GUT MICROBIOTA

Bifidobacterium infantis treatment promotes weight gain in Bangladeshi infants with severe acute malnutrition

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Disrupted development of the gut microbiota is a contributing cause of childhood malnutrition. *Bifidobacterium longum* subspecies *infantis* is a prominent early colonizer of the infant gut that consumes human milk oligosaccharides (HMOs). We found that the absolute abundance of *Bifidobacterium infantis* is lower in 3- to 24-month-old Bangladeshi infants with severe acute malnutrition (SAM) compared to their healthy age-matched counterparts. A single-blind, placebo-controlled trial (SYNERGIE) was conducted in 2- to 6-month-old Bangladeshi infants with SAM. A commercial U.S. donor–derived *B. infantis* strain (EVC001) was administered daily with or without the HMO lacto-*N*-neotetraose for 28 days. This intervention increased fecal *B. infantis* abundance in infants with SAM, although to levels still 10- to 100-fold lower than in untreated healthy controls. EVC001 treatment promoted weight gain that was associated with reduced intestinal inflammation markers in infants with SAM. We cultured fecal *B. infantis* strains from Bangladeshi infants and colonized gnotobiotic mice with these cultured strains. The gnotobiotic mice were fed a diet representative of that consumed by 6-month-old Bangladeshi infants, with or without HMO supplementation. One *B. infantis* strain, Bg_2D9, expressing two gene clusters involved in uptake and utilization of *N*-glycans and plant-derived polysaccharides, exhibited superior fitness over EVC001. The fitness advantage of Bg_2D9 was confirmed in a gnotobiotic mouse model of mother-to-infant gut microbiota transmission where dams received a pretreatment fecal community from a SAM infant in the SYNERGIE trial. Whether Bg_2D9 is superior to EVC001 for treating malnourished infants who consume a diet with limited breastmilk requires further clinical testing.

INTRODUCTION

The global burden of childhood undernutrition is great, causing 3.1 million deaths annually and accounting for 21% of life years lost among children younger than 5 years (*1*). More than 18 million children in this age range are affected by severe acute malnutrition (SAM), the most extreme form of undernutrition. SAM is responsible for nearly half of all undernutrition-related mortality; it is postulated to be caused by a number of factors, including low dietary energy intake and nutrient loss due to infection (*2*). SAM is manifest by markedly impaired ponderal growth (wasting) and is defined clinically by a mid-upper arm circumference (MUAC) of less than 11.5 cm and/or a weight-for-length (height) score that is greater than 3 SD below the mean value for a multinational World Health Organization (WHO) cohort of infants and children (WLZ < −3). SAM is associated with an enteropathy manifest as disruption of epithelial tight junctions and increased gut permeability, leading to intestinal and systemic inflammation (*3*). Four million children under 6 months of age are affected by SAM; they are particularly vulnerable, with increased immediate risk of infection and death, as well as long-term impairment of growth and cognitive development (*4*, *5*). Current management approaches include treatment with broad-spectrum antibiotics and nutritional supplementation with energy-dense therapeutic foods (*6*, *7*). Although these approaches reduce mortality rates (*8*), many children emerge with persistent moderate acute malnutrition (MAM) and remain susceptible to relapse to SAM.

The current study explores the role of bifidobacteria, notably, *Bifidobacterium longum* subspecies *infantis*, in the pathogenesis of SAM and as probiotic treatment. Recent work has shown that acute malnutrition in infants and children is associated with impaired development of their gut microbiota (*9*, *10*). Preclinical and clinical studies indicate that this disrupted codevelopment of the gut

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microbiota and host is a contributing cause, not simply an effect, of acute malnutrition (*11*, *12*). The gut microbiota of healthy breastfed infants in the first few months of life is dominated by members of the genus *Bifidobacterium* (*13*) including *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium longum* subspecies *longum*, and *Bifidobacterium infantis*. Among these bifidobacteria, *B. infantis* is uniquely adapted to the gut of the breastfed infant because it has five distinct gene clusters (commonly referred to as H1 to H5) that encode a variety of glycoside hydrolases and oligosaccharide transporters, which enable this subspecies to metabolize the full repertoire of the several hundred known human milk oligosaccharides (HMOs) (*14*–*16*). Observational studies of Bangladeshi infants have documented a positive association between the abundance of bifidobacteria in feces in the first 4 months of life and responses to oral and parenteral vaccines (*17*). Clinical studies of exclusively breastfed U.S. infants who received a probiotic strain of *B. infantis* (EVC001) revealed that colonization was associated with reductions in gut inflammation markers (*18*). Similar beneficial effects of *B. infantis* have been observed in preterm infants who are at elevated risk for nosocomial infections (*19*) and in experimental models of necrotizing enterocolitis (*20*).

A meta-analysis of HMOs in mothers representing different geographies, socioeconomic features, and stages of lactation identified LNT (lacto-*N*-tetraose) and LNnT (lacto-*N*-neotetraose) as among the most abundant structures present, accounting for up to 20% of total HMOs (*21*). More complex HMOs based on the LNT and LNnT core can comprise up to 70% of total HMOs and more than 90% in "nonsecretor" mothers who lack a functional fucosyltransferase 2 (*FUT2*) gene (*22*, *23*). *B. infantis* strains characterized to date can grow in vitro with HMOs as a sole carbon source; this phenotype is believed to involve internalization of the HMO by a specific adenosine triphosphate (ATP)–binding cassette (ABC) transporter and its subsequent catabolism by intracellular glycoside hydrolases to monosaccharides. With the exception of fucose, which is catabolized to pyruvate and 1,2-propanediol via the intermediate fuconolactone (*24*), monosaccharides from HMOs are substrates for the "bifid shunt" (fructose-6-phosphate phosphoketolase pathway), which produces ATP, acetate, and lactate as end products (*25*). Despite the broad conservation of the H1 gene cluster among *B. infantis* strains, strain-level variation exists for genes in the H5 cluster encoding the ABC transporter involved in uptake of LNT (*15*, *26*). Therefore, a reduced capacity to use LNT and its derivatives poses a competitive disadvantage to such *B. infantis* strains (*27*). At the same time, in many low-income settings, animal milk, gruels, and complementary foods are often introduced into the diet at an early age, creating a nutrient landscape that may not be optimal for *B. infantis* strains.

A general challenge in the field of human microbiome research is to identify alterations in microbial community composition and expressed functions, establish causal relationships between these configurational changes and disease pathogenesis, and then develop strategies for effectively repairing community function to restore health. If restoration involves administration of microbes, then decisions have to be made about which strains to select. The fitness of strains in the gut ecosystems of individuals will reflect a given strain's capacity to adapt to host habitat and environmental features; this may require searches for strains that have evolved in the microbial communities of the populations of individuals where treatment is being directed. The current study identifies *B. infantis* deficiency as a feature of the fecal microbiota of children with SAM compared to their age-matched healthy counterparts and offers an approach for identifying *B. infantis* strains that may be better suited for repairing the perturbed gut microbiota of infants presenting with SAM whose diets are rich in glycans derived from sources other than breastmilk.

RESULTS

B. infantis in the gut microbiota of Bangladeshi infants with SAM

B. infantis abundance was measured using a quantitative polymerase chain reaction (qPCR) assay directed to the *nanH2*/exo-α-sialidase gene (*Blon_2348*) in the H1 locus that is uniquely present in this subspecies (Fig. 1) (*28*). This qPCR assay was applied to DNA isolated from fecal samples collected from 3- to 24-month-old Bangladeshi children with SAM (n = 102) and age-matched Bangladeshi children without a wasting disorder (WLZ ≥ -2; n = 49; see table S1A for clinical characteristics). All children lived in Dhaka, Bangladesh, with many residing in Mirpur, an urban slum district within the city. Primers used to measure total bifidobacterial load were targeted to the 16*S* ribosomal RNA (rRNA) gene (*29*). The specificity of targeting for both sets of primers was confirmed using a reference collection of cultured gut bacterial strains with sequenced genomes (table S1, B and C).

In this cross-sectional study of healthy breastfed Bangladeshi infants and children, the highest levels of *B. infantis* were documented in feces at the end of the first postnatal month, with no statistically significant diminution in *B. infantis* absolute abundance through 12 months of age (blue points, Fig. 2A). During this period, ~75% of all bifidobacterial strains detected in fecal samples were *B. infantis* (*Blon_2348* positive); subsequently, its abundance declined progressively by >4 orders of magnitude between 12 and 24 months of age (Fig. 2A and table S1A). The overall abundance of members of the genus *Bifidobacterium* remained high throughout the 24-month period (Fig. 2B and table S1A), reflecting an increase in other bifidobacterial species (table S1D).

The *nanH2*-based qPCR assay showed that the absolute abundances of *B. infantis* were on average two to three orders of magnitude lower in 3- to 13-month-old Bangladeshi children with SAM (Fig. 2A, red points) compared to their healthy age-matched counterparts ($P < 0.05$, generalized additive model; Fig. 2A). No significant differences were evident between the ages of 14 and 24 months ($P > 0.05$; generalized additive model). Total bifidobacterial abundances were also significantly lower in fecal samples from 3- to 16-month-old children with SAM compared to their age-matched healthy counterparts ($P < 0.05$, generalized additive model; Fig. 2B). Compared to healthy infants, the fecal microbial communities of infants with SAM were dominated by pathobionts including *Escherichia*, *Shigella*, *Klebsiella*, and *Streptococcus* species (fig. S1, A and B, and table S1D).

Blon_2176, part of the H5 gene cluster, encodes the permease component of the ABC transporter for LNT, a prominent lacto-*N*-biose type I tetrasaccharide in breastmilk (*15*). A qPCR assay using primers directed against *Blon_2176* revealed that the mean absolute abundance of this gene was >1000-fold lower in both healthy infants and infants with SAM compared to the abundance of the *B. infantis* *Blon_2348* gene (Fig. 2, A and C). Moreover, there was a significant difference in the percentage of fecal samples from children with SAM compared to healthy Bangladeshi children who had detectable

