA was extracted from intestinal tissues using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and complementary DNA was synthesized using the iScript[™] cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). qRT-PCR was performed on the CFX96 Touch System (Bio-Rad) using SsoAdvanced[™] Universal SYBR[®] Green Supermix (Bio-Rad). glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the reference gene, and expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [46], with the CON group used as the reference.

For protein-level analysis, calprotectin was measured in fecal homogenates using a commercial ELISA kit (Fine Biotech, Ellicott City, MD, USA), and serum IgG levels were quantified using an ELISA kit (Immunology Consultants Laboratory, Portland, OR, USA), according to the manufacturers' protocols. Absorbance was recorded at 450 nm using a microplate reader (SpectraMax ABS Plus, Molecular Devices, San Jose, CA, USA), providing quantitative measures of intestinal inflammation and systemic immune response.

Statistical analysis

Clinical characteristics of infants were analyzed using univariate statistical methods. The Shapiro–Wilk test was used to assess the distribution of continuous variables. As several variables showed non-normal distributions and variance heterogeneity, continuous variables are presented as median with interquartile range (IQR). Comparisons between two groups were performed using the Wilcoxon rank-sum test, whereas comparisons among three groups were performed using the Kruskal–Wallis test. Categorical variables are presented as frequencies and percentages and were compared using Fisher exact test owing to sparse cell counts in several contingency tables. A two-sided p value < 0.05 was considered statistically significant. Microbiome data analyses were conducted using R software (v4.3.2, R Core Team, 2023) and GraphPad Prism (v10.0.0, GraphPad Software Inc., USA). Continuous data are presented as mean $\pm$ SD. For comparisons among three groups, ANOVA was performed, followed by Tukey's multiple comparisons test for post hoc pairwise analyses. Comparisons between two groups were conducted using the Student's t -test. Differentially abundant taxa were identified using LEfSe on the Galaxy platform. The Kruskal–Wallis test was used to detect significant differences in relative abundance, followed by pairwise Wilcoxon rank-sum tests. Linear discriminant analysis (LDA) was applied to estimate effect sizes, with an LDA score ≥ 2.0 considered significant [47]. Taxonomic differences were assessed using STAMP (v2.0) with Welch's t -test and false discovery rate (FDR)

correction. Results were visualized as extended error bar plots with mean proportions and 95% confidence intervals. Differential KEGG pathway abundance was analyzed using ALDEx2 (v1.36.0) with Welch's t-test, and effect sizes were calculated. Linear correlation between gut microbiota and immune related markers was estimated using Pearson's correlation test. Statistical significance was indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Results

Clinical characteristics of the clinical observational cohort
Clinical characteristics of the three groups are summarized in Table 1. No significant differences were observed in gestational age, birth weight among the three groups. Maternal and perinatal factors including sex, mode of delivery, chorioamnionitis, maternal antibiotic use, gestational diabetes mellitus (GDM), pregnancy-induced hypertension (PIH), and premature rupture of membranes (PROM) did not differ significantly among groups. Consistent with the clinical course of RDS, oxygen therapy and antibiotic use were significantly more frequent in the RDS groups (both $p < 0.001$), whereas enteral feeding rates and feeding type did not differ significantly ($p = 0.138$ and $p = 0.341$, respectively).

Gut microbiota alterations associated with RDS and SRT
Distinct alterations in gut microbial composition were observed among the three groups at both genus and species levels (Fig. 2a, b). In the RDS(S−) group, opportunistic pathogens including *Enterococcus* and *Staphylococcus*

were significantly increased, while beneficial taxa such as *Lactacaseibacillus* and *Bifidobacterium* were significantly decreased compared with the control group ($p < 0.05$; Fig. 2c). At the species level, *E. faecium* and *S. hominis* were notably elevated, whereas *B. longum* and *L. rhamnosus* were reduced in the RDS(S−) group (Fig. 2c). Following SRT, the RDS(S+) group showed microbial profiles more comparable to those of the control group, with partial normalization of both opportunistic and beneficial taxa.

Alpha diversity indices showed no statistically significant differences among groups, although a numerical reduction was observed in the RDS(S−) group with partial recovery in the RDS(S+) group (Fig. 2d). Beta diversity analysis revealed that the RDS(S+) group clustered more closely with the control group than with the RDS(S−) group (Fig. 2e), consistent with a partial shift in overall microbial community structure following SRT.

Co-occurrence network analysis further illustrated these patterns (Fig. 2f). In the control group, *Enterococcus*, *Staphylococcus*, and *Lactacaseibacillus* occupied central positions in the microbial network. This structured network was disrupted in the RDS(S−) group, with increased positive correlations among opportunistic taxa. In the RDS(S+) group, the network structure was partially restored toward that of the control group.

Clinical characteristics of the probiotic discovery cohort
Clinical characteristics of the two groups are summarized in Table 2. The RDS group had significantly lower gestational age (35.0 weeks [IQR: 33.0–35.0] vs. 38.6 weeks

Table 1 Clinical characteristics of participants in the clinical observational cohort

	Control group (n = 25)	RDS(S−) group (n = 7)	RDS(S+) group (n = 13)	p-value
GA (weeks, median [IQR])	38.7 [38.1, 39.0]	37.3 [36.0, 38.8]	38.0 [35.6, 38.4]	0.092
BW (gram, median [IQR])	3080 [2030,3390]	2860 [2069,3110]	3000 [2550,3170]	0.120
Male, n (%)	13 (52.0%)	5 (71.4%)	6 (46.2%)	0.599
Singleton, n (%)	23 (92.0%)	4 (57.1%)	11 (84.6%)	0.075
Cesarean section, n (%)	18 (72.0%)	5 (71.4%)	11 (84.6%)	0.634
Chorioamnionitis, n (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1.000
Maternal antibiotics, n (%)	3 (12.0%)	0 (0.0%)	0 (0.0%)	0.511
GDM, n (%)	5 (20.0%)	0 (0.0%)	0 (0.0%)	0.234
PIH, n (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1.000
PROM, n (%)	1 (4.0%)	2 (28.6%)	1 (7.7%)	0.135
Oxygen therapy, n (%)	0 (0.0%)	7 (100.0%)	13 (100.0%)	< 0.001
Antibiotics exposure, n (%)	0 (0.0%)	5 (71.4%)	13 (100.0%)	< 0.001
Enteral feeding, n (%)	25 (100.0%)	6 (85.7%)	12 (92.3%)	0.138
Breast milk feeding*, n (%)	0 (0.0%)	0 (0.0%)	1 (8.3%)	0.341

* Among infants receiving enteral feeding

Continuous variables are presented as median [IQR] and compared using the Kruskal–Wallis test. Categorical variables are presented as n (%) and compared using the Fisher exact test

All clinical variables assessed prior to fecal sample collection

RDS, respiratory distress syndrome; GA, gestational age; BW, birth weight; GDM, gestational diabetes mellitus; PIH, pregnancy-induced hypertension; PROM, premature rupture of membranes

