

SUPPLEMENTARY MATERIALS

SUPPLEMENTARY MATERIALS AND METHODS

HUMAN STUDIES

Collection of fecal samples from Bangladeshi cohorts

Fecal samples from healthy/non-wasted children that were used for qPCR assays and for culturing bifidobacterial strains were collected during the course of three studies that had been conducted at the icddr,b: (i) the MAL-ED birth cohort study of children aged 0-24 months (Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development; ClinicalTrials.gov identifier NCT02441426) (10); (ii) a cohort of healthy 12-24 month-old children enrolled in parallel with children with MAM as part of a pre-POC study of microbiota-directed complementary food (MDCF) prototypes (ClinicalTrials.gov identifier NCT03084731; ref. 11) and (iii) the NIH Birth Cohort Study (Field Studies of Amebiasis in Bangladesh; ClinicalTrials.gov identifier NCT02734264) (9,10). Two to 24-month-old infants/children with SAM were sampled prior to intervention in two icddr,b studies; (i) Development and Field Testing of Ready-to-Use-Therapeutic Foods Made of Local Ingredients in Bangladesh for the Treatment of Children with SAM (ClinicalTrials.gov Identifier, NCT01889329; ref. 11), and (ii) the SYNERGIE study described above. The research protocols for each of these studies were approved by the Ethical Review Committee at the icddr,b. Informed consent was obtained from the mother/guardian of each child. Use of biospecimens from each of the human studies for the analyses described in this report was approved by the Washington University Human Research Protection Office.

In the case of the completed NIH Birth Cohort and MAL-ED studies, fecal samples were placed in a cold box with ice packs within 1 hour of production by the donor and collected by field workers for transport back to the laboratory storage facility. For the 'Development and Field Testing of Ready-to-Use-Therapeutic Foods Made of Local Ingredients in Bangladesh for the Treatment of Children with SAM' study, the MDCF study healthy reference cohort, and the SYNERGIE trial, samples were flash frozen in liquid nitrogen-charged dry shippers (model CX-100, Taylor-Wharton) shortly after their production by the infant or child. Biospecimens were subsequently transported to the research laboratory, transferred to -80 °C freezers within 8 hours of collection and shipped on dry ice to Washington University School of Medicine in St. Louis where they were archived in a dedicated biospecimen repository at -80 °C.

V4-16S rRNA gene amplicon sequencing

SYNERGIE trial - DNA was extracted from fecal swab samples using the ZymoBIOMICS 96 MagBead DNA kit (Zymo Research). Extracted DNA was quantified using QuantIT dsDNA Assay kit, high sensitivity (ThermoFisher Scientific) according to the manufacturer's protocol. Variable region 4 of the 16S rRNA gene was amplified using barcoded 515F and 806R primers. Barcoded amplicons were sequenced (Illumina MiSeq, paired-end 250 nt reads). Datasets generated from the three treatment groups were demultiplexed, denoised, and amplicon sequence variants (ASVs) were identified using DADA2 (56). After merging, ASVs underwent taxonomic analysis using a pre-trained Naive Bayes classifier supplied by QIIME2 (v2019.7). The classifier was trained on the Greengenes 13_8_99% OTUs, trimmed to contain only the V4 region. MaAsLin 2 was used to compare the abundance of bacterial taxa between treatment groups (57).

Other Bangladeshi cohorts – Fecal samples were pulverized in liquid nitrogen and DNA extraction was performed by bead-beating with 500 µL of 0.1 mm diameter zirconia/silica beads in 500 µL phenol:chloroform:isoamyl alcohol (25:24:1), 210 µL 20% SDS, and 500 µL buffer A (200 mM NaCl, 200 mM Trizma base, 20 mM EDTA). Purified DNA (Qiaquick columns, Qiagen) was eluted in 70 µL Tris-EDTA (TE) buffer, quantified (Quant-iT dsDNA broad range kit; Invitrogen) and subjected to PCR and V4-16S rRNA gene amplicon sequencing as described above.

Quantification of gut inflammatory biomarkers

Calprotectin and Lipocalin-2 (LCN-2/NGAL) were quantified from 80 mg of feces diluted 1:10 in Meso Scale Discovery diluent using R-PLEX (Meso Scale Discovery, Rockville, MD). Fecal cytokine levels (IFN- β , IL-17A, IL-1 β and IL-6) were quantified using the U-Plex Inflammation Panel 1 Kit (human) according to the manufacturer's instructions. Plates were read on a Sector Imager 2400 using MSD Discovery Workbench analysis software. Standards and samples were measured in duplicate and blank values were subtracted from all readings. Assays were performed at least twice. Myeloperoxidase (MPO) was measured using commercially available ELISA kits (Alpco, Salem NH). A Kruskal-Wallis test with FDR correction was used to assess the effects of the different interventions on levels of biomarkers of gut inflammation at each study timepoint.