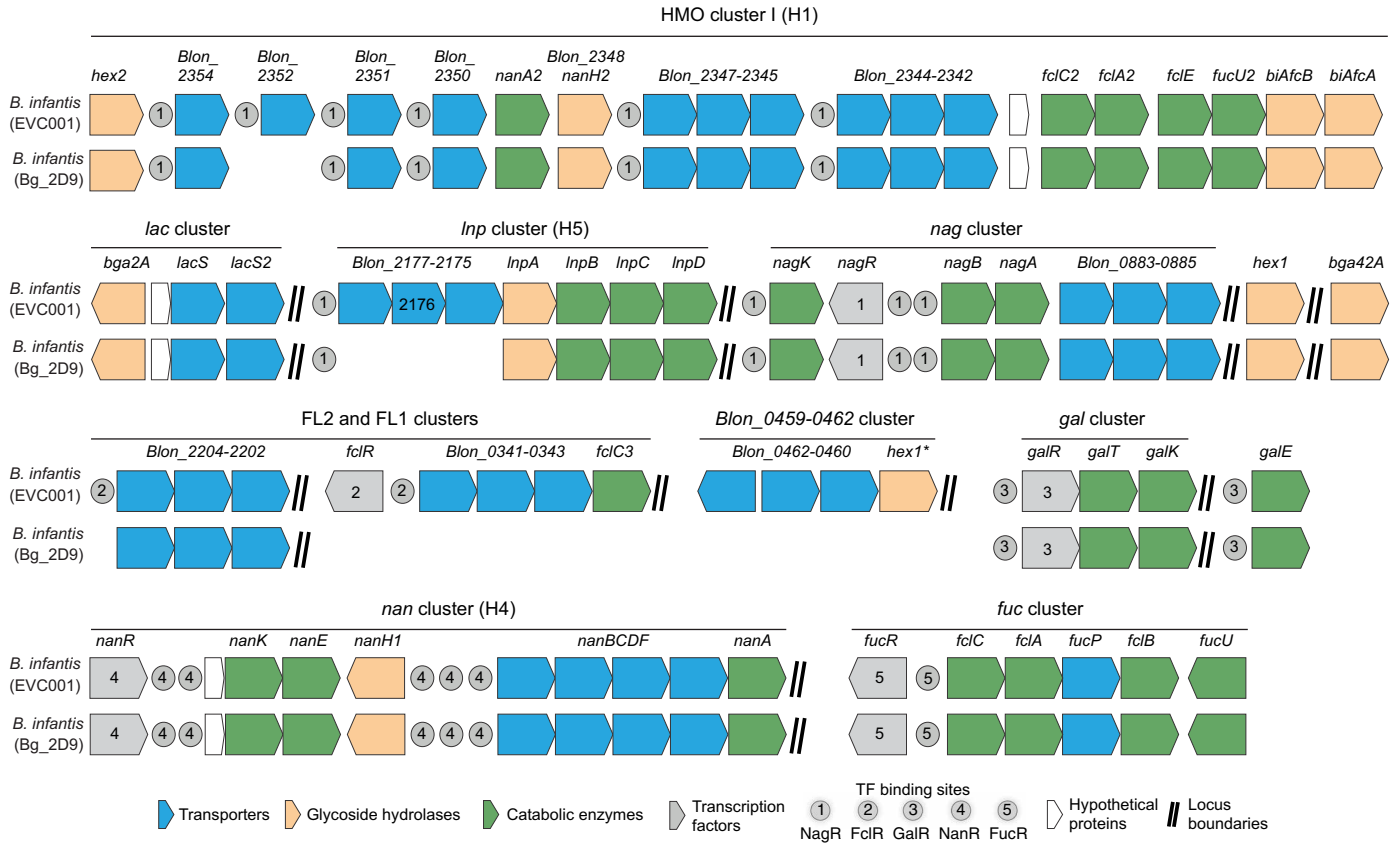

Fig. 1. HMO utilization genes in *B. infantis* EVC001 and Bg_2D9 strains. Shown is the reconstruction of loci involved in the utilization of HMOs in two *B. infantis* strains (see table S3). EVC001 is a U.S. donor–derived probiotic *B. infantis* strain; the Bg_2D9 strain was isolated from a 12-month-old healthy Bangladeshi child. Blon, *B. longum* locus tag; FL1/FL2, fucosyllactose 1 and fucosyllactose 2; Nan, *N*-acetylneuraminic acid; TF, transcription factor.

amounts of *Blon_2176* (38 versus 66%, respectively; $P < 0.05$, two-proportion Z test; table S1A).

Colonization of Bangladeshi infants with SAM by *B. infantis* EVC001

Given the deficiency of *B. infantis* in the fecal microbiota of infants with SAM, we performed a pilot single-blind, randomized clinical trial called SYNERGIE (SYNbiotic for Emergency Relief of Gut Instability and Enteropathy) to assess the extent to which EVC001 [a commercially available, U.S. infant–derived *B. infantis* strain with intact H1 to H5 gene clusters (*18*, *27*, *30*)] could colonize the gut in this population (Fig. 1). Infants with SAM between 2 and 6 months of age (mean, 4.1 ± 1.1 months; WLZ < −3, or bilateral pedal edema) were eligible for enrollment in the SYNERGIE trial after they had completed a standardized protocol for initial hospital-based management of SAM (see table S2A for CONSORT diagram). Sixty-two enrolled infants were subsequently randomly assigned to one of three treatment groups: once daily administration of 8×10^9 colony-forming units (CFU) of *B. infantis* EVC001 alone (probiotic arm; $n = 20$); the same dose of *B. infantis* EVC001 plus 1.6 g of the purified HMO LNnT (synbiotic arm; $n = 21$); lactose (625 mg/day; placebo arm; $n = 21$; Fig. 3A and table S2B).

At enrollment, there were no statistically significant differences between the three groups with respect to sociodemographic or clinical characteristics (table S2, C and D). Forty-two of the 62 infants had bilateral pedal edema; however, there were no statistically significant differences in the representation of this kwashiorkor-like phenotype among the three arms ($P = 0.54$; Kruskal-Wallis test; table S2D). Participants were treated with the probiotic, synbiotic, or placebo daily for 28 days, starting in hospital and continuing at home after discharge. They were subsequently followed for an additional 28 days after cessation of their treatment intervention. Hospital discharge occurred when an infant had achieved a WLZ better than −3 or, for those with kwashiorkor, when their edema had resolved. There were no statistically significant differences between the three groups in the duration of their hospital stay after treatment was initiated ($P = 0.89$; Kruskal-Wallis test; table S2D). Fecal samples were collected on day 1 (enrollment/baseline), day 28, and day 56 for analysis of the absolute abundance of *B. infantis* and for quantification of markers of intestinal inflammation. Anthropometry was performed on admission to the hospital of infants with SAM just before intervention with one of the two treatments or placebo (day 1), at the time of hospital discharge (day 28; end of treatment), and at study completion (day 56; table S2C).

EVC001 contains a 123–base pair (bp) sequence in *epsJ* (putative family 2 glycosyltransferase) that was not present in the genomes of six other *B. infantis* isolates that we had cultured from Bangladeshi children (table S3A). We designed qPCR primers targeting this region

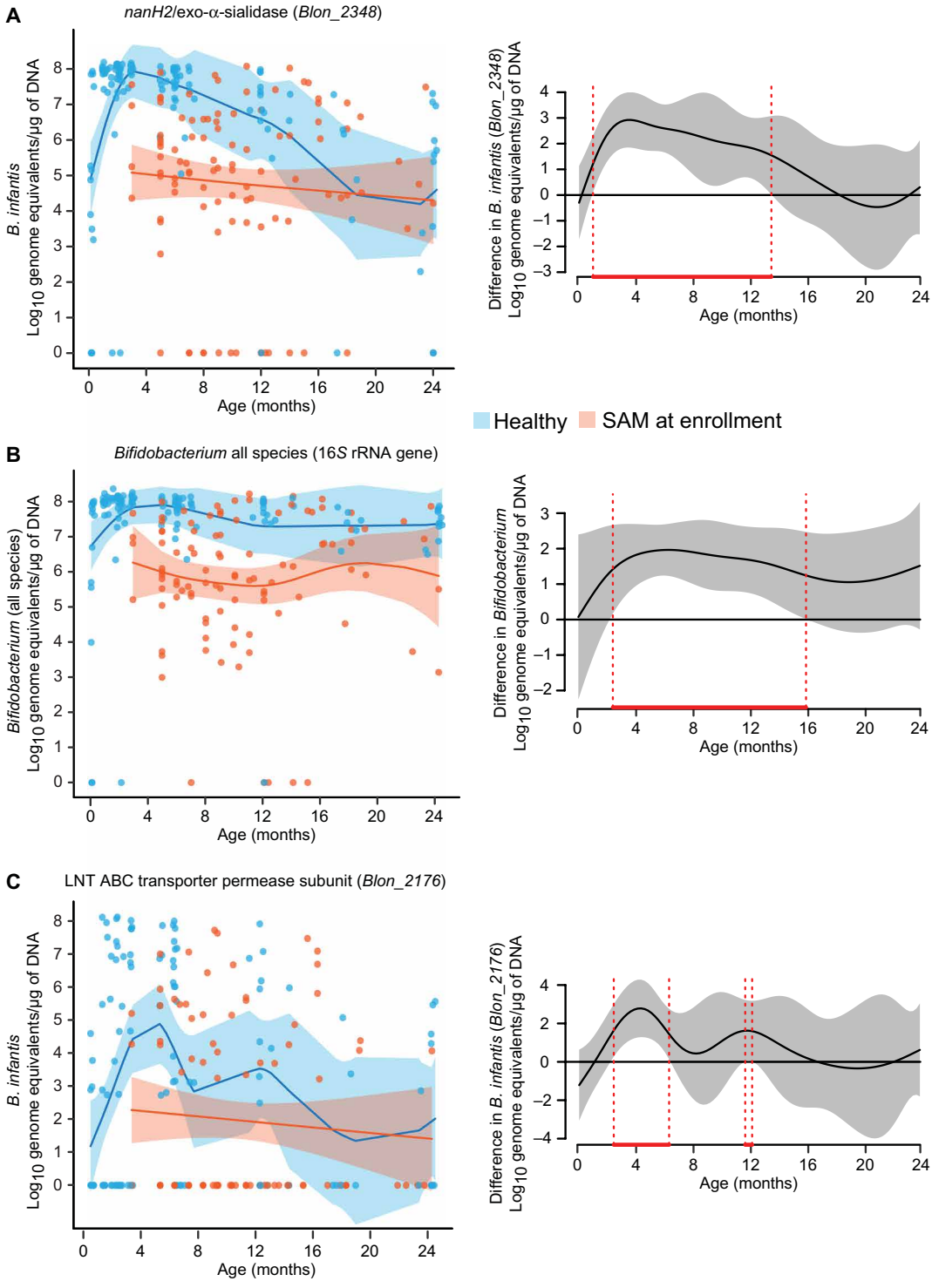

Fig. 2. *B. infantis* abundance in fecal samples from healthy Bangladeshi infants and those with SAM. (**A** to **C**) qPCR assay primers were directed against the NanH2 exo-α-sialidase (*Blon_2348*) gene of *B. infantis* (A), the 16*S* rRNA gene of all bifidobacterial species (B), and the LNT ABC transporter permease subunit (*Blon_2176*) gene of *B. infantis* (C). Fecal biospecimens were assayed from healthy children ($n = 130$ samples) and children with SAM ($n = 102$ samples). Scatterplots (left) display the absolute abundance of target genes as normalized to $\log_{10}$-transformed genome equivalents per microgram of fecal DNA as a function of age at the time of specimen collection. Samples from healthy infants and children are indicated by blue dots/shading, whereas those from individuals with SAM are denoted in red. A generalized additive model–derived best fit line (±2 SEM) is shown. Plot difference curves (right) depict statistically significant differences in the best fit lines (areas bounded by red dashed vertical lines), between the two models (healthy and SAM; see table S1).