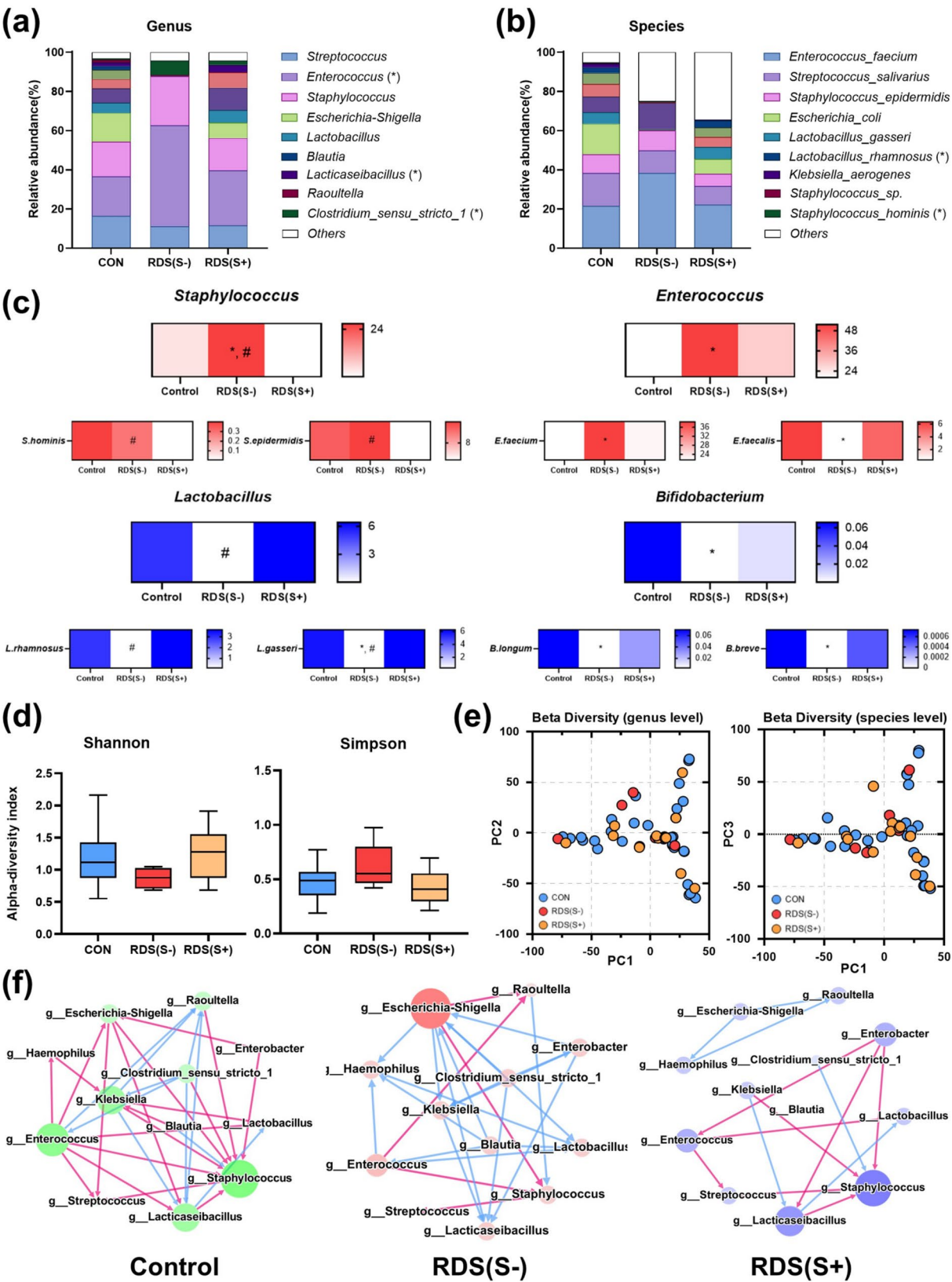


Fig. 2 (See legend on next page.)

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Fig. 2 Gut microbiota alterations associated with RDS and SRT. **(a–b)** Relative abundance of gut bacterial taxa at genus and species levels across the control, RDS(S–), and RDS(S+) groups. **(c)** Heatmap comparisons of relative abundance for key opportunistic (*Staphylococcus*, *Enterococcus*) and beneficial (*Lactobacillus*, *Bifidobacterium*) taxa and their constituent species; * $p < 0.05$ vs. control group; # $p < 0.05$ vs. RDS(S+) group. **(d)** Alpha diversity assessed by Shannon and Simpson indices. Boxes represent interquartile range; whiskers indicate minimum and maximum values. **(e)** Beta diversity visualized by principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity at genus and species levels. **(f)** Co-occurrence network analysis of microbial interactions in the control, RDS(S–), and RDS(S+) groups; node size reflects relative abundance, pink lines indicate positive correlations, and blue lines indicate negative correlations. Microbiome relative abundance data are presented as mean $\pm$ SD. Alpha diversity indices are presented as median (IQR). Control, $n = 25$; RDS(S–), $n = 7$; RDS(S+), $n = 13$. RDS, respiratory distress syndrome; SRT, surfactant replacement therapy

[IQR: 37.7–38.9], $p < 0.001$) and birth weight (2,100 g [IQR: 1,658–2,305] vs. 3,080 g [IQR: 2,950–3,200], $p < 0.001$) compared with the CON group. A significantly lower proportion of singleton births was observed in the RDS group (39.1% vs. 94.1%, $p < 0.001$). Maternal and perinatal factors including sex, mode of delivery, chorioamnionitis, maternal antibiotic use, GDM, PIH, and PROM did not differ significantly between groups. Consistent with the clinical course of RDS, oxygen therapy and antibiotic use were more frequent in the RDS group (both $p < 0.001$), and the rate of enteral feeding was lower ($p = 0.005$), although the proportion of breast milk feeding among those receiving enteral nutrition did not differ significantly ($p = 1.000$).

Gut microbiota characterization in CON and RDS groups

Alpha diversity measured by the Shannon index showed no significant difference between groups (Fig. 3a), reflecting comparable microbial richness. Beta diversity analysis revealed a tendency toward compositional separation between groups, with greater inter-individual variation observed in the RDS group (Fig. 3b). At the genus level, *Lactobacillus* and *Limosilactobacillus* were more abundant in the CON group, while *Enterococcus* and *Klebsiella* were enriched in the RDS group (Fig. 3c). At the species level, *L. rhamnosus*, *L. fermentum*, and *L. gasseri* were more abundant in the CON group, whereas *E. faecium* and *Klebsiella aerogenes* were enriched in the RDS group (Fig. 3d). LEfSe analysis confirmed distinct taxonomic differences between groups (LDA score > 2.0), with *B. longum*, *L. fermentum*, and *L. gasseri* enriched in the CON group and *E. faecium* and *Veillonella parvula* enriched in the RDS group (Fig. 3e, f).

Culturomics identified 528 bacterial isolates across 54 distinct species, of which 13 belonged to beneficial genera including *Bifidobacterium* and *Lactobacillus* (Fig. 3g). Metagenomic sequencing identified 223 species while culturomics yielded 54 species, with 28 species identified by both approaches (Fig. 3h).

Identification and characterization of candidate probiotic strains

Based on metagenomic analysis and LEfSe, *B. longum*, *L. reuteri*, *L. fermentum*, and *L. gasseri* were identified as CON-enriched taxa and selected as isolation targets (Fig. 3d, e). Of these, *B. longum*, *L. fermentum*, and *L.*

gasseri were successfully isolated from CON group fecal samples, while *L. reuteri* was not recovered. Key probiotic properties were evaluated for all three isolates, including acid and bile tolerance, antimicrobial activity, adhesion capacity, hemolytic activity, and BSH activity (Supplementary Table 1). Based on superior performance in acid/bile tolerance and adhesion capacity as primary selection criteria, *L. fermentum* SLAM_LAF05 and *B. longum* SLAM_BIL02 were selected as candidate probiotic strains. Both strains exhibited γ -hemolysis and complied with EFSA antibiotic resistance guidelines (Supplementary Table 2).