***In silico* reconstruction of HMO and non-HMO carbohydrate utilization pathways in *B. infantis* strains cultured from Bangladeshi donors**

Culturing - Fecal samples, collected from 6-24-month-old Bangladeshi children who had been enrolled in the MDCF, MAL-ED and SAM clinical studies (see table S3A for the origin of each isolate), were pulverized in liquid nitrogen and a ~0.1 g aliquot of each sample was transferred to an anaerobic Coy chamber (Coy Laboratory Products; atmosphere of 75% N₂, 20% CO₂, and 5% H₂). Samples were diluted 1:10 (wt/vol) with reduced PBS (PBS/ 0.05% L-cysteine-HCl) in 50 mL conical plastic tubes containing 5 mL of 2 mm-diameter glass beads (VWR). Tubes were gently vortexed and the resulting slurry was passed through a 100 μ m-pore diameter nylon cell strainer (BD Falcon). A 500 μ L aliquot of each clarified fecal sample was added to 4.5 mL of PBS and a 1:10, 1:100, 1:1000, and 1:10,000 dilution series was prepared in PBS. LYBHI (brain-heart infusion medium supplemented with 0.5% yeast extract) agar plates were streaked with 100 μ L of each dilution. Plates were incubated for 2-3 days at 37 °C under anaerobic conditions. Colonies were picked into 96 deep-well plates (Thermo Fisher Scientific) containing 600 μ L of Wilkins-Chalgren broth and incubated overnight at 37 °C. (Isolate stocks were prepared by combining 50 μ L of culture with 50 μ L of PBS/30% glycerol in shallow 96-well plates. Stocks were then frozen at -80 °C for future use). A 500 μ L aliquot of each culture was transferred to 2 mL screw cap tubes and pelleted by centrifugation. The resulting supernatant was discarded and DNA was extracted from pellets with phenol:chloroform. V4-16S rRNA gene amplicons were generated by PCR and sequenced (Illumina MiSeq; paired-end 250 nt reads). Clonal isolates whose V4-16S rRNA gene amplicon sequences shared \geq 97% sequence identity with bifidobacteria were subjected to full-length 16S rRNA gene sequencing using primers 8F and 1391R (58).

Identification of unique strains by genome sequencing - Cryopreserved stocks of bacteria were brought into the Coy chamber, struck onto MRS agar plates for single colonies, incubated overnight at 37 °C under anaerobic conditions and replated on MRS-agar. A single colony was picked into 6 mL of MRS broth and incubated at 37 °C to late log phase. Genomic DNA was isolated from cell pellets (11), libraries were prepared for shotgun sequencing (TruSeq Nano DNA Library Prep Kit, Illumina). Libraries were pooled and multiplex sequencing was performed (Illumina Nextseq instrument; paired-end 150 bp reads). Raw reads were demultiplexed (bc12fastq) and pre-processed to remove low-quality bases and reads (trim_galore, v0.4.5). Quality-controlled reads were then subsampled to a depth of ~100-fold coverage using bbtools (v38.26). Paired-end reads corresponding to each genome were assembled using Spades with the careful option (v3.13.0). Isolates sharing \geq 99% nucleotide sequence identity in their full length 16S rRNA genes and \leq 96% nucleotide sequence identity throughout their genomes [NUCmer (59)] were defined as unique strains.

Pacbio and Illumina hybrid assemblies - Cryopreserved stocks of organisms for long-read sequencing/assembly were struck onto Brain Heart Infusion (BHI) medium and incubated overnight. Single colonies were picked, inoculated into 6 mL of liquid MRS medium, and incubated for 2 days. Turbid cultures were transferred to 15 mL conical tubes and pelleted by centrifugation. DNA was recovered using a high molecular weight genomic DNA extraction kit (MagAttract HMW, Qiagen). Purified DNA was prepared for long-read sequencing using the SMRTbell Template Prep Kit (v1.0,

PacBio) and Barcoded Adapter Kit (v8A, PacBio) and whole genome sequencing was performed [PacBio Sequel System; read length, 3681±861 (mean±SD) nt]. Sequencing reads were demultiplexed and converted from raw bam to fastq format (SMRT Tools software, v5.1.0 or 6.0.0). Short reads generated from the Illumina sequencer and long reads for each isolate were co-assembled using Unicycler (v0.4.7). For both short-read and hybrid assemblies, assembly quality statistics were generated using Quast (v4.5). Open reading frames were identified and annotated using Prokka (v1.12). Additional functional annotation was added based on homology to entries in the microbial community SEED (mcSEED) database (11,32).

In silico reconstructions and phenotype predictions - Subsystems-based, context-driven functional assignments of genes, curation and reconstruction of bifidobacterial carbohydrate metabolic pathways were performed in the web-based mcSEED environment, a private clone of the publicly available SEED platform (33). The mcSEED platform includes: (i) 336 genomes representing 15 species of bifidobacteria isolated from the human gut and (ii) a collection of curated subsystems capturing utilization of mono-, oligo-, polysaccharides and other carbohydrates in bifidobacteria. Data on functional roles (transporters, glycoside hydrolases, catabolic enzymes, transcriptional regulators) involved in bifidobacterial sugar metabolism were collected by extensive literature searches using PaperBLAST (60), and by exporting information from the Carbohydrate Active Enzyme (CAZy) database (61), Transporter Classification (TCDB) (62) and RegPrecise (63) databases. Reconstruction of regulons and prediction of transcription factor binding sites was performed as described previously (64).