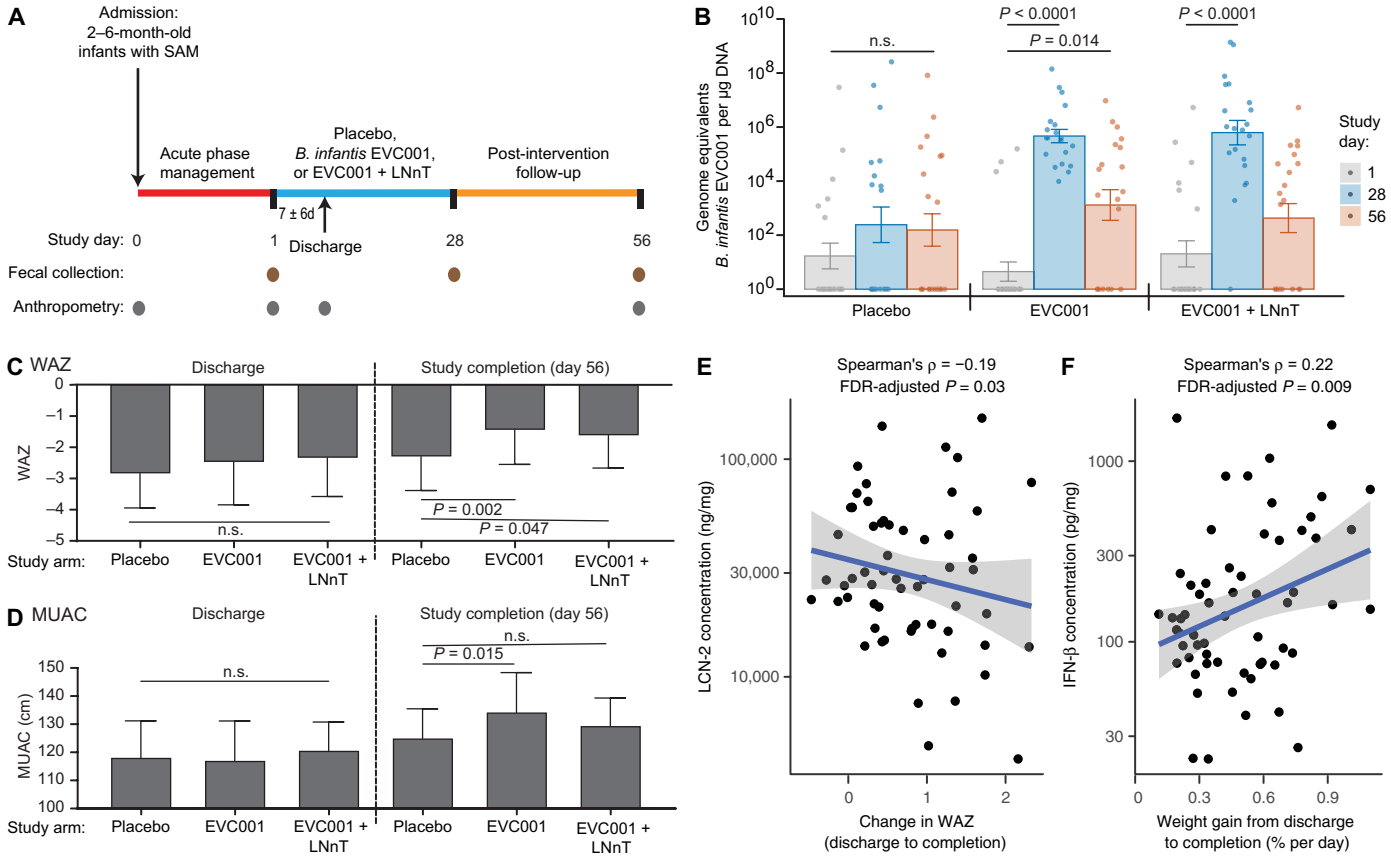

Fig. 3. SYNERGIE clinical trial. (**A**) Shown is the design of the SYNERGIE clinical study. (**B**) The absolute abundance of *B. infantis* EVC001 in fecal samples collected from trial participants was measured using qPCR primers directed to a region of the *epsJ* gene. Fecal samples were collected on the indicated study days from participants in each arm of the study: placebo (lactose), probiotic (EVC001), and synbiotic (EVC001 + LNnT). Each dot represents a study participant. Barplots represent mean values ± SEM; $n = 20$ to 21 participants per study arm. The *P* values indicated for within-group comparisons were determined using Dunn's test with Benjamini-Hochberg correction. n.s., not significant. (**C** and **D**) Effect of the interventions on weight-for-age *z* scores (WAZ) (C) and mid-upper arm circumference (MUAC) (D) at the end of the study (day 56) compared to the time of hospital discharge. Bar plots represent group means; error bars represent SD. *P* values were calculated using Mann-Whitney *U* test. (**E**) Spearman correlation between the concentration of fecal lipocalin-2 (LCN-2) and the change in WAZ from hospital discharge to study completion. (**F**) Spearman correlation between the concentration of fecal interferon-β (IFN-β) and the rate of weight gain in infants between discharge and study completion [Spearman's ρ and FDR-adjusted *P* values for each correlation are shown in (E) and (F)].

(table S1B) and confirmed their specificity for EVC001 using a panel of 31 sequenced human gut–derived strains, including 16 bifidobacteria (table S1C). On the basis of this qPCR assay, we observed a statistically significant increase in the abundance of EVC001 in the fecal microbiota at day 28 compared to baseline in infants with SAM who received EVC001 alone and in those who received EVC001 plus LNnT ($P < 0.0001$ and $P < 0.0001$, respectively; Dunn's test with Benjamini-Hochberg correction; Fig. 3B). At day 28, amounts of EVC001 in feces from infants in the EVC001 and EVC001 + LNnT groups were significantly higher than in infants in the placebo group ($P = 0.004$ and $P = 0.001$, respectively; Dunn's test with Benjamini-Hochberg correction). By day 56, the abundance of EVC001 had fallen in both the probiotic and synbiotic treatment groups compared to day 28, although it remained significantly higher than before intervention in the probiotic (EVC001 alone) arm ($P = 0.014$; Dunn's test with Benjamini-Hochberg correction (Fig. 3B). However, peak colonization by the probiotic EVC001 strain in this pilot study of infants with SAM was one to two orders of magnitude lower than the average total abundance of *B. infantis* in age-matched healthy children living in the same community (Fig. 2A and table S1A).

Gut bacterial community composition was assessed by sequencing of PCR amplicons generated from variable region 4 of 16*S* rRNA genes present in the fecal samples collected before and after the interventions (table S2E). No significant differences were noted in the changes in the relative abundance of specific bacterial taxa between treatment groups from baseline to day 28 ($P > 0.05$, Kruskal-Wallis test with Benjamini-Hochberg correction). In all groups, Enterobacteriaceae were significantly reduced from baseline to day 28 ($P < 0.001$, Mann-Whitney *U* test with Benjamini-Hochberg correction), falling in the EVC001 group from a relative abundance of 79 ± 29% (means ± SD) at baseline to 42 ± 26% at day 28.

Effects of colonization with EVC001 on host phenotypes

Because of the presence of bipedal edema in 42 of the 62 infants (68%) at enrollment, it was not possible to compare weights between groups until edema had resolved at the time of hospital discharge. At discharge, there were no statistically significant differences in weight-for-age *z* scores (WAZ) or MUAC between the intervention groups ($P = 0.361$ and $P = 0.624$, respectively, Kruskal-Wallis test; table S2F). However, at study completion (day 56), WAZ and

MUAC in infants treated with EVC001 were significantly greater than for infants in the placebo arm, indicating an improvement in weight gain ($P = 0.002$, WAZ; $P = 0.015$, MUAC; Mann-Whitney U test; Fig. 3, C and D, and table S2F). WAZ was also significantly higher in children receiving EVC001 + LNnT compared to children in the placebo arm at study completion ($P = 0.047$; Mann-Whitney U test; Fig. 3C and table S2F). At the end of the 2-month study, WLZ had improved in all groups, although the differences between treatment groups were not statistically significant ($P = 0.22$, Kruskal-Wallis test; table S2F). Last, at study completion, linear growth [defined by length (height)–for-age z score] trended higher in children who had received EVC001 compared to those in the placebo arm ($P = 0.08$, Kruskal-Wallis test; table S2F).

A previous study showed that stable colonization of healthy breastfed infants in the United States with EVC001 resulted in reduced fecal markers associated with gut inflammation and intestinal permeability compared to a control group that did not receive EVC001 (*18*). Therefore, we measured lipocalin-2 (LCN-2), myeloperoxidase, calprotectin, and several cytokines [interferon-β (IFN-β), interleukin-17A (IL-17A), IL-1β, and IL-6] in fecal samples collected from study participants on study day 1, at the time of hospital discharge, and on days 28 and 56 (table S2G). We did not observe statistically significant differences in these markers between the groups in this pilot study. However, the amount of LCN-2 was negatively correlated with the change in WAZ from hospital discharge to study completion [Spearman's $\rho = -0.19$, false discovery rate (FDR)–adjusted $P = 0.03$; Fig. 3E]. In addition, we observed a significant positive correlation between the amount of IFN-β at day 28 (*31*) and the rate of weight gain in infants with SAM between discharge and study completion ($\rho = 0.22$, FDR-adjusted $P = 0.009$; Fig. 3F).

More than half of the infants with SAM in the SYNERGIE study were not receiving any breastmilk at the time of hospital admission: 15 of 21 (71%) of the children who were subsequently randomized to the synbiotic arm, 8 of 20 (40%) in the probiotic arm, and 11 of 21 (52%) in the placebo arm. Even among those infants who were receiving breastmilk at the time of admission, consumption was only 18 ± 13% of the recommended daily volume for age-matched healthy infants (table S2C). This raised the question of whether the limited persistence of colonization with the EVC001 strain compared to that previously observed in exclusively breastfed healthy U.S. infants reflected the reduced prevalence of breastfeeding and lower quantities of breastmilk consumption by these Bangladeshi infants with SAM. Therefore, we next examined gut microbial communities of Bangladeshi children to search for *B. infantis* strains with a competitive advantage over EVC001.

In silico and in vitro characterization of Bangladeshi *B. infantis* strains

We characterized the genomic features of 10 *B. infantis* strains: 6 we had cultured from fecal samples collected from three healthy and one undernourished infants/children, aged 6 to 24 months, living in Mirpur; 2 strains were from Malawian infants (MC1 and MC2); 1 strain was a U.S. donor–derived type strain [American Type Culture Collection (ATCC) 15697] and EVC001 (table S3A). We used microbial community SEED (mcSEED), which has been used for manual curation of >2600 bacterial taxa cultured from the human gut microbiota (*32*). mcSEED "subsystems" encompass functions (enzymes, transporters, and transcriptional regulators) that reflect current knowledge of specific metabolic pathways or groups of pathways represented in this set of human gut bacterial genomes (*33*). mcSEED metabolic modules are lists of genes that can be more granular than a subsystem (e.g., uptake of a nutrient separately from its metabolism) (*32*, *33*). mcSEED-based in silico metabolic reconstructions allowed us to predict the ability of these strains to synthesize amino acids and B vitamins and use various carbohydrates (table S3, B to D).

Table S3 (E and F) summarizes the results of our in silico analysis of the ability of each strain to import and use HMOs. All these *B. infantis* strains have transporters for prominent neutral/fucosylated HMOs, with the notable exception of LNT (table S3E). Unlike the U.S. donor–derived ATCC 15697 type strain and EVC001, only one of the Bangladeshi strains (Bg_463) had an ortholog of the canonical, biochemically characterized LNT transporter, Blon_2175-2177 (*26*). In contrast, all strains had a transporter for LNnT, the prominent type II [Gal(β1-4)GlcNAc-containing] isomer of LNT (Blon_2342-2344 or Blon_2345-2347).