Whole-genome sequencing revealed genome sizes of 2,077,674 bp (GC content 51.51%, 2,033 CDS) for *L. fermentum* SLAM_LAF05 and 2,582,071 bp (GC content 60.19%, 2,137 CDS) for *B. longum* SLAM_BIL02 (Fig. 4a). Genomic annotation identified distinct probiotic-associated gene profiles in both strains (Fig. 4b). *L. fermentum* SLAM_LAF05 harbored genes related to stress resistance, acid and bile tolerance, EPS biosynthesis, immune modulation, adhesion, bacteriocin production, and carbohydrate metabolism. *B. longum* SLAM_BIL02 possessed genes involved in acid and bile tolerance, carbohydrate metabolism, EPS biosynthesis, and bacteriocin production, with fewer genes associated with adhesion and stress response.

A Random Forest classification model based on the relative abundances of the two strains achieved an AUC of 0.88 ± 0.15 in distinguishing CON from RDS groups, indicating good discriminatory performance (Fig. 4c).

Probiotic supplementation modulates gut microbiota in the FIMM model

To evaluate the effects of candidate probiotic strains on RDS-associated gut microbiota in vitro, fecal samples from the CON, RDS, and RDS+PRO groups were analyzed using the FIMM system. Shannon alpha diversity was significantly higher in the RDS+PRO group compared to both the CON ($p < 0.01$) and RDS groups ($p < 0.05$), while no significant difference was observed between CON and RDS (Fig. 5a). Beta diversity analysis demonstrated distinct clustering of all three groups, with statistically significant compositional differences confirmed by PERMANOVA ($p = 0.0037$; Fig. 5b).

Taxonomic analysis revealed that probiotic supplementation in the RDS+PRO group was associated with

Table 2 Clinical characteristics of participants in the probiotics discovery cohort

	CON group (n = 17)	RDS group (n = 23)	p-value
GA (weeks, median [IQR])	38.6 [37.7, 38.9]	35.0 [33.0, 35.0]	< 0.001
BW (gram, median [IQR])	3080 [2950, 3200]	2100 [1658, 2305]	< 0.001
Male, n (%)	13 (56.5%)	11 (47.8%)	0.768
Singleton, n (%)	16 (94.1%)	9 (39.1%)	< 0.001
Cesarean section, n (%)	12 (70.6%)	18 (78.3%)	0.717
Chorioamnionitis, n (%)	0 (0.0%)	0 (0.0%)	1.000
Maternal antibiotics, n (%)	2 (11.8%)	6 (26.1%)	0.428
GDM, n (%)	4 (23.5%)	2 (8.7%)	0.373
PIH, n (%)	1 (5.9%)	2 (8.7%)	1.000
PROM, n (%)	2 (11.8%)	5 (21.7%)	0.438
Oxygen therapy, n (%)	0 (0.0%)	21 (91.3%)	< 0.001
Antibiotic exposure, n (%)	0 (0.0%)	18 (78.3%)	< 0.001
Enteral feeding, n (%)	17 (100.0%)	14 (60.9%)	0.005
Breast milk feeding*, n (%)	2 (11.8%)	1 (7.1%)	1.000

* Among infants receiving enteral feeding

Continuous variables are presented as median [IQR] and compared using the Wilcoxon rank-sum test. Categorical variables are presented as n (%) and compared using the Fisher exact test

All clinical variables assessed prior to fecal sample collection

RDS, respiratory distress syndrome; GA, gestational age; BW, birth weight; GDM, gestational diabetes mellitus; PIH, pregnancy-induced hypertension; PROM, premature rupture of membranes

increased abundance of beneficial taxa including *Lactobacillus* and *Bifidobacterium*, and decreased abundance of opportunistic pathogens including *Enterococcus*, *Staphylococcus*, and *Clostridioides* compared with the RDS group (Fig. 5c, d). Comparisons between CON and RDS, and between CON and RDS+PRO, further supported these compositional differences (Fig. 5e, f).

Co-occurrence network analysis revealed distinct microbial interaction patterns across groups (Fig. 5g). In the RDS group, *Lactobacillus* showed markedly reduced node size and minimal network connections, with the network dominated by opportunistic taxa including *Staphylococcus*, *Enterococcus*, and *Clostridioides*. Following probiotic supplementation, the RDS+PRO group showed partial restoration of network structure, with *Bifidobacterium* retaining notable associations and the overall pattern becoming more comparable to that of the CON group.

Functional prediction using PICRUSt2 identified distinct KEGG pathway profiles between the RDS+PRO and RDS groups (Fig. 5h). The RDS+PRO group showed enrichment of pathways related to nutrient metabolism, including protein digestion and absorption, vitamin metabolism, and bile acid biosynthesis. In contrast, the RDS group showed higher predicted activity in the bacterial invasion of epithelial cells pathway.

Probiotic supplementation modulates gut microbiota in the FMT mouse model

To evaluate the in vivo effects of RDS-associated gut microbiota and probiotic supplementation, FMT was performed in antibiotic-depleted C57BL/6 mice using fecal samples derived from CON or RDS infants; the RDS+PRO group additionally received daily oral gavage of candidate probiotic strains (Fig. 6a). The RDS group demonstrated significantly lower body weight relative to the CON group ($p < 0.01$), while the RDS+PRO group exhibited a significant increase in body weight compared to the RDS group ($p < 0.01$; Fig. 6b).

Shannon alpha diversity did not differ significantly among the three groups (Fig. 6c). Beta diversity analysis using Bray–Curtis dissimilarity revealed distinct inter-group clustering, with PERMANOVA confirming statistically significant differences in overall community composition ($p = 0.0005$; Fig. 6d).

At the genus level, *Bifidobacterium* abundance was significantly greater in both the CON and RDS+PRO groups relative to the RDS group ($p < 0.0001$), whereas *Enterococcus* and *Escherichia-Shigella* were significantly enriched in the RDS group compared with both CON and RDS+PRO groups ($p < 0.0001$ and $p < 0.01$, respectively; Fig. 6f). *Akkermansia* abundance was significantly higher in the CON group than in the RDS