Pan-genome analysis - A pan-genome analysis of the five *B. infantis* strains used in the gnotobiotic mouse experiments was performed using Roary 3.12.0 (65). Genomes in the gff format with mcSEED-derived annotations were inputted and the pangenome with a 95% minimum percentage identity cut-off for Blastp (66) was generated using the following command:

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roary -p 16 -e -n -i 95 -f 95 percent *.gff
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A total of 267 genes unique to *B. longum* subsp. *infantis* Bg40721_2D9_SN_2018 were manually screened and genomic clusters corresponding to carbohydrate metabolism were identified.

***In vitro* growth studies**

B. infantis strains were streaked from frozen stocks onto BHI blood agar plates which were incubated for 48 hours at 37 °C under anaerobic conditions. Three colonies of each strain were used to generate three individual overnight monocultures cultures in 1 mL of low-carbohydrate minimal MRS medium (lcMRS) in a 96-well plate. 10 mL of an aqueous stock solution of filter-sterilized 10% (w/v) glucose was added to 40 mL of lcMRS to make lcMRS + glucose medium. 50 µL of the culture was added to 1 mL of lcMRS + glucose medium in a 96-well plate and these subcultures were incubated under anaerobic conditions for 16 hours at 37 °C. The OD₆₀₀ of the subcultures was then recorded and each subculture was adjusted to an OD₆₀₀ of 0.3 in lcMRS + glucose broth in a fresh 96-well plate. Lactose or different HMOs [lacto-N-tetraose (LNT; Evolve Biosystems), lacto-N-neotetraose (LNnT; Glycom A/S), 2'fucosyllactose (2'FL; Glycosyn), 3'sialyllactose (3'SL; Genechem) and 6' sialyllactose (6'SL; Genechem)] were dissolved in distilled water at 100 g/L and filter sterilized. A 30 µL aliquot of each HMO stock solution was added to 120 µL of lcMRS and mixed (final HMO concentration 2% w/v). 5 µL of the OD₆₀₀ standardized subcultures were used to inoculate the lcMRS + carbohydrate medium into 96-well plates. Growth at 37 °C under anaerobic conditions was monitored over 30 hours by measuring OD₆₀₀ every 15 minutes using a Gen5 Microplate Reader (Biotek). Each experiment was conducted with three biological replicates. The significance of observed differences in OD₆₀₀ values at 30 hours were calculated using a one-way ANOVA with a Dunnett's post hoc test (using strain EVC001 as the reference control group).

GNOTOBIOLIC MOUSE EXPERIMENTS

Construction of the Mirpur-6 diet

Based on extensive knowledge of Bangladeshi complementary feeding practices, including quantitative 24-hour dietary recall surveys conducted at the Mirpur site, a 'Mirpur-6 diet' was prepared by Dyets, Inc. (Bethlehem, PA) to be representative of the contribution of milk and complementary foods consumed by 6-month-old infants living in this locale. Rice (parboiled, long grain) and red lentils (masoor dal) were each cooked separately with an equal weight of water at 100 °C in a steam-jacketed kettle until partially cooked (still firm) and then set aside. Market fresh potatoes, spinach and yellow onions were washed, chopped in a vertical cutter mixer and cooked in the kettle without added water at 70 °C until soft. Sweet pumpkin (Calabaza variety) was chopped and boiled in the steam-jacketed kettle until soft and then strained. At this point, all of the cooked ingredients were combined, whole bovine milk powder (Franklin Farms East, Bethlehem, PA), soybean oil, salt, turmeric and garlic were added and the resulting diet was mixed extensively and allowed to cool (see **table S4A** for the proportions of each ingredient). Diets were dried on trays overnight at 30 °C, and pelleted by extrusion (½" diameter; California Pellet Mill, CL5). Dried pellets were weighed into 250g portions, placed in a paper bag with an inner wax-lining which, in turn, was placed in a plastic bag. The plastic bag was vacuumed sealed and its contents were sterilized by gamma irradiation (30-50 kGy; Sterigenics). Sterility was confirmed using culture-based assays as described in ref. 11. Nutritional analysis of the diet was performed (Nestlé Purina Analytical Laboratories) (**table S4B**).

Colonization of mice

In vivo competition between B. infantis strains in gnotobiotic mice - The four *B. infantis* strains that had been isolated from Bangladeshi children were combined with EVC001 prior to gavage. For one arm of the experiment, this consortium of *B. infantis* strains was supplemented with a *B. bifidum* strain recovered from a 12-month-old healthy Bangladeshi child (*Bifidobacterium bifidum*_41221_3D10; **table S3A**). Frozen stocks of the cultured strains were thawed inside the anaerobic Coy chamber and 100 µL of the stock was spread on agar plates containing MRS agar and 0.05% L-cysteine-HCl. Plates were incubated at 37 °C under anaerobic conditions for 48 h. Single colonies were handpicked and transferred into 5 mL of MRS broth. Liquid cultures were subsequently incubated at 37 °C under anaerobic conditions for 24 h, after which time a 100 µL aliquot was withdrawn to measure OD₆₀₀. All monocultures were then normalized to the lowest OD₆₀₀ among the strains (0.6) and equal volumes of each organism were pooled. Glass crimp vials (Wheaton) were filled with 800 µL of 1:1 mixture of sterile PBS/30% glycerol/0.05% L-cysteine hydrochloride and the pooled strains, sealed and immediately stored in -80 °C until use (within a week).