We subsequently performed a series of in vitro growth experiments using four Bangladesh *B. infantis* strains plus EVC001. Monocultures were grown for 30 hours under anaerobic conditions at 37°C in a low-carbohydrate DeMan-Rogosa-Sharpe medium that was supplemented with individual purified HMOs prominently represented in breastmilk [LNT, LNnT, 2'-fucosyllactose (2'-FL), and 3'-sialyllactose and 6'-sialyllactose (3'-SL and 6'-SL)]; lactose served as a positive control ($n = 3$ replicates for each strain under each condition). Each of the strains tested exhibited slower growth rates in the presence of sialylated HMOs (3'-SL and 6'-SL) compared to neutral or fucosylated carbohydrate structures (fig. S2) [to date, no SL-specific transporter has been characterized in bifidobacteria, although the exo-α-sialidase NanH2 (Blon_2348, GH33) that is ubiquitous among *B. infantis* strains cleaves the sialic acid residue from both 3'-SL and 6'-SL]. We found that strains predicted to have the full complement of downstream catabolic enzymes and transporters for either LNnT or 2'-FL [Blon_2345-2347 and Blon_2202-2204, respectively; (*26*, *34*)] were able to grow in the presence of the corresponding HMO (table S3, E and F). An unanticipated result was the comparable growth of strains in the presence of LNT (fig. S2A), suggesting that the three Bangladeshi strains that lacked the Blon_2175-2177 ABC transporter have an alternative mechanism for LNT uptake/utilization whose efficiency is comparable to the canonical LNT transporter, at least in vitro in the absence of competition. On the basis of these in vitro observations, we performed a follow-up in vivo study involving gnotobiotic mice colonized with a consortium of *B. infantis* strains where this canonical LNT transporter was either present (EVC001 and Bg_463) or absent (Bg_2D9, Bg_1G8, and PS064).

In vivo competition between Bangladeshi *B. infantis* strains and EVC001 in gnotobiotic mice fed HMO-supplemented Bangladeshi diets

To compare the in vivo fitness of the Bangladeshi *B. infantis* strains, 5-week-old germ-free C57BL/6J mice were started on a sterilized bovine milk powder/rice-lentil–based chow similar to the diet consumed by 6-month-old children living in the Mirpur slum of Dhaka (table S4). After 2 days of consumption of this diet (Mirpur-6), the drinking water of one group of animals was supplemented with LNT (12.5 g/liter), a second group received LNnT (12.5 g/liter), and a third control group received unsupplemented water. All mice in these

three arms (n = 6 to 7 per treatment group) were subsequently colonized with the consortium of five *B. infantis* strains followed 2 days later by a second gavage with the same consortium (Fig. 4A). In a fourth arm, mice were colonized with the five-member *B. infantis* consortium plus a *B. bifidum* strain (Bg_3D10; table S3A) that we isolated from the fecal microbiota of a healthy 12-month-old Mirpur child; these mice were treated with LNnT-supplemented drinking water (Fig. 4A). Analysis of the genome of this *B. bifidum* strain indicated that it contained genes encoding a membrane-bound extracellular lacto-*N*-biosidase I (LnbB) and an extracellular exo-β-(1-3)-*N*-acetylglucosaminidase (BbhI; table S3F). These enzymes endow it with the capacity to degrade LNT and LNnT (*35*, *36*), resulting in the release of lacto-*N*-biose and lactose. We reasoned that the presence of *B. bifidum* could potentially limit the amount of LNnT available to other microbial consumers. Mice in all four treatment groups were fed the Mirpur-6 diet ad libitum for 4 weeks. Fecal samples were collected every two days, and water, with or without HMO supplementation, was replenished daily.

There were no significant differences in body weights (measured every 2 days) between the four treatment groups [$P > 0.05$, two-way repeated-measures analysis of variance (ANOVA); table S5A]. The absolute abundance of each *B. infantis* strain in each of the treatment groups was determined on experimental days 4, 8, 12, 18, and 26 by short read shotgun sequencing of fecal DNA. The Bg_2D9 strain,

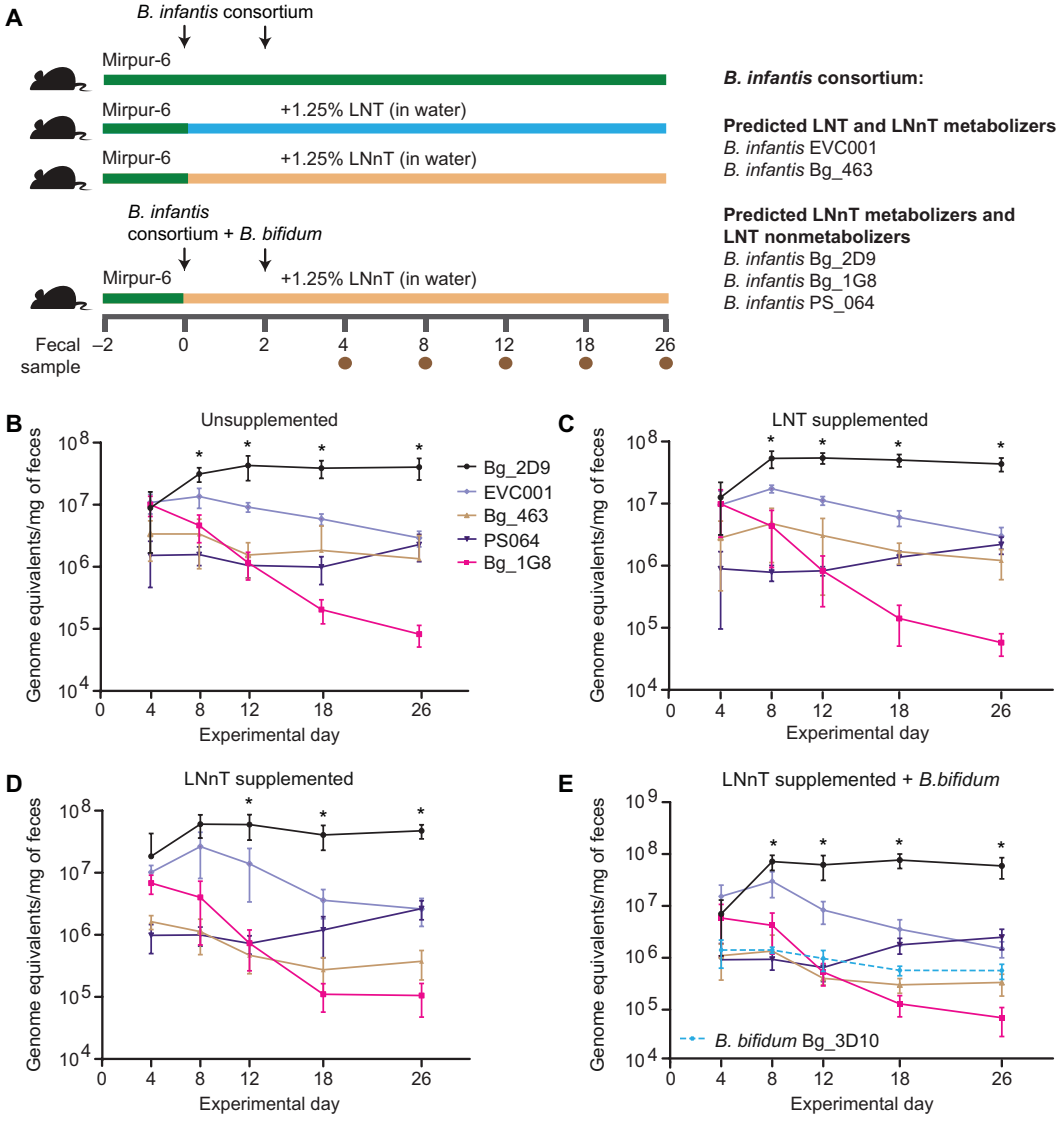

Fig. 4. In vivo competition between *B. infantis* strains in gnotobiotic mice. (**A**) Shown is the experimental design of the study examining the fitness of five *B. infantis* strains in gnotobiotic mice consuming the Mirpur-6 diet ± LNT or LNnT. (**B** to **D**) Shown are results of competition experiments involving the five-member consortium of *B. infantis* strains introduced into gnotobiotic mice. (**E**) The same five-member *B. infantis* consortium shown in (B) to (D) was introduced together with a *B. bifidum* strain isolated from a healthy 12-month-old Bangladeshi child into gnotobiotic mice. Absolute abundances of the different strains, as a function of time and HMO supplementation, were determined by short read shotgun sequencing of fecal DNA. Mean values ± SD are plotted; n = 6 to 7 mice per group; n = 1 experiment. Time points where the absolute abundance of the *B. infantis* strain Bg_2D9 was higher than other consortium members were identified using a mixed-effects linear model followed by Tukey's multiple comparison test. *$P_{adj} < 0.05$.

which, as noted above, was isolated from a healthy 12-month-old child from Mirpur and lacked the LNT transporter (Blon_2175-2177), became the dominant member over time, achieving an absolute abundance on day 18 that was ≥10-fold higher than that for each of the other strains in the three groups that had received the five-strain consortium (FDR-adjusted $P < 0.01$, mixed-effects model followed by Tukey's multiple comparison test; Fig. 4, B to D, and table S5B). During the first 18 days of colonization, EVC001 was the second most abundant strain, after which time it no longer exhibited a competitive advantage over the other three strains (Fig. 4, B to D). In the six-member community containing *B. bifidum*, Bg_2D9 dominated the community by day 8 and maintained its higher absolute abundance compared to each of the other strains for the duration of the experiment (Fig. 4E and table S5B).

Comparison of the Bg_2D9 genome to those of other consortium members

To identify features that might explain the competitive advantage of the Bg_2D9 strain, we compared its genome to the genomes of each of the other four *B. infantis* strains in the consortium. Using a minimum threshold of 95% sequence identity for orthologous genes, we identified 267 genes that were unique to the Bg_2D9 strain (Fig. 5A). Most of these 267 genes encoded small hypothetical proteins or were mobile elements, but they included members of a predicted β-glucoside utilization gene cluster (*bgl*) and an *N*-glycan utilization gene cluster (*ngl*; Fig. 5A and table S3, G to I).

The *bgl* cluster contained three glycoside hydrolases, an ABC transport system, and a TetR family transcriptional regulator (*bglT*). Among 34 published *B. infantis* genomes (*37*), only 3 (Bg_2D9, Bg_2C3, and Malawi_MC1; all described here) have this locus (table S3I). Given that β-1,3–linked glucosides are common constituents of plant cell wall polysaccharides and about 60% by weight of the Mirpur-6 diet is plant based (table S4A), we reasoned that a unique β-glucoside utilization cluster in Bg_2D9 could provide one explanation for its fitness advantage over the other members of the *B. infantis* consortium introduced into gnotobiotic mice.