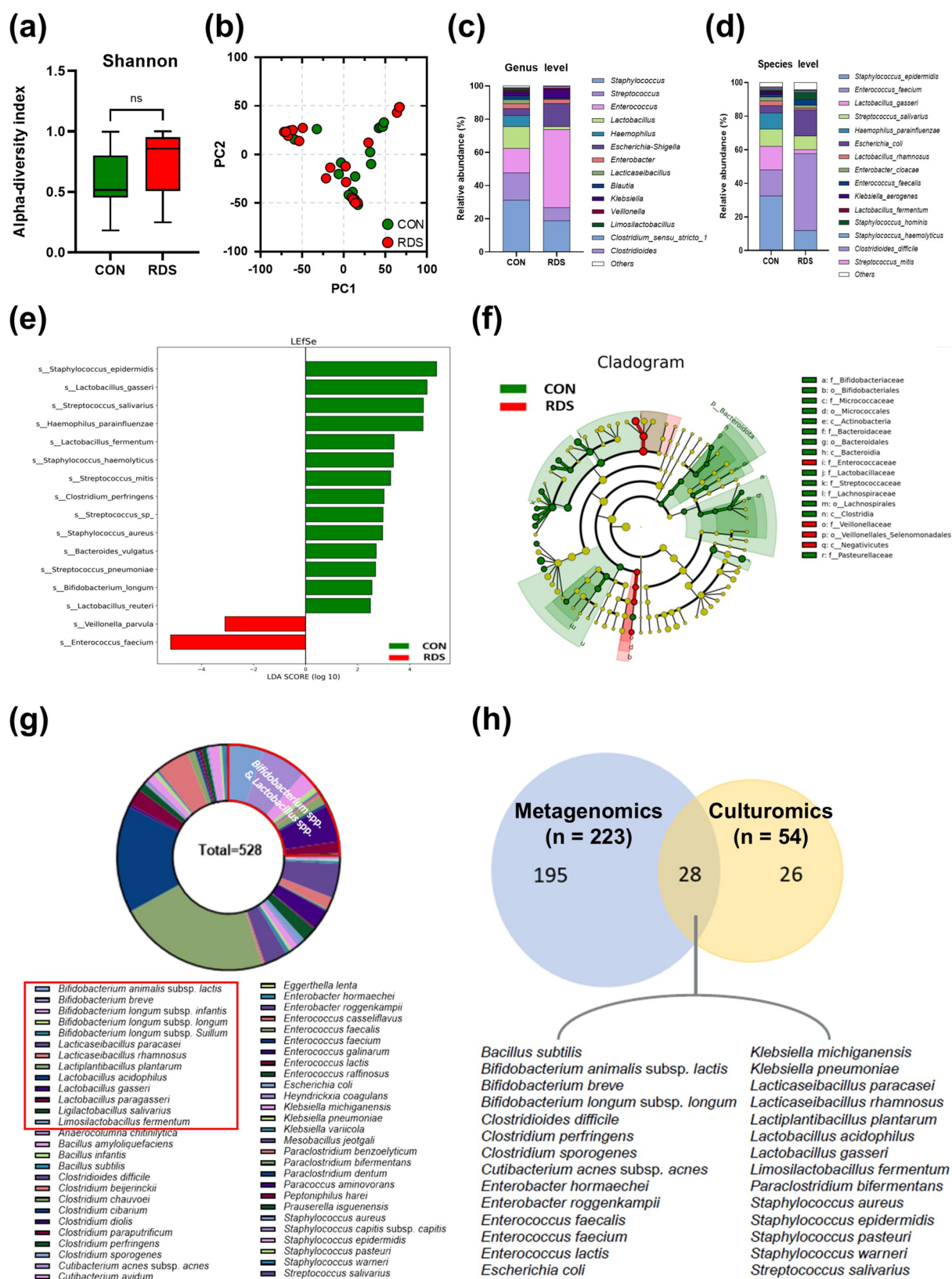


Fig. 3 (See legend on next page.)

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Fig. 3 Gut microbiota characterization in the probiotic discovery cohort. **(a)** Alpha diversity assessed by the Shannon index. Boxes represent interquartile range; whiskers indicate minimum and maximum values. **(b)** Beta diversity visualized by PCoA based on Bray–Curtis dissimilarity. **(c–d)** Relative abundance of bacterial taxa at genus and species levels. **(e)** LEfSe analysis showing differentially abundant species between CON and RDS groups; LDA score threshold > 2.0. **(f)** Cladogram illustrating phylogenetic distribution of differentially abundant taxa; CON-enriched lineages are shown in green and RDS-enriched lineages in red. **(g)** Distribution of 528 bacterial isolates identified by culturomics across 54 species; species belonging to *Bifidobacterium* and *Lactobacillus* genera are highlighted. **(h)** Venn diagram comparing species identified by metagenomic sequencing ($n = 223$) and culturomics ($n = 54$), with 28 species commonly identified by both approaches. Microbiome relative abundance data are presented as mean $\pm$ SD. Alpha diversity index is presented as median (IQR). CON, $n = 17$; RDS, $n = 23$. ns, not significant; CON, control; RDS, respiratory distress syndrome; PCoA, principal coordinate analysis; LEfSe, linear discriminant analysis effect size; LDA, linear discriminant analysis

group ($p < 0.05$), without reaching statistical significance between the RDS+PRO and other groups (Fig. 6f). No statistically significant differences in *Lactobacillus* abundance were observed among groups (Fig. 6f).

Co-occurrence network analysis demonstrated distinct patterns of microbial interaction across groups (Fig. 6g). In the RDS group, node sizes corresponding to beneficial taxa including *Akkermansia*, *Lactobacillus*, and *Bifidobacterium* were markedly attenuated, while *Enterococcus* and *Clostridioides* exhibited increased centrality and connectivity. In the RDS+PRO group, the network architecture more closely resembled that of the CON group, with *Akkermansia*, *Lactobacillus*, and *Bifidobacterium* contributing more evenly to the overall network structure.

Probiotic supplementation modulates gut barrier integrity and immune responses

To assess the effects of FMT and probiotic supplementation on gut barrier function and immune responses, qRT-PCR and ELISA analyses were performed in the FMT mouse model. Both ZO-1 and MUC2 expression were significantly reduced in the RDS group compared to the CON group ($p < 0.0001$ and $p < 0.001$, respectively), while the RDS+PRO group demonstrated significantly higher expression of both markers relative to the RDS group ($p < 0.0001$ and $p < 0.05$, respectively; Fig. 7a). Reg3g expression was significantly elevated in the RDS+PRO group compared to both the RDS ($p < 0.001$) and CON groups ($p < 0.01$; Fig. 7a).

No significant differences in IL-10 expression were observed among groups (Fig. 7b). TNF- α expression was significantly higher in the RDS group relative to both the CON ($p < 0.01$) and RDS+PRO groups ($p < 0.05$), with no significant difference between CON and RDS+PRO (Fig. 7b). Fecal calprotectin levels were significantly lower in the RDS+PRO group compared to both the RDS ($p < 0.0001$) and CON groups ($p < 0.01$), while the RDS group showed significantly higher levels than the CON group ($p < 0.01$; Fig. 7c). Serum IgG levels were significantly lower in the RDS group compared to both the CON and RDS+PRO groups ($p < 0.0001$), with no

significant difference between CON and RDS+PRO (Fig. 7c).

Spearman correlation analysis demonstrated that beneficial taxa including *Bifidobacterium*, *Lactobacillus*, and *Akkermansia* were positively correlated with ZO-1, MUC2, Reg3g, and IgG, and negatively correlated with calprotectin and TNF- α , whereas *Enterococcus* and *Escherichia-Shigella* exhibited opposing patterns (Fig. 7d). Linear regression analysis further revealed significant negative correlations between the abundances of *L. fermentum* and *B. longum* and fecal calprotectin levels ($p < 0.001$ and $p = 0.008$, respectively), and significant positive correlations with Reg3g expression ($p < 0.001$ and $p = 0.005$, respectively; Fig. 7e). *L. fermentum* additionally demonstrated a significant positive correlation with serum IgG levels ($p = 0.017$), while *B. longum* did not reach statistical significance ($p = 0.211$).

Discussion

In this study, we examined the relationship between RDS and early gut microbiome composition in neonates through a two-arm translational approach. In the observational arm, the gut microbiota of late preterm and term infants with RDS showed distinct alterations compared with healthy controls, characterized by depletion of beneficial taxa and enrichment of opportunistic pathogens. Among infants who received SRT, gut microbial profiles showed a partial shift toward those of the control group, consistent with a potential association between pulmonary recovery and gut microbial dynamics. Building on these clinical observations, candidate probiotic strains identified from healthy infant fecal microbiota suggested the potential to modulate RDS-associated dysbiosis and support gut barrier integrity and immune responses in complementary in vitro and in vivo experimental models. Dysbiosis in preterm infants is well documented in the early neonatal period [48–51]. This study extends this context to infants with RDS as a specific clinical condition, as RDS represents one of the most prevalent morbidities in this population [2, 52]. Our findings demonstrate that late preterm and term infants with