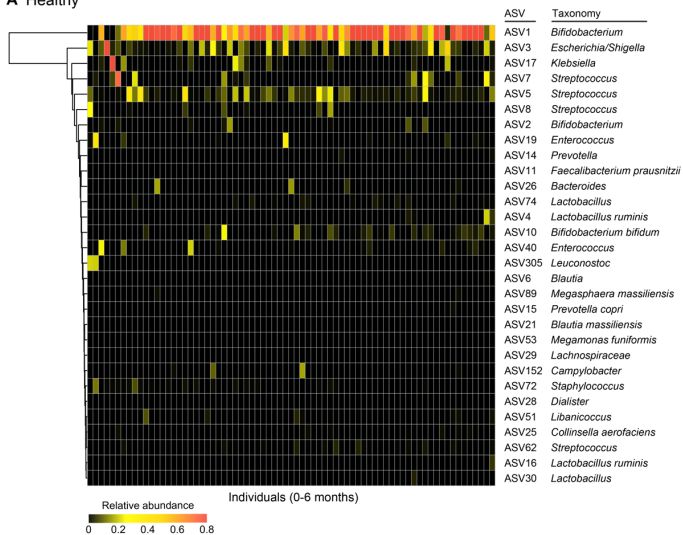
Five-week-old germ-free male C57BL/6 mice, (bred in our gnotobiotic facility and whose feces are collected and cultured under aerobic and anaerobic growth conditions prior to each experiment to confirm absence of contamination) were fed the Mirpur-6 diet for 2 days prior to gavage with a defined consortium; this was followed by a second gavage of the same consortium two days later. Fecal specimens were collected every 48 hours from all animals in all treatment groups. Throughout the experiment, all animals in each treatment group (n=3-4 mice/cage; 6-7 animals/group) were provided the Mirpur-6 diet *ad libitum*. Mice received autoclaved water with or without LNT or LNnT: the dose of LNT or LNnT was equivalent to that consumed if the Mirpur-6 diet had been supplemented with 12.5 g/L (1.25%) HMO. Non-fasted animals were euthanized on experimental day 28.

Intergenerational model of B. infantis strain transmission - A fecal sample, collected prior to intervention from a 5-month-old child in the SYNERGIE trial, was flash frozen in liquid nitrogen and maintained at -80°C prior to shipping to Washington University in St. Louis (see **table S2E** for bacterial community composition of the SAM microbiota prior to intervention on study day 1). After pulverization, a 100 mg aliquot was clarified in 6 mL of PBS/15% glycerol 0.025% L-cysteine hydrochloride using a 40 µm nylon cell strainer (BD Falcon, USA) and stored at -80° C. Four 15-week-old germ-free dams (n=two mice per cage; 2 cages) that had been fed breeder chow (LabDiet® 5021; PMI Nutrition International,

LLC) prior to parturition were switched to the Mirpur-6 diet on postpartum day 2 and gavaged with the microbiota sample from the SAM donor on postpartum days 4 and 7. On postpartum day 11, one cage with two dams and 12 pups was transferred to a second isolator. Separate MRS broth cultures of *B. infantis* Bg_2D9 and EVC001 grown at 37 °C under anaerobic conditions were adjusted to $OD_{600}=0.7$ and mixed at a 1:1 ratio just prior to gavage of dams in the second isolator on postpartum days 12 and 14. Pups were fully weaned on postnatal day 21 (P21). Between group differences in pup weight over time were assessed for statistical significance using a two-way repeated measures ANOVA followed by Šidák's multiple comparison test. Fecal samples were collected from the mice on P21, P28, and P35. DNA was isolated and bacterial community composition was assessed by sequencing of PCR amplicons generated from variable region 4 of 16S rRNA genes using the Illumina MiSeq instrument [$16,258.2 \pm 2,170.4$ (mean \pm SD) 250 nt paired end reads/sample]. Data were processed and analyzed with the DADA2 pipeline and Phyloseq package in R (see above). qPCR based determination of the absolute abundances of *B. infantis* Bg_2D9 and EVC001 was performed using fecal DNA and strain specific primers (*nglA* and *epsJ* respectively) as detailed above except TaqMan Universal Master Mix II containing uracil N-glycosylase (UNG, Applied Biosystems) was used and the assays were run in single-plex (10 μ L total reaction volume). A two-tailed Wilcoxon matched-pairs signed rank test was used to determine the statistical significance of differences in the abundances of EVC001 and Bg_2D9 at each timepoint.