Asparagine-linked glycans (*N*-glycans) have structural similarities to HMOs and are abundant in human and bovine milk where they decorate numerous proteins including lactoferrin and immunoglobulins (*38*). Previous reports have indicated that *B. infantis* ATCC 15697

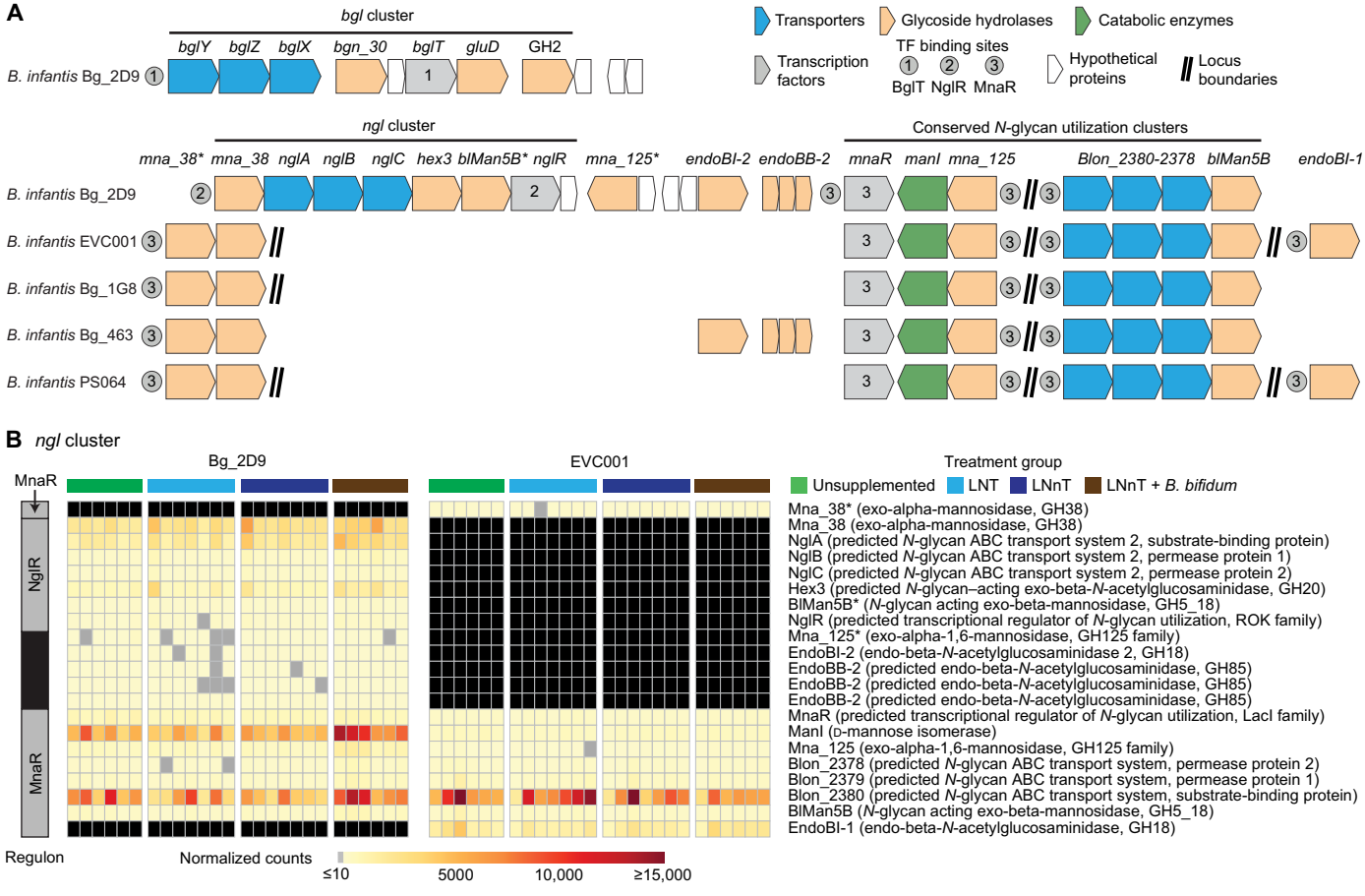

Fig. 5. Carbohydrate utilization gene clusters in *B. infantis* strains. (**A**) Shown are a β-glucoside utilization (*bgl*) gene cluster in Bg_2D9 and the *N*-glycan utilization (*ngl*) cluster in *B. infantis* strains of the five-member consortium introduced into gnotobiotic mice. Predicted transcription factor binding sites are denoted by gray circles. Gene locus tags and annotations are given in table S3. (**B**) Expression of *ngl* cluster genes in the *B. infantis* Bg_2D9 and EVC001 strains. Mice fed either the Mirpur-6 diet, the Mirpur-6 diet + 1.25% LNT (in their drinking water), or the Mirpur-6 diet + 1.25% LNnT (in their drinking water) were colonized with a five-member consortium of *B. infantis* strains including Bg_2D9 and EVC001. In a fourth group, *B. bifidum* was included with the five-member consortium, and mice were given drinking water supplemented with 1.25% LNnT. The black bar in the leftmost column indicates that the transcription factor has not been characterized. Cells in the heatmap that are colored black denote the absence of an ortholog in a strain, and cells colored gray depict low expression (≤10 DESeq2-normalized read counts).

and EVC001 are capable of using a wide array of *N*-glycans both in vitro and in vivo (*39*, *40*). We identified a *ngl* cluster in the Bg_2D9 genome that contained two endo-β-*N*-acetylglucosaminidases, EndoBI-2 and EndoBB-2 (Fig. 5A and table S3G). The *ngl* cluster also contained genes encoding an ABC transport system predicted to transport *N*-glycans, glycoside hydrolases involved in degradation of *N*-glycans, and a transcriptional regulator NglR (Fig. 5A). Extending our analysis to 336 bifidobacterial genomes including 34 *B. infantis* isolates (*37*) revealed that the Bg_2D9 strain was one of only six that contained the *ngl* cluster (table S3I). EVC001 was predicted to have *N*-glycan metabolizing capabilities through an alternative pathway that includes EndoBI-1, a β-mannosidase (BlMan5B), and another predicted *N*-glycan transporter (Blon_2378-2380) (*41*), plus α-mannosidase, Mna_125, and mannose isomerase (ManI; Fig. 5A).

Analysis of gene expression in consortium members

We subsequently performed microbial RNA sequencing (RNA-seq) of cecal contents harvested at the time of euthanasia of gnotobiotic mice colonized with the five *B. infantis* strains with or without *B. bifidum*. We examined expression of the β-glucoside utilization (*bgl*) and *N*-glycan utilization (*ngl*) loci, as well as other genes involved in LNT and LNnT utilization, as a function of the presence or absence of these HMOs in the drinking water. Transcript counts were mapped to the genomes of consortium members and then normalized and analyzed to identify differentially expressed genes. All eight genes comprising the *bgl* cluster were expressed in the Bg_2D9 strain, but there was no difference in their expression based on the presence or absence of either LNT or LNnT (fig. S3A and tables S5C and S6). The *bgl* cluster comprises a regulon controlled by a TetR family regulator, BglT (Fig. 5A and table S7). We postulated that this regulator may have responded to β-gluco-oligosaccharides originating from the grain components (rice and lentil) of the Mirpur-6 diet (*42*).

There were no statistically significant effects of HMO supplementation on expression of genes in the *ngl* cluster of Bg_2D9 that are regulated by NglR or MnaR (Fig. 5B and tables S5C, S6, and S7). Compared to animals receiving the Mirpur-6 diet alone, supplementation with LNT produced a statistically significant increase in expression ($\log_2$-fold difference > 1.5 and FDR-adjusted $P < 0.05$) of genes in the H1 cluster that encoded six type II HMO transporter proteins (fig. S3B, and tables S5C and S6). In LNnT-supplemented mice, with the exception of Blon_2345, expression of these genes was also significantly elevated in Bg_2D9 compared to their expression in animals receiving unsupplemented water ($\log_2$-fold difference > 1.5; FDR-adjusted $P < 0.05$; fig. S3B and tables S5C and S6). The absence of statistically significant induction of expression of other glycan transporter genes in the presence of LNT raises the possibility that this H1 cluster encodes a transport system that might also import LNT in addition to LNnT.

Looking beyond these two loci in Bg_2D9, we noted that LNnT supplementation significantly increased the expression of several genes required for HMO metabolism including *nagA* (*N*-acetylglucosamine-6-phosphate deacetylase) and *nagB* (glucosamine-6-phosphate deaminase), which are involved in *N*-acetylglucosamine (GlcNAc) catabolism; the *lnp* cluster (H5) involved in lacto-*N*-biose/galacto-*N*-biose catabolism; and the predicted HMO transporters Blon_2350 and Blon_2351 in the H1 cluster ($\log_2$-fold difference > 1.5 and FDR-adjusted $P < 0.05$; fig. S3B and tables S5C and S6). In contrast, expression of genes involved in sialic acid utilization including components of the *nan* cluster (H4) sialic acid ABC transporter (*nanB* and *nanD*) and *N*-acetylneuraminate lyase (*nanA*) was reduced significantly in Bg_2D9 in both LNT-supplemented and LNnT-supplemented animals ($\log_2$-fold difference > 1.2, FDR-adjusted $P < 0.05$; fig. S3B and tables S5C and S6).

Last, when comparing the LNnT-supplemented groups of gnotobiotic mice with or without *B. bifidum*, expression of most genes involved in HMO and *N*-glycan metabolism did not differ substantially (fig. S3B and tables S5C and S6). However, we observed a significant increase in the expression of H4 cluster genes involved in sialic acid catabolism in the Bg_2D9 genome (Fig. 1) when *B. bifidum* was present ($\log_2$-fold difference > 1.8, FDR-adjusted $P < 0.5$; fig. S3B and tables S5C and S6). Consistent with these observations, *B. bifidum* has been reported to produce an extracellular sialidase (table S3F) that can liberate sialylated glycans present in bovine milk, which would then be available for cross-feeding of other members of the microbial community (*43*, *44*). Collectively, our analyses indicate that among the strains evaluated, Bg_2D9 had the biggest endowment of glycoside hydrolases and candidate transporters for *N*-glycan utilization.

Prevalence of *B. infantis* strains having the *nglA* transporter gene in Bangladeshi children

We returned to the collection of fecal DNA samples from the cross-sectional survey of Bangladeshi infants and children described in table S1A and applied a qPCR assay that used primers against the *nglA* component of the ABC transport system identified in the genome of the *B. infantis* Bg_2D9 strain [see table S1 (B and C) for primer design and the results of specificity tests]. This assay disclosed that the absolute abundance of *nglA* was on average two to three orders of magnitude lower in 3- to 13-month-old children with SAM compared to their healthy age-matched counterparts ($P < 0.05$, generalized additive model; red-bounded region in Fig. 6A), whereas no significant differences were evident between 14- and 24-month-old children ($P > 0.05$; generalized additive model). Of the samples for which sufficient DNA was available to assay, 55 of 117 (47%) from healthy children and only 18 of 83 (22%) from children with SAM were positive for *nglA* ($P < 0.05$, two-proportion Z test; table S1A). There were no fecal samples that were positive for *nglA* and negative for the characteristic *B. infantis Blon_2348*/*nanH2* sialidase gene, suggesting that when detected, *nglA* was present within genomes belonging to strains of *B. infantis*.