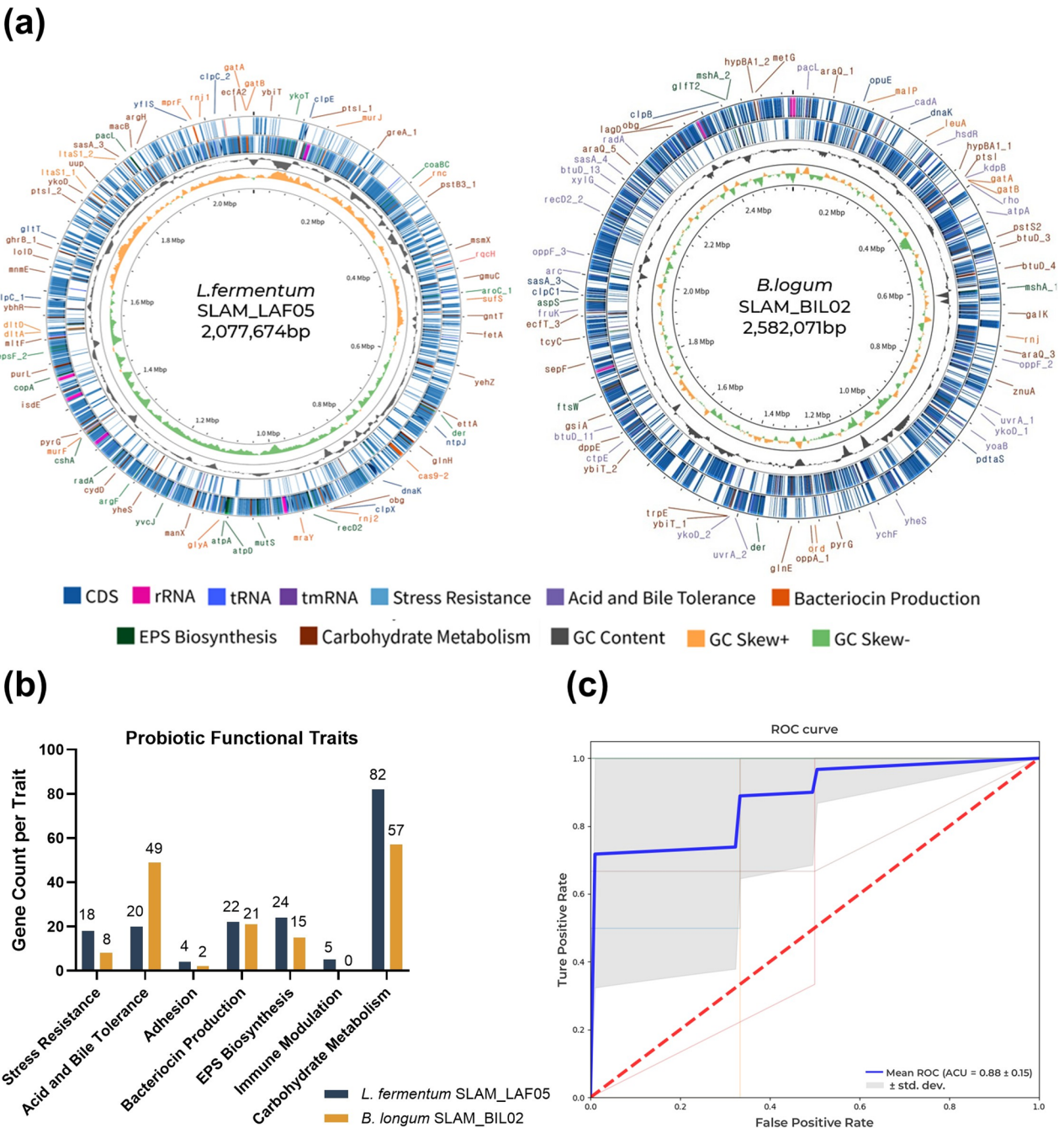


Fig. 4 Genomic characterization and discriminatory performance of candidate probiotic strains. **(a)** Circular genome maps of *L. fermentum* SLAM_LAF05 (2,077,674 bp) and *B. longum* SLAM_BIL02 (2,582,071 bp) illustrating genomic organization and functional gene annotations. **(b)** Gene counts per probiotic functional trait for *L. fermentum* SLAM_LAF05 and *B. longum* SLAM_BIL02. **(c)** Receiver operating characteristic (ROC) curve of a Random Forest classification model based on the relative abundances of the two strains, demonstrating discriminatory performance between CON and RDS groups (mean AUC = 0.88 ± 0.15). Model performance was assessed using 10-fold cross-validation. CON, *n* = 17; RDS, *n* = 23. CON, control; RDS, respiratory distress syndrome; CDS, coding sequences; EPS, exopolysaccharide; ROC, receiver operating characteristic; AUC, area under the curve

RDS exhibit gut microbiota alterations characterized by depletion of beneficial commensals and enrichment of opportunistic pathogens, with pathogenic taxa gaining ecological dominance in network analysis. Interestingly, infants who received SRT showed a partial shift in gut microbial profiles toward those of healthy controls.

Rather than reflecting a direct effect of surfactant, this observation may be more plausibly interpreted in the context of the broader systemic improvements conferred by restoration of respiratory function following SRT, including enhanced oxygenation, restoration of bowel perfusion, stabilization of host homeostatic pathways,