SUPPLEMENTARY FIGURES

A Healthy



B SAM

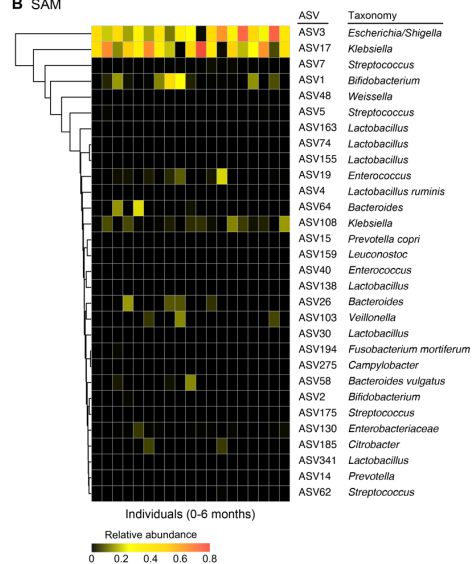
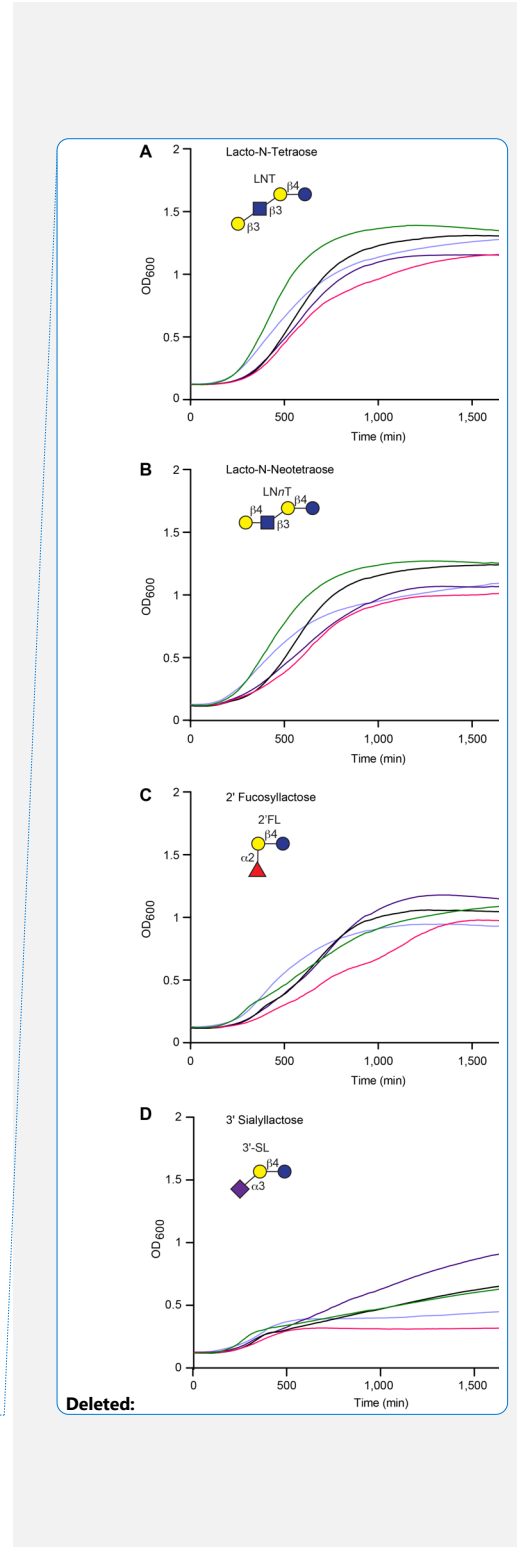
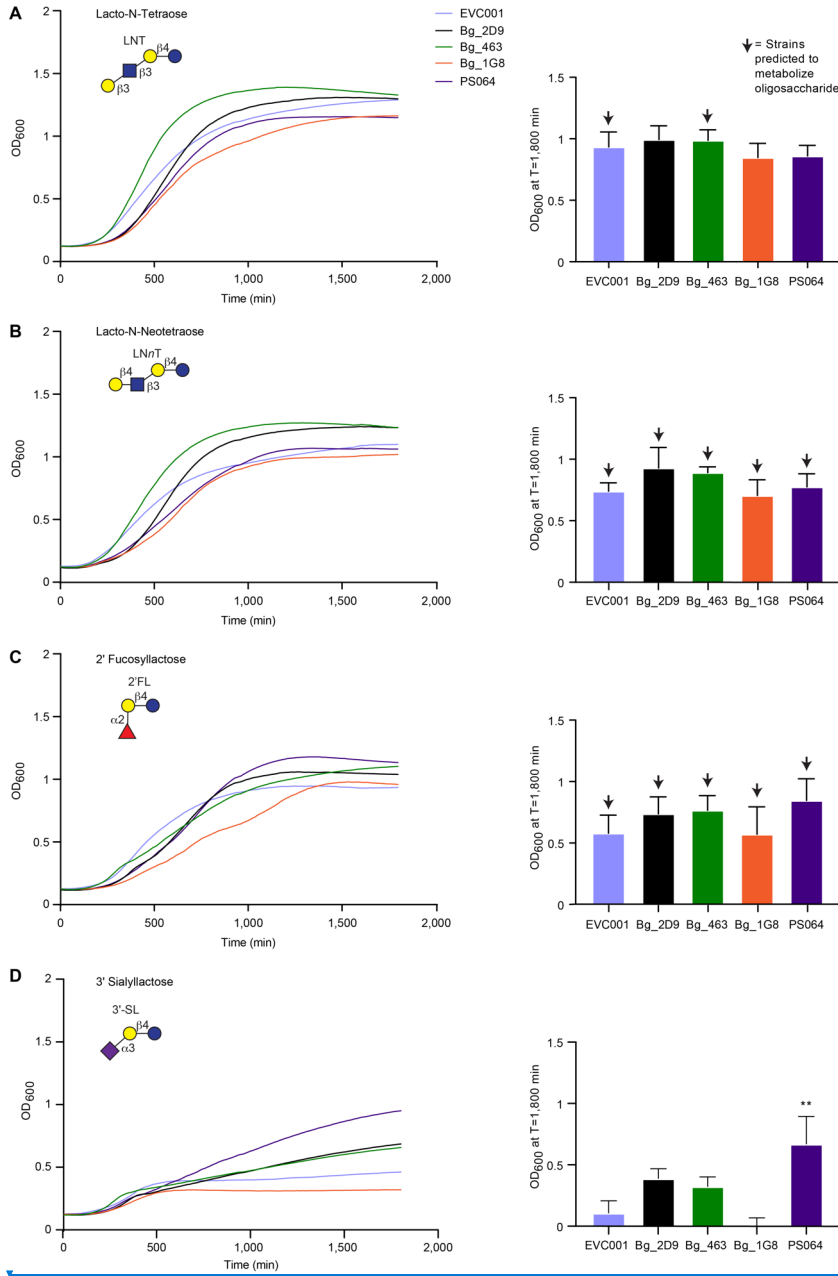