Competition between *B. infantis* Bg_2D9 and EVC001 in gnotobiotic mice harboring a fecal microbiota from an infant with SAM

We subsequently used gnotobiotic mice to test the relative capacities of *B. infantis* Bg_2D9 and EVC001 to establish themselves in a fecal microbiota sample obtained from a 5-month-old infant with SAM in the SYNERGIE trial before the probiotic intervention (the experimental design is summarized in Fig. 6B). Germ-free pregnant C57BL/6J dams were initially housed in the same isolator that contained two cages with two dams per cage. Animals were fed a standard breeder chow. On day 2 after parturition, both groups of dams were switched to the Mirpur-6 diet. On postpartum day 4, both dams in each group were gavaged with the fecal microbial community from the infant with SAM. The gavage was repeated 3 days later, and on day 11, one of the two cages was moved to a

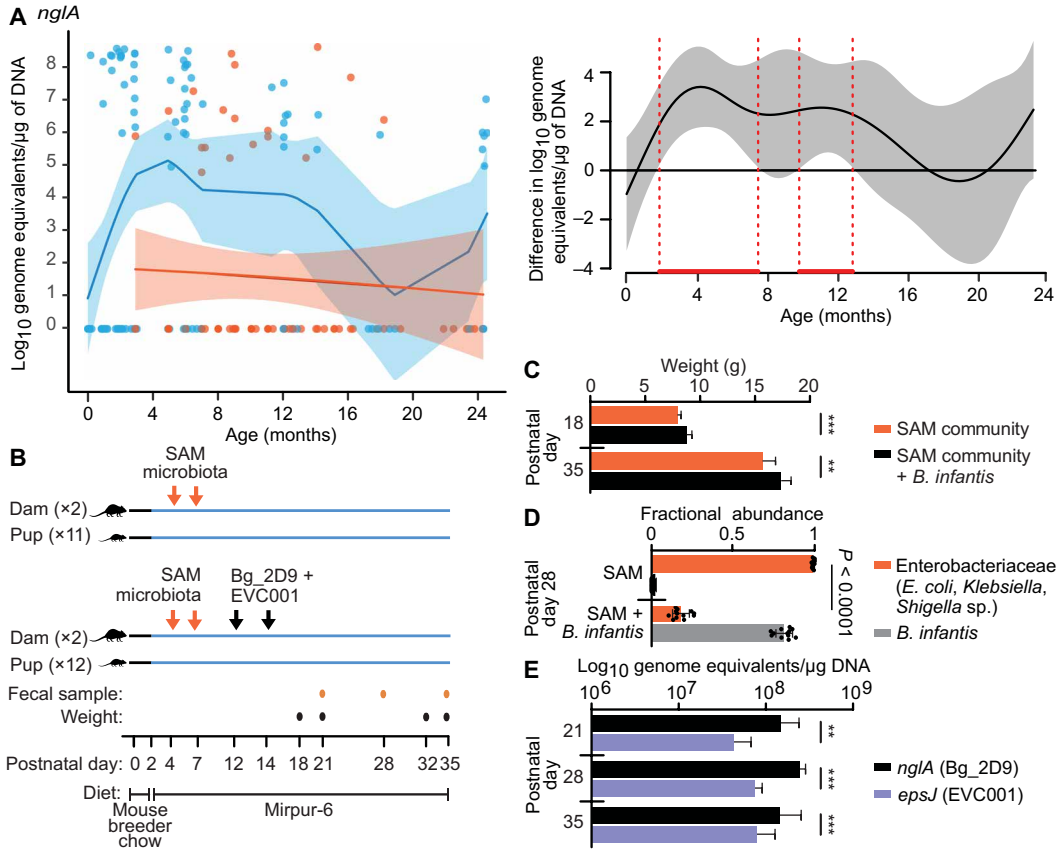

Fig. 6. Colonization by *B. infantis* Bg_2D9 of gnotobiotic mice harboring a SAM microbiota. (**A**) The absolute abundance of the *nglA* subunit of the *B. infantis* *N*-glycan ABC transport system (NglABC) was measured in fecal samples from Bangladeshi children using qPCR. Fecal biospecimens from healthy children ($n = 117$ samples) and children with SAM ($n = 83$ samples) were assayed. The scatterplot (left) displays the absolute abundance of *nglA* as a function of age at the time of specimen collection. Samples from healthy infants and children are indicated by blue points/shading, whereas those from individuals with SAM are denoted in red. A generalized additive model–derived best fit line (±2 SEM) is shown. A plot difference curve (right) showing statistically significant differences in the best fit lines between the two models (healthy and SAM) is indicated by areas bounded by red dashed vertical lines. (**B**) Shown is the experimental design of the gnotobiotic mouse study examining colonization of the gut microbiota of pups whose mothers received a fecal microbiota sample from a SAM donor with or without *B. infantis* strains Bg_2D9 and EVC001. (**C**) Shown is the weight of pups on postnatal days 18 and 35 (means ± SD; $n = 11$ pups in the SAM-only group and $n = 12$ pups in the SAM plus *B. infantis* group, $n = 1$ experiment). ***$P < 0.001$; **$P < 0.01$, two-way repeated-measures ANOVA followed by Šidák's multiple comparison test (table S5D). (**D**) Shown are the results of a 16*S* rRNA–based analysis of the relative abundances of ASVs assigned to Enterobacteriaceae and bifidobacteria present in the fecal microbiota of P28 pups in the two treatment groups. (**E**) Shown are the absolute abundances of Bg_2D9 and EVC001 in feces collected from pups at P21, P28, and P35 as defined by qPCR using strain-specific primers targeting the *nglA* and *epsJ* genes, respectively. ***$P < 0.001$; **$P < 0.01$, two-tailed Wilcoxon matched-pairs signed-rank test.

separate gnotobiotic isolator. On postpartum days 12 and 14, another type of gavage was performed, this one consisting of a mixture containing equivalent concentrations of Bg_2D9 and EVC001 that was administered to both dams in one of the two groups; both dams in the other group received a sham gavage. Pups (n = 11 to 12 per treatment group) were maintained with their dams until postnatal day 23 (P23; time of weaning), after which they were given the Mirpur-6 diet, exclusively.

Pups were weighed on P18, P21, P32, and P35 (day of euthanasia). Animals in the *B. infantis*–treated group had significantly greater weights at all time points compared to pups whose mothers had only received the SAM microbiota ($P < 0.01$, two-way repeated-measures ANOVA with Šidák's correction for multiple comparisons; Fig. 6C and table S5D).

Fecal samples were collected from the four dams on P11, P21, and P28 and from their pups on P21, P28, and P35. Sequencing amplicons generated from the 16*S* rRNA genes present in fecal samples from the four dams collected before the *B. infantis* gavage on P11 revealed that they were colonized almost exclusively (>99% relative abundance) by Enterobacteriaceae. Specifically, there were amplicon sequence variants (ASVs) belonging to *Enterococcus* (relative abundance of 61 ± 11%; mean ± SD), *Escherichia/Shigella* (24 ± 12%), and *Klebsiella* (14 ± 7%). In the dams that received *B. infantis* gavages, Enterobacteriaceae were reduced to 64 ± 9% relative abundance in postpartum day 28 fecal samples, with bifidobacteria accounting for 35 ± 9% of their communities (Fig. 6D, table S5E). No bifidobacteria were detected in dams (or their pups) that did not receive the *B. infantis* gavage. Suppression of Enterobacteriaceae was even more pronounced in weaned (P28) pups of the *B. infantis* recipients: 18 ± 5% relative abundance for *Enterococcus*, *Escherichia/Shigella*, and *Klebsiella* ASVs combined compared to 99 ± 1% aggregate relative abundance of these taxa in pups of mothers that received only the SAM microbiota (Fig. 6D and table S5E). These data are consistent with our observations in the SYNERGIE trial, although the

magnitude of suppression of Enterobacteriaceae achieved was larger in the mouse study where *B. infantis* achieved about two orders of magnitude greater absolute abundance than in the probiotic-treated infants in the SYNERGIE trial.

Strain-specific qPCR revealed that dams that had received the *B. infantis* gavage were colonized with both strains at P35, with Bg_2D9 being significantly more abundant than EVC001 (8.17 versus 7.67 $\log_{10}$ genome equivalents/μg of fecal DNA; $P = 0.012$, paired t test). Pups of mothers that had received the Bg_2D9 plus EVC001 strain mixture were colonized with high amounts (>8 $\log_{10}$ genome equivalents/μg of DNA) of *B. infantis* at the earliest time point sampled (P21; Fig. 6E). The absolute abundance of Bg_2D9 attained was significantly greater than that for EVC001, and the difference persisted until euthanasia (P35) ($P < 0.01$, two-tailed Wilcoxon matched-pairs signed-rank test). Thus, this maternal-pup transmission model provided preclinical evidence of the superior competitiveness of the Bg_2D9 strain over the EVC001 strain in the context of a donor SAM microbiota and the Mirpur-6 diet.

DISCUSSION

Disrupted codevelopment of the gut microbiota and its host during early postnatal life is one manifestation of MAM and SAM. A recent randomized controlled trial of a microbiota-directed complementary food formulation designed to repair features of disrupted gut microbiota development evident in 12- to 18-month-old children with MAM living in an urban slum in Dhaka, Bangladesh demonstrated improvements in weight gain that were superior to those obtained with a standard nutritional supplement (*12*). Gut microbiota repair was accompanied by increases in 70 plasma proteins and mediators of various aspects of musculoskeletal and brain development, as well as decreases in proteins involved in inflammatory responses. The SYNERGIE clinical study described here focused on the more severe form of wasting, SAM, manifest in younger (2- to 6-month-old) infants living in the same urban slum in Dhaka, Bangladesh. Compared to chronologically age-matched healthy infants, those with SAM exhibited lower absolute abundances of a key early colonizer of the gut that is a highly efficient consumer of HMOs, *B. longum* subsp. *infantis*. The SYNERGIE trial examined the extent to which treatment with EVC001, a commercially available *B. infantis* strain isolated from a healthy infant in the United States, with or without LNnT supplementation, could colonize the gut microbiota of the Bangladeshi infants with SAM. Daily treatment with the probiotic alone, synbiotic (EVC001 plus LNnT), or placebo (lactose) began after participants had completed an acute phase management protocol for SAM (*45*) and continued for 28 days, followed by an additional 28 days of follow-up. Despite the relatively short exposure period and small cohort size, engraftment of EVC001 was similar in the probiotic and synbiotic arms and was associated with statistically significant improvements in ponderal growth (WAZ and MUAC) and changes in fecal markers indicating reduced gut inflammation. In addition, we observed a positive correlation between fecal IFN-β and the rate of weight gain. A recent study of EVC001-supplemented U.S. infants also revealed suppression of intestinal inflammation markers, a response that was associated with a higher abundance of bifidobacteria in their feces. Moreover, in vitro assays of fecal water from these EVC001-treated U.S. infants disclosed a shift in T cell polarization away from a T helper 2 (T_H2) state toward a T_H1 state, with accompanying suppression of T_H17 cytokines and an increase in IFN-β (*46*). These results raise the possibility that increased intestinal IFN-β may be one factor that contributed to weight gain in children treated with EVC001 in the SYNERGIE study.

The mean absolute abundance of *B. infantis* EVC001 achieved in the SYNERGIE study was ~10- to 100-fold lower than that found in age-matched, untreated, healthy breastfed children from the same locale and was not significantly improved by coadministration of LNnT. Breastmilk consumption by the infants with SAM in our pilot study was low, suggesting that other bifidogenic factors found in breastmilk may have been limiting for robust colonization by EVC001. Thus, alternative approaches to restoring *B. infantis* in the gut microbiota of such infants to levels found in healthy infants might be expected to provide even greater clinical responses than were obtained in our study. As described here, one strategy for achieving this goal involved searching for *B. infantis* strains adapted to the gut environments of infants living in impoverished communities. This search was guided by the reality that whereas exclusive breastfeeding of infants is recommended by the WHO for the first 6 months, in many low-income settings, gruels, animal milk, and complementary foods are often introduced into the diet at an early age for economic or cultural reasons. Given that this dietary landscape may not be optimal for *B. infantis* strains adapted to thrive in the gut microbiota of infants who are exclusively/predominantly breastfed, we conducted an in vivo screen of a consortium of *B. infantis* strains cultured from the fecal microbiota of Bangladeshi infants living in the same impoverished slum community. This screen was executed in gnotobiotic mice fed a diet that reflected the early complementary feeding practices of these children. The staged in vivo competition experiments in gnotobiotic mice revealed a strain (Bg_2D9) isolated from a healthy 12-month-old child that expressed a distinctive repertoire of carbohydrate using genes and had a marked fitness advantage over the other, predominantly milk-adapted, strains lacking these genes. Two expressed gene clusters in particular distinguished this isolate from the others. One gene cluster encoded enzymes that use β-1,3–linked glucosides, which are common constituents of plant cell wall polysaccharides. The other gene cluster encoded components needed for utilization of *N*-linked glycans represented in human and bovine milk proteins. These latter components included two endo-β-*N*-acetylglucosaminidases for releasing sugar moieties of *N*-glycans, an ABC transport system, as well as multiple intracellular glycoside hydrolases involved in using HMOs that may also contribute to metabolism of complex *N*-glycans (fig. S4). We postulate that these endo-β-*N*-acetylglucosaminidases (EndoBI-2 and EndoBB-2) may be able to release sugar moieties from *N*-glycans, which are further transported into the cell via NglABC or Blon_2378-2380. Internalized sugar moieties would then be degraded from their nonreducing ends by the orchestrated action of exo-acting glycoside hydrolases; these glycoside hydrolases include Bga2A, Hex1, Hex2, NanH2, BiAfcA, and BiAfcB, which act on linkages found in both HMOs and complex *N*-glycans containing GlcNAc, fucose, and NANa residues.