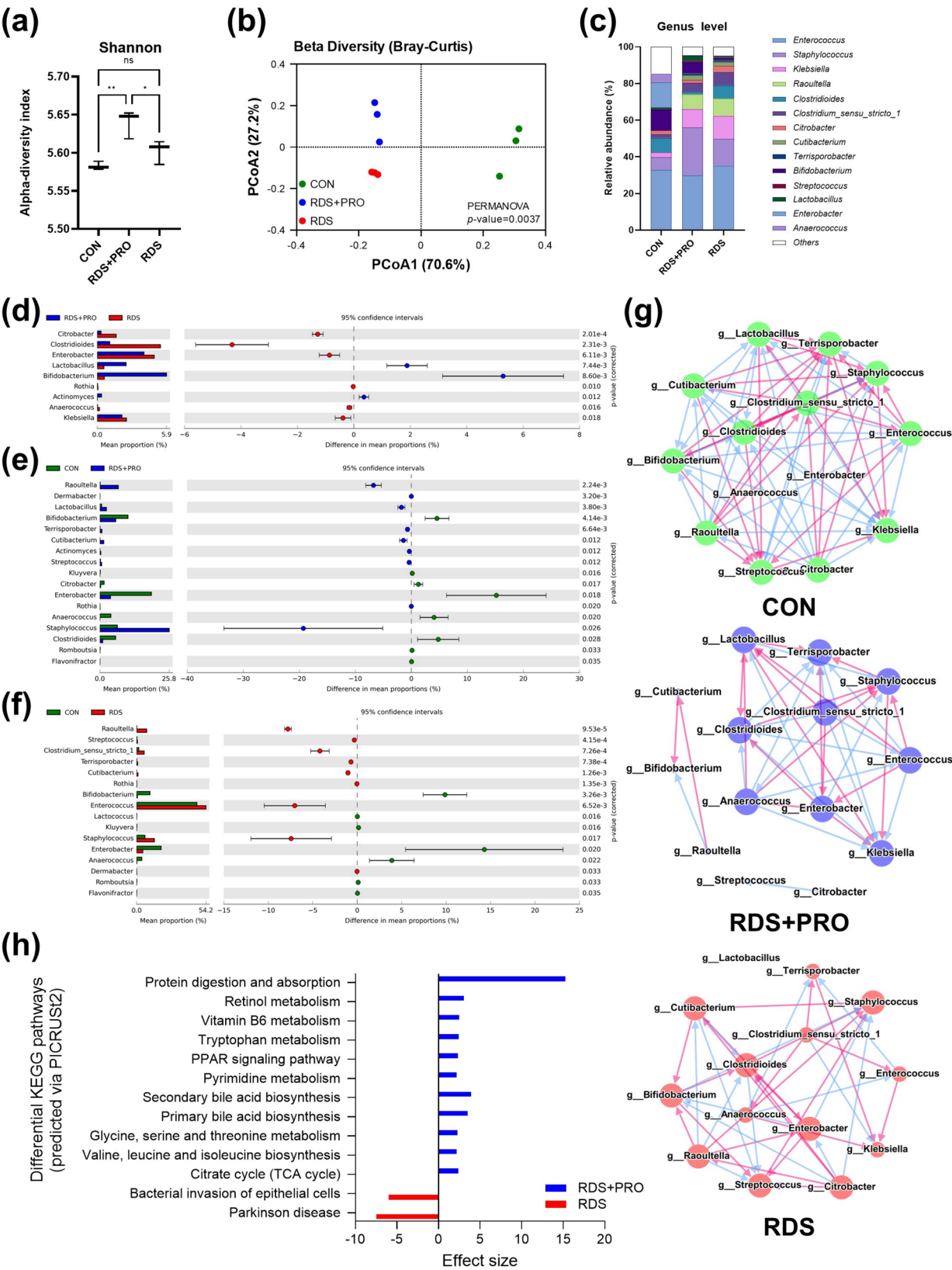


Fig. 5 (See legend on next page.)

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Fig. 5 Probiotic supplementation modulates gut microbiota in the FIMM model. **(a)** Alpha diversity assessed by the Shannon index; $**p < 0.01$; $*p < 0.05$. **(b)** Beta diversity visualized by PCoA based on Bray–Curtis dissimilarity; PERMANOVA $p = 0.0037$. **(c)** Relative abundance of bacterial taxa at genus level. **(d–f)** Extended error bar plots from STAMP analysis (Welch's t-test with FDR correction) comparing mean proportions with 95% confidence intervals between **(d)** RDS + PRO and RDS, **(e)** CON and RDS + PRO, and **(f)** CON and RDS groups. **(g)** Co-occurrence network analysis of microbial interactions in CON, RDS + PRO, and RDS groups; node size reflects relative abundance, pink lines indicate positive correlations, and blue lines indicate negative correlations. **(h)** Differential KEGG pathway abundance between RDS + PRO and RDS groups predicted by PICRUST2; bars represent effect sizes. Data are presented as mean $\pm$ SD. $n = 3$ per group. CON, control; RDS, respiratory distress syndrome; RDS + PRO, RDS group with probiotic supplementation; FIMM, fermenter for intestinal microbiota model; PCoA, principal coordinate analysis; STAMP, statistical analysis of metagenomic profiles; KEGG, Kyoto Encyclopedia of Genes and Genomes

and potential immunomodulatory effects mediated by surfactant-associated proteins [53]. These systemic changes may collectively create a more favorable intestinal environment conducive to microbial recovery. This interpretation aligns with the concept of gut–lung axis bidirectionality, wherein pulmonary insults such as hypoxia and respiratory failure may aggravate intestinal dysbiosis, and conversely, improvements in pulmonary status may reciprocally influence the gut microbial environment [6, 7, 54].

The interpretation of these findings requires acknowledgment of potential confounding factors inherent to the prospective nature of this study. In the clinical observational cohort, where groups were composed to be as clinically comparable as possible to isolate the effects of RDS and SRT on microbial composition, gestational age, birth weight, and maternal and perinatal factors did not differ significantly among the three groups. Oxygen therapy and antibiotic exposure were more frequent in the RDS groups, reflecting the greater clinical severity inherent to this population rather than independent confounding variables. Notably, despite higher antibiotic exposure, a known driver of gut dysbiosis, infants who received SRT exhibited microbial profiles more comparable to those of healthy controls, further supporting the role of respiratory status in shaping gut microbial composition as discussed above. In contrast to the observational cohort, the probiotic discovery cohort showed more pronounced differences in gestational age, birth weight, and clinical exposures between the CON and RDS groups, reflecting the inclusion of additionally recruited infants with typical RDS characteristics. Rather than representing a limitation, this profile may more accurately reflect the clinical population in whom probiotic intervention would realistically be considered. Furthermore, as fecal samples from this cohort were directly used for both candidate strain isolation and as the dysbiotic samples in the FIMM and FMT mouse models, the more pronounced

RDS characteristics of this group may have enhanced the translational relevance of the experimental findings by providing gut microbiota more representative of the target clinical population.

The candidate probiotic strains identified in this study were derived through direct comparison of gut microbiota between healthy term infants and those with RDS, with strains enriched in the healthy infant gut selected as isolation targets considering the disease-specific microbial context. Among the isolated species, *L. fermentum* SLAM_LAF05 and *B. longum* SLAM_BIL02 were selected based on their consistently superior performance across acid and bile tolerance and adhesion capacity, properties commonly evaluated to assess the probiotic potential of microorganisms in the gastrointestinal tract [55, 56]. *L. gasseri* isolates were not selected, as no single isolate demonstrated consistent performance across both criteria simultaneously. Whole-genome sequencing, recommended as a comprehensive approach for probiotic strain identification and characterization [57], further confirmed the presence of genes associated with key probiotic functions including stress resistance, EPS biosynthesis, and immune modulation, and both strains demonstrated antibiotic resistance profiles compliant with EFSA safety guidelines [57].

The biological relevance of these strains is supported by prior studies in neonatal contexts. *L. fermentum* has been shown to modulate intestinal immune responses in neonatal epithelial cells through TLR9 activation and reduction of pro-inflammatory IL-6 [58], while *B. longum* and related subspecies have demonstrated gut barrier reinforcement and attenuation of pro-inflammatory cytokines in preterm animal models [59]. In the present study, supplementation with LAF05 and BIL02 was associated with upregulation of ZO-1 and MUC2, tight junction and mucin proteins whose disruption is implicated in intestinal barrier dysfunction in preterm neonates [60, 61], alongside upregulation of Reg3g and reduction