fig. S1. Relative abundances of the 30 most abundant Amplicon Sequence Variants (ASVs) in the fecal microbiota of Bangladeshi infants who exhibited healthy growth (A) or who had SAM (B). Each column represents an individual participant.



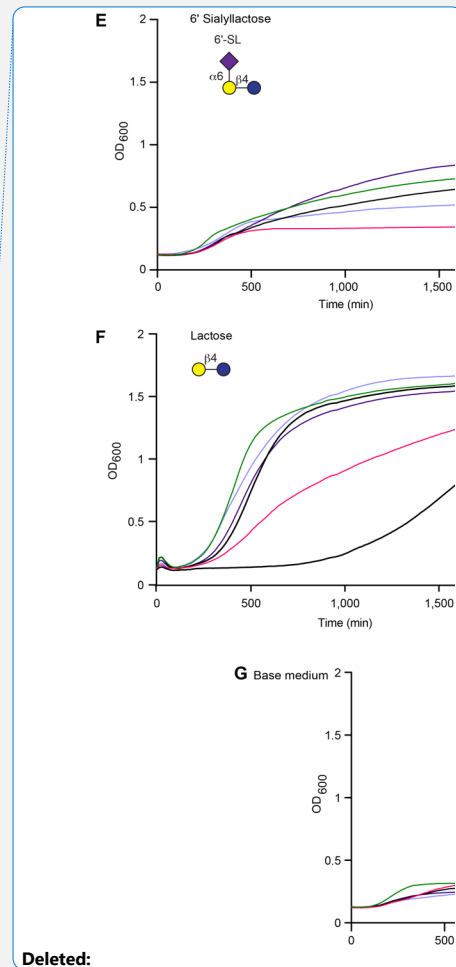
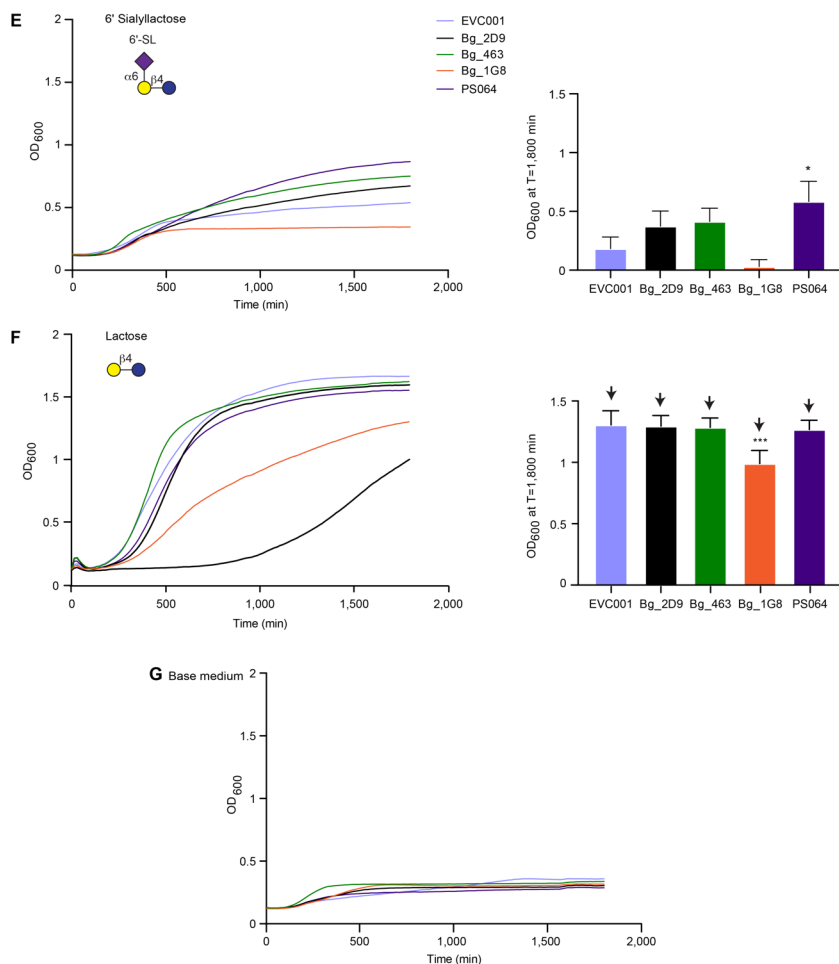