The fitness advantage of the *B. infantis* Bg_2D9 strain in gnotobiotic mice was maintained even when the diet was supplemented with LNT and LNnT, suggesting that the abundant nonhuman milk glycans (e.g., β-1,3–linked glucosides and *N*-linked glycans) present in the Mirpur-6 diet may have a dominant effect on shaping the structure of this defined gut microbial community. An alternative possibility is that the Bg_2D9 strain has acquired an enhanced capacity to

use various host mucin glycan degradation products, which play an important role in localizing beneficial mutualists to the intestinal mucosa (*47*).

One limitation of our clinical study was that we did not include an arm with LNnT alone. This was due to our concern that in the absence of a "consumer" such as *B. infantis*, a high dose of this prebiotic may cause gastrointestinal intolerance or other adverse events in an already vulnerable study population. A limitation of the preclinical studies was that although the *ngl* and *bgl* gene clusters in Bg_2D9 appear to be involved in the uptake and utilization of *N*-glycans and plant-derived polysaccharides, establishing that they are important for the fitness of Bg_2D9 in vivo requires testing their functions using targeted genetic deletion and complementation studies. Moreover, experimental tests of the carbohydrate structures that they are able to use are needed. Lastly, the degree to which these or related genomic features are represented in other *B. infantis* isolates recovered from various populations where there is early postnatal consumption of substantial amounts of dietary plant glycans is currently unknown.

Our follow-on studies involved an intergenerational gnotobiotic mouse model of microbial transmission from dam to pups. These experiments revealed that when administered to dams that had previously been colonized with a SAM community, Bg_2D9 became the dominant member of the gut microbiota of their offspring; this dominance was associated with suppression of Enterobacteriaceae and superior weight gain in the pups. Translating these preclinical results into studies to assess the clinical benefits of Bg_2D9 on restoration of healthy growth in infants with SAM will require the development of stable probiotic formulations that can be administered in low-income settings and tested in larger, sufficiently powered trials. Our finding that two of the most prominent HMOs, LNT and LNnT, did not produce marked effects on the fitness of *B. infantis* Bg_2D9 in the staged competition experiments in gnotobiotic mice suggests that Bg_2D9 could be effective as a probiotic without the need for HMO supplements and the added cost that they bring. In addition, the potential for maternal-infant transmission of *B. infantis* raises the question of whether Bg_2D9 could be administered as a probiotic to women during pregnancy. Future clinical studies should ideally include a mechanistic component using multiomics approaches to define the gut microbiota and host physiologic state before, during, and after probiotic treatment (*11*, *12*). The resulting datasets would enable the role of Bg_2D9 and its effects on gut microbiota repair and health to be more clearly and comprehensively characterized. Last, the analysis of *B. infantis* EVC001 and Bg_2D9 strains illustrates how searching for different strains that have evolved to adapt to host habitats with different environmental exposures could yield single-strain or multistrain probiotics or synbiotics with broader host ranges, higher engraftment efficiencies, and thus more generalizable and impactful health benefits.

MATERIALS AND METHODS

Study design

We undertook a three-arm, single-blind, parallel assignment trial, SYNERGIE, which was designed to assess the extent of colonization of the SAM infant gut microbiota by the *B. infantis* EVC001 strain administered daily for 28 days, either alone or in combination with an HMO (LNnT), as compared to a placebo treatment (lactose). The primary outcome measure was the abundance of *B. infantis* in the feces of study participants as measured by qPCR after 28 days of supplementation (EVC001 versus placebo and EVC001 + LNnT versus placebo). Secondary outcome measures included assessment of the bacterial composition of the gut microbiota of participants, changes in their anthropometric indices, and changes in markers of intestinal inflammation after 28 days of treatment. The goal of SYNERGIE was to generate data to justify whether a larger, appropriately powered trial to assess the impact of treatment on clinical outcomes should be conducted.

Gnotobiotic mouse studies were designed to compare the fitness of EVC001 with several other *B. infantis* strains that had been cultured from the fecal microbiota of Bangladeshi infants. Animals were fed a diet (Mirpur-6) representative of that consumed by 6-month-old Bangladeshi children living in an urban slum in Dhaka and given drinking water with or without supplementation with two HMOs, LNT or LNnT. *B. infantis* strains were introduced into germ-free mice in two different formats. The first format involved recently weaned animals that received a consortium of five *B. infantis* strains with or without *B. bifidum*. The second format was designed to mimic mother-to-infant transmission; it entailed initial colonization of female germ-free mice (dams) with an intact uncultured fecal microbiota obtained prior to intervention from an infant with SAM in the SYNERGIE trial, followed by simultaneous gavage of *B. infantis* EVC001 and Bg_2D9. Transfer of the *B. infantis* strains and their absolute abundance was defined by qPCR of fecal DNA. Dams were randomly assigned to each treatment group. All data were generated from all samples without knowledge of treatment group. No data were excluded, and there were no outliers.

Human study

The SYNERGIE study was approved by the Institutional Review Board of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) (protocol no. PR-17112) and registered at ClinicalTrials.gov ("Pilot of a Prebiotic and Probiotic Trial in Young Infants With Severe Acute Malnutrition" NCT03666572). The study was conducted between September 2018 and March 2020 as a single-blind randomized trial involving 2- to 6-month-old infants of either sex presenting with a WLZ score of <−3 or bilateral pedal edema, who had completed an acute phase management protocol for SAM (*45*) in the in-patient ward of Dhaka Hospital at icddr,b. Other inclusion criteria were that the child's caregiver (i) was willing to provide consent for enrollment, (ii) lived locally (<15 km), and (iii) was willing to stay in the Nutritional Rehabilitation Unit (NRU) of Dhaka Hospital for the duration of the hospital stay (~15 days). Infants were excluded if they had septic shock or pneumonia requiring assisted ventilation, acute kidney injury, congenital defects interfering with feeding such as cleft palate, chromosomal anomalies, jaundice, tuberculosis, or ongoing maternal antibiotic usage for breastfeeding infants.

Each participant was assigned to receive the standard-of-care diet (F-75 formula during the acute phase treatment of SAM, F-100 formula during hospital stay for nutritional rehabilitation, and standardized infant formula upon discharge). After acute phase management, infants were transferred to the NRU, enrolled, and randomized to receive either 8×10^9 CFU of *B. infantis* EVC001, 8×10^9 CFU of *B. infantis* EVC001 plus LNnT, or placebo (lactose) for 4 weeks, after which time they were followed for four additional weeks. Randomization was performed in a 1:1 ratio using stratification by age (2 to 3.9 versus 4 to 6 months) to ensure equal allocation

of infants by age group. Treatments were assigned using random permuted blocks of three. The enrollment target was 54 participants, with 18 infants (9 from each age bin) allocated to each arm.

B. infantis EVC001 was administered as a single daily dose mixed with 5 ml of milk (breastmilk or F-100). Each sachet containing 1.6 g of LNnT was mixed with 200 ml of F-100 (WHO, 2002) and administered daily after completion of the antibiotic component of their in-patient acute phase management protocol. The protocol for discharge from the NRU required each participant to achieve a WLZ of ≥−3 (ideally, ≥−2) and, in the case of infants with edematous malnutrition, resolution of their edema. While at home, 1.6 g of LNnT was given twice daily by the caregiver, each time mixed with 120 ml of breastmilk or infant formula (Mead Johnson) without prebiotics or HMOs. Refrigerated storage of the probiotic, consumption of LNnT, and morbidity were all monitored twice a week by field research assistants. Parents/caregivers in the study were blinded to treatment assignment; icddr,b study staff were not blinded because they needed to prepare the LNnT in the infant formula while the infants were hospitalized.

The amount of breastmilk consumed was measured by the test weighing method (i.e., weighing the infant before and after feeding) at the time of enrollment. Breastfeeding was encouraged between feeds throughout the study. Infants who were not breastfed received infant formula at home in addition to their allotted LNnT supplements. A group of nonmalnourished infants (WLZ ≥ −1) who were hospitalized with infections and treated with antibiotics were also given EVC001 in the SYNERGIE study; the results of this analysis will be reported separately.

Fecal samples and anthropometric data were obtained before the start of supplementation (day 1), at the end of supplementation (day 28), and 4 weeks after cessation of treatment (day 56). To preserve bacterial community composition at the time of fecal collection, fecal swabs for DNA-based assays were placed in prelabeled, buffered lysis tubes (Zymo Research) that were flash-frozen in liquid nitrogen within 20 min of defecation (*48*). Fecal specimens used for measurement of intestinal inflammation markers were flash-frozen without added buffer. Samples were stored at −80°C before being shipped to Evolve BioSystems Inc. (Davis, CA) where assays of EVC001 colonization and markers of intestinal inflammation were performed. Data were analyzed from 21 participants who completed the synbiotic arm, 20 from the probiotic arm, and 21 from the placebo arm.

qPCR assays of the absolute abundance of *B. infantis* in human fecal samples

PCR primers

A multiplex qPCR assay was developed to quantify *B. infantis* using primers directed at *Blon_2348* (*28*), *Blon_2176*, and *Bifidobacterium* spp. 16*S* rRNA (*29*). A fourth assay (primer/probe combination) was designed to quantify the absolute abundance of EVC001. The EVC001 genome was first fragmented in silico into 20-bp k-mers; all tiled k-mers were aligned with the genomes of sequenced *B. infantis* strains isolated from Bangladeshi children [plus the *B. breve* type strain (ATCC 15700) as an additional control for specificity] to identify sequences (k-mers) containing ≥2 base mismatches. Contiguous k-mers were stitched together, leading to the identification of a 123-bp sequence in *epsJ* (locus tag N_00627) that was represented in the EVC001 genome but rarely present in Bangladeshi samples. We also developed a qPCR assay directed against a 95-bp target in the *nglA* gene that was present in *B. infantis* Bg_2D9 but not EVC001 (table S3I).

Custom TaqMan probes (Applied Biosystems) with FAM (*Blon_2348*), VIC (*Blon_2176*), and JUN (*Bifidobacterium* spp.) dyes and nonfluorescent QSY quencher were obtained from Thermo Fisher Scientific. Primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA). Forward and reverse primer sets were tested in silico using the National Center for Biotechnology Information (NCBI) Primer-BLAST program with default settings against the NCBI nonredundant nucleotide database to predict specificity and cross-reactivity. The Oligoanalyzer tool (IDT) was used to assess the melting temperature compatibility of primers for multiplexing. To determine sensitivity, a standard curve was generated for the *Blon_2348* and *Blon_2176* qPCR assays with seven 10-fold dilutions of DNA purified from *B. infantis* ATCC 15697.