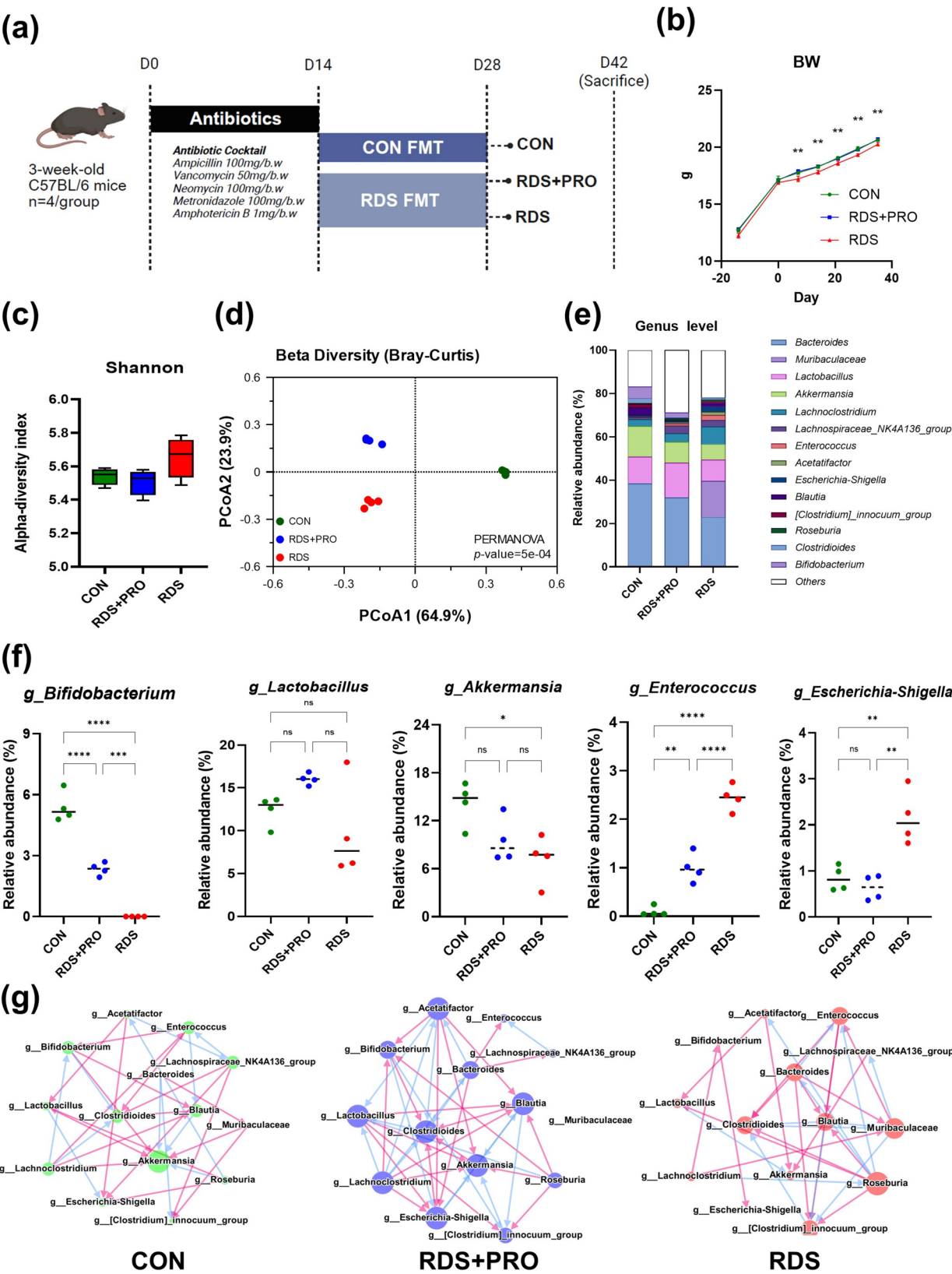


Fig. 6 (See legend on next page.)

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Fig. 6 Probiotic supplementation modulates gut microbiota in the FMT mouse model. **(a)** Experimental timeline of the FMT mouse model. **(b)** Body weight changes over the experimental period. **(c)** Alpha diversity assessed by the Shannon index. **(d)** Beta diversity visualized by PCoA based on Bray–Curtis dissimilarity; PERMANOVA $p=0.0005$. **(e)** Relative abundance of bacterial taxa at genus level. **(f)** Relative abundance of key genera: *Bifidobacterium*, *Lactobacillus*, *Akkermansia*, *Enterococcus*, and *Escherichia-Shigella*; **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. **(g)** Co-occurrence network analysis of microbial interactions in CON, RDS + PRO, and RDS groups; node size reflects relative abundance, pink lines indicate positive correlations, and blue lines indicate negative correlations. Microbiome relative abundance and body weight data are presented as mean $\pm$ SD. Alpha diversity index is presented as median (IQR). $n=4$ per group. ns, not significant; CON, control; RDS, respiratory distress syndrome; RDS + PRO, RDS group with probiotic supplementation; FMT, fecal microbiota transplantation; PCoA, principal coordinate analysis

of fecal calprotectin, which may reflect intestinal inflammation in preterm neonates [62, 63]. Improvement in serum IgG levels further suggests a probiotic-associated enhancement of systemic immune responses, which, in the context of the gut-lung axis, may represent a pathway through which microbiota-targeted intervention could contribute to immune defense relevant to neonatal respiratory disease [10, 64, 65]. Taken together, these findings propose *L. fermentum* SLAM_LAF05 and *B. longum* SLAM_BIL02 as candidate probiotic strains warranting further investigation in the context of RDS and its associated complications.

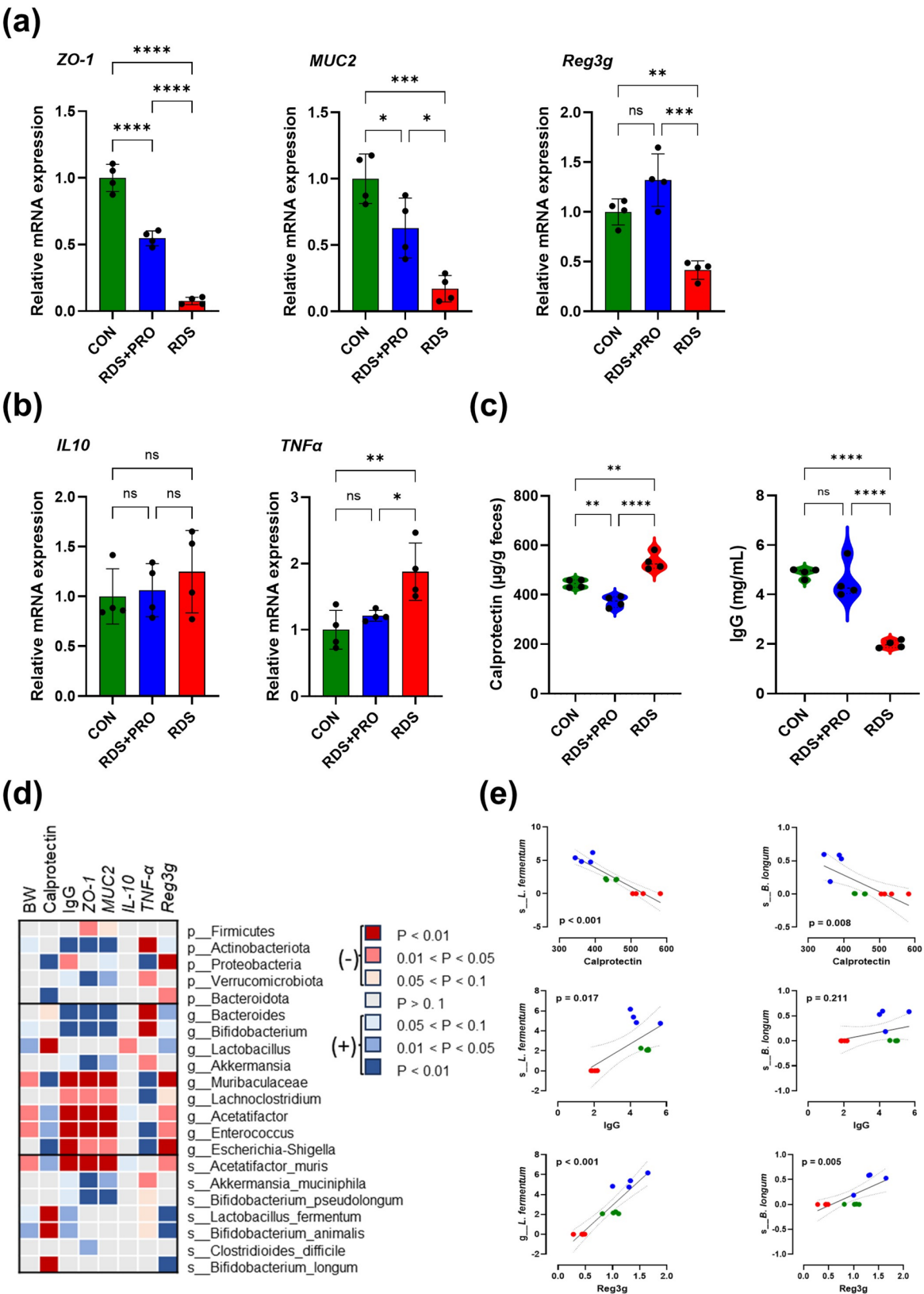
These findings suggest that gut microbiome alterations may represent a clinically relevant dimension of the pathophysiology of RDS. Microbiota-targeted approaches therefore warrant further exploration as a potential complementary strategy in the management of this population. The disease-specific approach to probiotic candidate identification employed in this study may provide a contextually grounded basis for future probiotic development. Further investigations are warranted in several directions. Longitudinal microbiome sampling encompassing the period before and after SRT would better delineate the temporal dynamics of gut microbial changes in relation to disease course and treatment response. Direct profiling of the lung microbiome via tracheal aspirate, feasible in intubated infants, would provide additional characterization of gut-lung axis dynamics in this population. Validation in larger, multi-center cohorts incorporating a broader range of perinatal variables would strengthen the generalizability of clinical observations. Finally, evaluation of the candidate probiotic strains in RDS-specific in vivo disease models with direct assessment of pulmonary outcomes and functional mechanistic pathways represents a critical step toward establishing the translational potential of this microbiota-targeted approach.