fig. S2. *In vitro* growth phenotypes of *B. infantis* strains in defined low-carbohydrate MRS medium in the presence or absence of different HMOs. (A-F) Growth as a function of the indicated carbon source. Left panels present growth curves over the 30-hour period of sampling; panels on the right indicate OD₆₀₀ after 30 hours of culture (n=3 cultures/condition). (G) Growth of the different strains over 30 hours in the absence of HMO supplementation. Downward pointing arrows indicate strains that are predicted to utilize a given HMO (based on the presence of a corresponding transporter and requisite metabolic enzymes). Results are representative of N=3 independent experiments each performed in triplicate. Mean values ± SD are plotted in the right panels. *, P<0.05 (one-way ANOVA with a Dunnett's post hoc test, using EVC001 as the comparator).

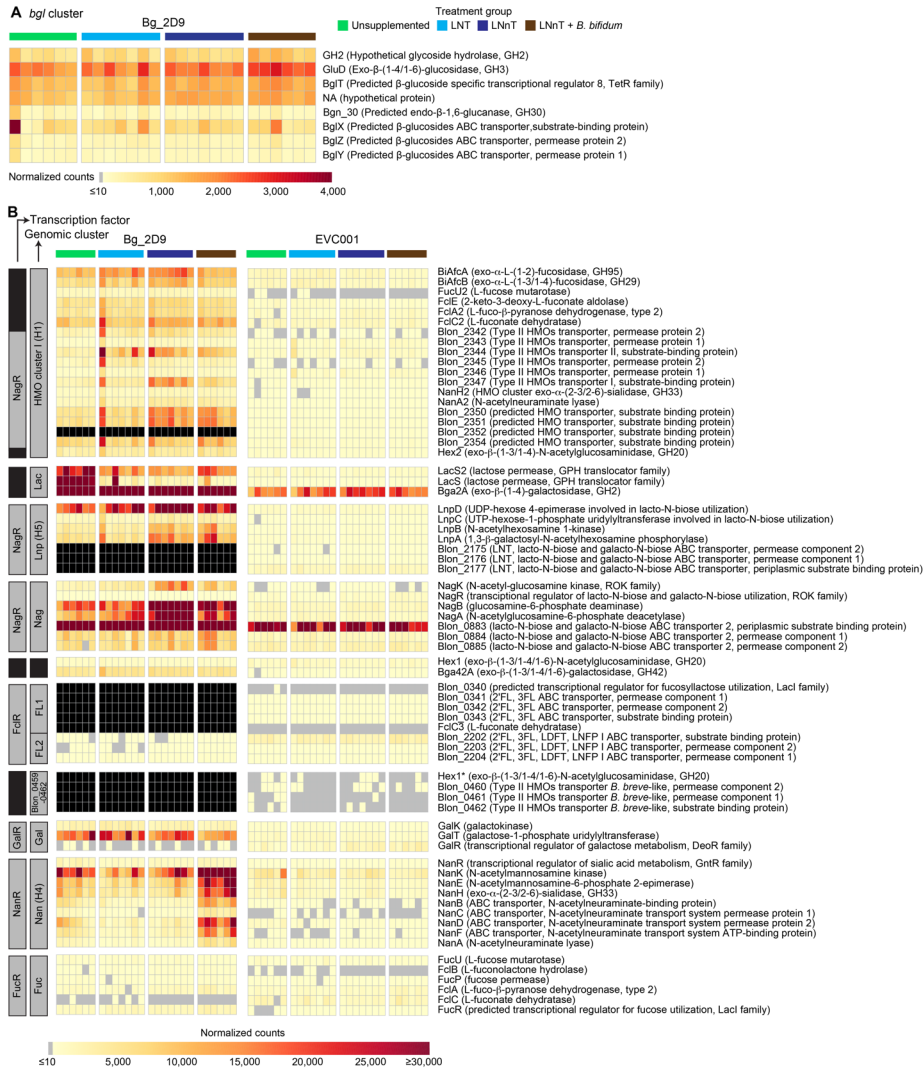


fig. S3. Expression of *bgl* cluster and HMO utilization genes in *B. infantis* Bg_2D9 and EVC001. Mice fed either Mirpur-6, Mirpur-6 + 1.25% LNT (in their drinking water) or Mirpur-6 + 1.25% LNT were colonized with the consortium of five *B. infantis* strains. In the fourth arm, *B. bifidum* was included in the consortium and mice were given drinking water supplemented with 1.25% LNT. (A,B) Heatmaps show DESeq2-normalized transcript counts mapped to genes in the *bgl* cluster in Bg_2D9 and EVC001, in each mouse in each treatment group (panel A), and to genes involved in HMO utilization (panel B). Each column represents an individual animal (n=6-7 mice/group; N=1 experiment). Black shading in the

left-most column indicates that a transcription factor has not been defined for the corresponding gene. Black colored cells in the heatmap indicate absence of an orthologous gene, while cells colored grey denote low levels of expression of the gene (≤ 10 DESeq2-normalized read counts).