A similar approach was used for the *epsJ* and *nglA* assays, except that DNA purified from *B. infantis* Bg_2D9 and EVC001, respectively, was used to generate the standard curves. To ascertain the specificity of the assays, the multiplex qPCR panel was run with purified nucleic acids from 16 *Bifidobacterium* strains and 15 non-*Bifidobacterium* human gut bacterial isolates selected on the basis of their 16*S* rRNA gene sequence similarity to members of *Bifidobacterium* (table S1C).

Conditions for PCR

PCR reaction mixtures contained (i) 900 nmol of forward and reverse primers and 250 nmol of TaqMan probes for *Blon_2348*, *Blon_2176*, and *nglA* assays and 150 nmol both 16*S* rRNA gene primers for *Bifidobacterium* sp.; (ii) 5 μl of TaqMan Multiplex Master Mix (Universal Mastermix for *nglA*); (iii) 2.5 μl of genomic DNA [≤7.5 ng of total DNA mass; prepared from fecal samples as previously described; (*11*)]; and (iv) nuclease-free water to make up a total reaction volume of 10 microliters. Assays were performed in duplicate in a 384-well plate format using an Applied Biosystems QuantStudio 6 Flex qPCR instrument. Temperature parameters were as follows: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. With the exception of the *nglA* assay, standard curves were generated for every PCR plate run using seven serial 10-fold dilutions of purified DNA from *B. infantis* type strain (ATCC15697). For the *nglA* assay, standard curves were generated using seven serial 10-fold dilutions of purified DNA from *B. infantis* Bg_2D9.

A singleplex qPCR assay was run using the *epsJ* primers. PCR reactions consisted of (i) 900 nmol of forward and reverse primers and 250 nmol of FAM-labeled TaqMan probe, (ii) 10 μl of TaqMan Universal Master Mix II containing uracil *N*-glycosylase (Applied Biosystems), (iii) 5 μl of a given fecal genomic DNA preparation (diluted 1:10), and (iv) nuclease-free water to make up a total reaction volume of 20 μl. Assays were performed in duplicate in 96-well plate format using a QuantStudio 3 instrument (Thermo Fisher Scientific). Cycling parameters were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, and 60°C for 30 s. Standard curves were generated for each PCR plate using six 10-fold dilutions of genomic DNA extracted from known CFU counts of *B. infantis* EVC001.

Analysis of data

On the basis of the slope and intercept derived from linear regression for cycle thresholds against copy number for the reference, the abundance of each of the three targets was calculated for each sample on the plate. Amplification efficiencies were calculated from the

slope [94.3 ± 2.1% (means ± SD) for the genus *Bifidobacterium* 16*S* rRNA gene assay, 89.6 ± 3.1% for the *Blon_2348* assay, 87.8 ± 2.3% for the *Blon_2176* assay, and 85.7 ± 4.1% for the *nglA* assay]. Coefficient of determination (R^2) values for each standard curve exceeded 0.99. Raw data were normalized for input DNA concentration and expressed in genome equivalents per microgram of fecal DNA [note that because in silico alignment of the 16*S* rRNA gene primers against the genome of *B. infantis* ATCC 15697 using the NCBI Primer-BLAST program (*49*) identified four identical target sequences, calculation of total *Bifidobacterium* abundance in fecal samples was based on the assumption that four copies of this gene are present in each *B. infantis* genome, whereas abundances of the other PCR targets (*Blon_2348*, *Blon_2176*, *epsJ*, and *nglA*) were each based on a single copy/genome]. Because of non-normal distribution (Shapiro-Wilk normality test, $P < 0.001$), qPCR target abundance data were $\log_{10}$-transformed, and Mann-Whitney *U* test was used to determine statistical significance of differences between healthy and SAM children in age bins of 3 to 12, 12 to 18, and 18 to 24 months. A pseudocount of 1 was added to all counts before $\log_{10}$ transformation to convert values below the limit of detection to 0. Log_{10}-transformed individual qPCR target abundance data were fitted with a generalized additive model using the "gam" function from the "mgcv 1.8-31" package in R as a Gaussian family. A generalized cross-validation method was used for smoothing, and age at the time of sample collection was used as a predictor to compare the healthy versus SAM models. The "plot_diff" function from "itsadug 2.4" package in R was used to generate difference curves for the two groups. Log_{10}-transformed *epsJ* qPCR values generated from fecal samples collected during the SYNERGIE study were compared across each time point within treatment arms using Dunn's test of multiple comparisons with Benjamini-Hochberg adjustment in R ("dunn_test" function from the "rstatix 3.6.2" package).

Gnotobiotic mouse studies

All mouse experiments were carried out using protocols approved by the Washington University in St. Louis Institutional Animal Care and Use Committee (protocol no. 20-0322). Mice were housed in plastic flexible-film gnotobiotic isolators (Class Biologically Clean Ltd., Madison, WI) at 23°C under a strict 12-hour light cycle (lights on at 0600 hours). Cages contained autoclaved paper "shepherd shacks" for environmental enrichment.

Quantifying absolute abundances of *B. infantis* and *B. bifidum* strains in mice colonized with the bifidobacterial consortium

The absolute abundances of *B. infantis* strains in fecal samples collected from colonized mice in the experiment described in Fig. 4A were defined by short read shotgun sequencing of community DNA [COPRO-Seq; (*50*)]. To determine absolute abundance, 6.7×10^6 cells of *Alicyclobacillus acidiphilus* DSM 14558 and 29.8×10^6 cells of *Agrobacterium radiobacter* DSM 30147 were added to each weighed frozen fecal pellet collected from each animal on study days 4, 8, 12, 18, and 26 (*51*). Fecal pellets were then subjected to bead beating for 4 min (Mini-BeadBeater-8, BioSpec) in a mixture containing 500 μl of extraction buffer [200 mM NaCl, 200 mM tris (pH 8), and 20 mM EDTA], 210 μl of 20% SDS, 500 μl of phenol/chloroform/isoamyl alcohol (pH 7.9) (25:24:1; Ambion), and 250 μl of 0.1-mm-diameter zirconia beads (BioSpec Products). Samples were centrifuged at 4°C for 4 min at 3220*g*. The aqueous phase was collected; nucleic acids were purified using QIAquick columns (Qiagen) and eluted from the columns into 10 mM tris-Cl (pH 8.5). DNA concentration was quantified (Quant-iT dsDNA assay kit, broad sensitivity; Thermo Fisher Scientific) and adjusted to 0.75 ng/μl with UltraPure water (Milli-Q). COPRO-Seq libraries were prepared using the Nextera DNA Library Prep kit protocol (Illumina) and custom barcoded primers (*52*). Barcoded libraries were sequenced on an Illumina NextSeq instrument [75-nucleotide (nt) unidirectional reads; $2.71 \pm 1.38 \times 10^6$ reads per sample (mean ± SD)].

Reads were demultiplexed and mapped to the sequenced whole genomes of the five *Bifidobacterium* strains plus five "distractor" genomes (*Lactobacillus ruminis* ATCC 27782, *Olsenella uli* DSM 7084, *Pasteurella multocida* USDA-ARS-USMARC 60385, *Prevotella dentalis* DSM 3688, and *Staphylococcus saprophyticus* ATCC 15305). The proportion of total reads mapping to the five distractor genomes for each sample was used to set a conservative threshold (mean + 2 SD) for colonization of an organism in an animal. For each member of the community, absolute abundance was calculated by multiplying the normalized counts of strains by the abundance of *A. acidiphilus* (cell number per normalized count) divided by the sample weight (*53*). Mixed-effects linear models followed by Tukey's post hoc test (GraphPad Prism, version 8) were applied to test for significant interaction of time and abundance of the strains.

Microbial RNA-seq

Cecal contents harvested at the time of euthanasia from gnotobiotic mice in the experiment described in Fig. 4A were flash-frozen in liquid nitrogen and stored at −80°C. For RNA extraction, cecal samples were kept on ice, and the following reagents were added in the following order: (i) 250 μl of acid-washed glass beads (212 to 300 μm diameter; Millipore Sigma; catalog number G1277), (ii) 500 μl of 2× buffer B (200 mM NaCl and 20 mM EDTA), (iii) 210 μl of 20% SDS, and (iv) 500 μl of phenol:chloroform:isoamyl alcohol (25:24:1, pH 4.5; Thermo Fisher Scientific). The mixture was homogenized using a bead beater (Mini-BeadBeater-8, BioSpec) at room temperature for a total of 5 min, with a pause for 2 min on ice after the first 3 min. The mixture was centrifuged (7000*g* for 10 min at 4°C), and RNA was isolated from 500 μl of the aqueous phase using a previously described protocol (*54*). RNA integrity and fragment size were assessed (4200 TapeStation System; Agilent) followed by elimination of genomic DNA by using two sequential deoxyribonuclease (DNase) treatments [Baseline-ZERO DNase (Lucigen) and Turbo DNase (Invitrogen)]. The absence of genomic DNA was verified by qPCR using primers against *Bifidobacterium* spp. 16*S* rRNA gene (*29*). Total RNA was purified (MEGAclear Transcription Clean-Up Kit; Thermo Fisher Scientific) and quantified (Qubit RNA BR Assay Kit. Invitrogen). A 1 μg aliquot was subsequently depleted of rRNA using the Ribo-Zero (Epidemiology/Bacteria) Kit (Illumina) followed by ethanol precipitation. A The SMARTer Stranded RNA-Seq Kit (Takara Bio USA) was used to prepare double-stranded complementary DNA and indexed libraries. Libraries were sequenced using an Illumina NextSeq platform [70-nt unidirectional reads; $5.3 \times 10^7 \pm 2.8 \times 10^6$ reads per sample (mean ± SD); $n = 26$ samples]. The first 5 cycles of sequencing were omitted because this library preparation strategy introduces three nontemplated deoxyguanines. Reads were demultiplexed, checked for quality using FastQC, and were mapped to the genomes of the members of the consortia. Transcript counts were normalized and analyzed using the DESeq2 package in R [version 4.0.2; (*55*)] at the level of

individual strains. For each strain, raw count data were fitted to a negative binomial generalized linear model using the DESeq2 workflow to identify statistically significant differences in gene expression (log_2-fold difference and Wald test *P* value) in the following groups of mice: (i) LNT supplemented versus unsupplemented, (ii) LNnT supplemented versus unsupplemented, and (iii) LNnT supplemented and colonized with or without *B. bifidum*.

Statistical analysis

Clinical data from the SYNERGIE study were entered into pretested case report forms and analyzed using SPSS (20.0 version; Armonk, NY). Demographic, clinical, and socioeconomic data were expressed as median and interquartile range for asymmetric quantitative data. For categorical data, frequency with proportional estimates was used. Kruskal-Wallis *H* test was used to assess the statistical significance of differences between the three treatment arms. Mann-Whitney *U* tests were used to determine statistically significant differences in anthropometric measures between pairs of treatment groups at the indicated time points. The statistical significance of differences in EVC001 engraftment over time within treatment groups was determined using Dunn's test of multiple comparisons with Benjamini-Hochberg adjustment in R (dunn_test function from the rstatix 3.6.2 package). Kruskal-Wallis test with FDR correction was used to assess the statistical significance of the effects of the different interventions on markers of gut inflammation at each time point. Correlations between fecal markers and anthropometric parameters were calculated as Spearman's ρ with FDR-adjusted *P* values.

SUPPLEMENTARY MATERIALS

www.science.org/doi/10.1126/scitranslmed.abk1107
Materials and Methods
Figs. S1 to S4
Tables S1 to S7
MDAR Reproducibility Checklist
References (*56*–*66*)

View/request a protocol for this paper from *Bio-protocol*.

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