This study has several limitations. First, as a prospective study conducted in a real-world clinical setting, complete control of therapeutic interventions was not feasible. Oxygen therapy, antibiotic administration, and other clinical exposures are inherent to the clinical course of RDS and cannot be independently manipulated. To minimize the influence of postnatal exposures on gut microbial composition, fecal samples were collected within the first 48 h of life, representing the

earliest practicable sampling time point. Second, the observational design precludes causal inference, and the findings should be interpreted as associations rather than mechanistic relationships. Direct characterization of the lung microbiome was not performed, as tracheal aspirate sampling is only accessible in infants requiring invasive ventilation, limiting its application to a subset of the study population. Third, this study was conducted at a single center with a relatively small clinical cohort, and the absence of longitudinal sampling limits assessment of the temporal trajectory of gut microbial changes in relation to disease course and treatment. Fourth, the in vitro and in vivo experimental models were exploratory in nature, with small group sizes reflecting the hypothesis-generating scope of this work. Adequately powered studies with formal sample size calculations will be required in future investigations. Finally, respiratory outcomes and histological analysis were not evaluated in the present animal model, as the FMT mouse model was designed to assess the effects of candidate probiotic strains on RDS-associated gut microbiota rather than to replicate RDS disease pathophysiology. Dedicated RDS-specific in vivo models addressing pulmonary outcomes and functional mechanistic pathways will be necessary to advance this line of investigation.

Conclusions

This study represents an early translational effort to systematically characterize gut microbiome alterations in neonates with RDS and to evaluate the biological plausibility of microbiota-targeted intervention through a disease-specific probiotic approach. The identification of RDS-associated gut dysbiosis and its partial resolution following SRT underscores the potential clinical relevance of the gut-lung axis in neonatal respiratory disease. The derivation of candidate probiotic strains through direct comparison of gut microbiota between healthy and RDS-affected infants provides a disease-informed foundation that may complement existing approaches to neonatal microbiome support. Taken together, these findings offer a new perspective on the interplay between gut microbial dynamics and the early postnatal respiratory course and provide a basis for future investigations into microbiota-targeted strategies to support neonatal health.



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Fig. 7 Probiotic supplementation modulates gut barrier integrity and immune responses in the FMT mouse model. **(a)** Relative mRNA expression of gut barrier markers ZO-1, MUC2, and Reg3g; **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. **(b)** Relative mRNA expression of inflammatory cytokines IL-10 and TNF- α . **(c)** Fecal calprotectin levels ($\mu\text{g/g}$ feces) and serum IgG levels (mg/mL). **(d)** Spearman correlation heatmap illustrating associations between microbial taxa and host markers; red indicates negative correlation and blue indicates positive correlation, with color intensity reflecting statistical significance. **(e)** Linear regression plots showing correlations between the abundances of *L. fermentum* and *B. longum* and fecal calprotectin, serum IgG, and intestinal Reg3g expression; p values derived from Pearson's correlation test. Bar graphs represent mean $\pm$ SD with individual data points. Violin plots show full data distribution with individual data points. $n = 4$ per group. ns, not significant; CON, control; RDS, respiratory distress syndrome; RDS + PRO, RDS group with probiotic supplementation; FMT, fecal microbiota transplantation

Abbreviations

ANOVA	Analysis of variance
ASV	Amplicon sequence variant
AUC	Area under the curve
BHI	Brain heart infusion
BSH	Bile salt hydrolase
CDS	Coding sequence
CFU	Colony-forming unit
CON	Control
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EPS	Exopolysaccharide
FIMM	Fermenter for intestinal microbiota model
FMT	Fecal microbiota transplantation
GDM	Gestational diabetes mellitus
IgG	Immunoglobulin G
IL-10	Interleukin-10
KEGG	Kyoto Encyclopedia of Genes and Genomes
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
MIC	Minimum inhibitory concentration
MRS	De Man, Rogosa & Sharpe
MUC2	Mucin 2
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PCoA	Principal coordinate analysis
PICRUSt2	Phylogenetic investigation of communities by reconstruction of unobserved states 2
PIH	Pregnancy-induced hypertension
PERMANOVA	Permutational multivariate analysis of variance
PROM	Premature rupture of membranes
qRT-PCR	Quantitative real-time PCR
RDS	Respiratory distress syndrome
Reg3g	Regenerating islet-derived protein 3 gamma
ROC	Receiver operating characteristic
SD	Standard deviation
SP	Surfactant protein
STAMP	Statistical analysis of metagenomic profiles
SRT	Surfactant replacement therapy
TNF- α	Tumor necrosis factor-alpha
VFDB	Virulence factor database
ZO-1	Zonula occludens-1

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12967-026-08462-x>.

Supplementary Material 1

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Author contributions

E.S. Conceptualization, Data curation, Investigation, Methodology, Validation, Visualization, Writing - original draft. S.H.K. Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. M.-J.K. Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. J.K.H. Data

curation, Visualization, Writing - review & editing. G.M.: Data curation, Investigation, Resources, Validation. Y.S.C. Supervision, Writing - review & editing. J.-K.H. Supervision, Writing - review & editing. Y.K.: Investigation, Supervision, Funding acquisition, Writing - review & editing. B.-H.J. Data curation, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing - review & editing. H.-K.P. Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing - review & editing. All authors have full access to all the data in the study and accept responsibility to submit for publication.

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Data availability

The raw sequence data are accessible through NCBI's Sequence Read Archive, under the BioProject ID PRNJA1234853 (Gut microbiome), and PRNJA1277480 (FIMM and FMT mouse). This study did not generate custom code. Analysis were performed using established software and packages.

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Hanyang University Medical Center (IRB No. 2023-04-047). Written informed consent to participate in this study was obtained from the parents or legal guardians of all enrolled infants. All methods were performed in accordance with the principles of the Declaration of Helsinki and relevant guidelines and regulations. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC No. SNU-250108-3) of the College of Pharmacy, Seoul National University.

Consent for publication

Written informed consent for publication was obtained from the parents or legal guardians of all enrolled infants.

Competing interests

The authors have no conflicts of interest to declare.

Author details

¹Department of Agricultural Biotechnology and Research Institute of Agriculture and Life Science, Seoul National University, Seoul 08826, Republic of Korea

²Department of Pediatrics, Hanyang University College of Medicine, Seoul 04763, Republic of Korea

³Department of Pediatrics, Samsung Medical Center, Sungkyunkwan University School of Medicine, 06351 Seoul, Republic of Korea

⁴Department of Forest Products and Biotechnology, Kookmin University, 02707 Seoul, Republic of Korea

⁵Department of Earth Resources and Environmental Engineering, Hanyang University, Seoul 04763, Republic of Korea

⁶Department of Obstetrics and Gynecology, Hanyang University College of Medicine, 04763 Seoul, Republic of Korea

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