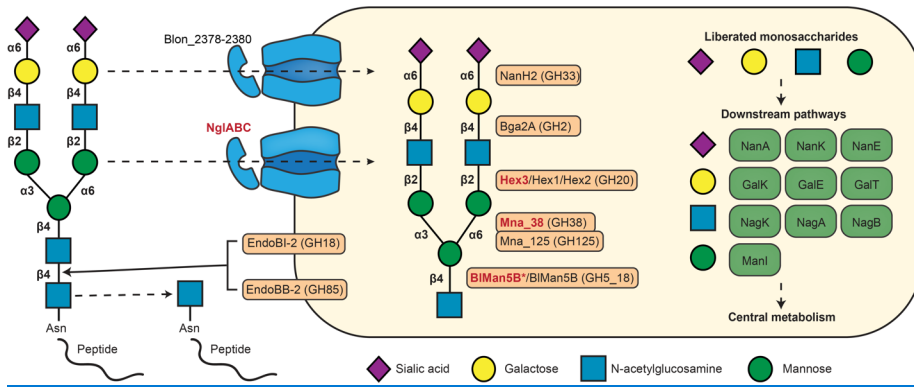


fig. S4. Proposed scheme for *N*-glycan utilization by *B. infantis* Bg 2D9. Breakdown of glycosidic linkages by specific glycoside hydrolases (GHs) is indicated in orange boxes. Proteins encoded by genes in the *ngl* cluster are highlighted in red.

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SUPPLEMENTARY TABLES

table S1. Metadata related to infants/children whose fecal samples were used for quantification of the abundance of *B. infantis*, all bifidobacteria and other bacterial taxa, and the design of qPCR primers for the studies described in Figs. 2, 3, 4 and 6. (A) Clinical characteristics of donors and quantification of bifidobacterial genes in their fecal samples by qPCR. (B) Characteristics of qPCR primers and probes used in this study. (C) Specificity of the qPCR assays. (D) Relative abundance of 16S rRNA gene amplicon sequence variants in fecal samples from which bifidobacterial gene qPCR data was generated.

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table S2. SYNERGIE clinical trial evaluating engraftment of *B. infantis* strain (EVC001) administered with or without LNnT to infants with SAM. (A) CONSORT flow diagram. (B) Supplements used in the study. (C) Clinical metadata for study participants. (D) Socio-demographic, anthropometric and clinical characteristics of infants assigned to the three intervention groups. (E) Fecal V4-16S rRNA gene amplicon sequencing data aggregated from children in each of the three treatment arms over the course of the trial. (F) Effect of the interventions on weight-for-age z-score (WAZ) and mid-upper arm circumference (MUAC). (G) Effect of the interventions on intestinal biomarkers of inflammation and their correlation with levels of *B. infantis*.

table S3. Bifidobacterial strains used in the *in vitro* growth and gnotobiotic mouse experiments and their predicted capacities to (i) synthesize amino acids, vitamins and cofactors and (ii) utilize HMOs and other carbohydrates. (A) Origin of bifidobacterial strains and genome assembly characteristics. (B) Predicted capacity to synthesize amino acids. (C) Predicted capacity to produce vitamins and cofactors. (D) Predicted capacity to utilize various carbohydrates. (E) Representation of HMO transporters. (F) Representation of glycoside hydrolases involved in HMO utilization. (G) Representation of glycoside hydrolases and transporters involved in *N*-glycan utilization. (H) Representation of glycoside hydrolases and transporters in the *bgl* cluster. (I) Representation of *ngl* and *bgl* loci in 336 bifidobacterial genomes.

table S4. Mirpur-6 diet used in the gnotobiotic mouse experiments described in Fig. 4 and Fig. 6. (A) Composition. (B) Nutritional analysis.

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table S5. Body weights, absolute abundances of *B. infantis* strains and relative abundances of ASVs in the fecal microbiota and microbial RNA-Seq datasets generated from the cecal contents of gnotobiotic mice in the experiments described in Fig. 4A and Fig. 6B. (A) Body weights (grams) (refers to mice in Fig. 4A). (B) Absolute abundances of *B. infantis* strains (genome equivalents/mg feces) in the fecal microbiota collected from mice in each of the experimental groups over the course of the experiment (Fig. 4A). (C) Log2-fold changes in the expression of genes involved in glycan utilization in each of the *B. infantis* strains as a result of LNT or LNnT supplementation of the Mirpur-6 diet (Fig. 4A). (D) Body weights of pups whose mothers had been colonized with intact SAM microbiota with or without *B. infantis* strains Bg_2D9 and EVC001 (Fig. 6B,C). (E) Fractional abundance of amplicon sequence variants (ASVs) in fecal samples obtained from dams/pups colonized with intact SAM microbiota, with or without *B. infantis* Bg_2D9 and EVC001 (Fig. 6B).

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table S6. DESeq-normalized transcript counts mapped to individual strains in each of the mice in each treatment group in the experiment described in Fig. 4A.

table S7. Predicted transcription factor binding sites in the promoter regions of genes involved in glycan utilization in *B. infantis* strains introduced into gnotobiotic mice in the experiment described in Fig. 4A.

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