Persistent gut microbiota immaturity in malnourished Bangladeshi children

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Therapeutic food interventions have reduced mortality in children with severe acute malnutrition (SAM), but incomplete restoration of healthy growth remains a major problem2-3. The relationships between the type of nutritional intervention, the gut microbiota, and therapeutic responses are unclear. In the current study, bacterial species whose proportional representation define a healthy gut microbiota as it assembles during the first two postnatal years were identified by applying a machine-learning-based approach to 16S ribosomal RNA data sets generated from monthly faecal samples obtained from birth onwards in a cohort of children living in an urban slum of Dhaka, Bangladesh, who exhibited consistently healthy growth. These age-discriminatory bacterial species were incorporated into a model that computes a ‘relative microbiota maturity index’ and ‘microbiota-for-age Z-score’ that compare postnatal assembly (defined here as maturation) of a child’s faecal microbiota relative to healthy children of similar chronologic age. The model was applied to twins and triplets (to test for associations of these indices with genetic and environmental factors, including diarrhoea), children with SAM enrolled in a randomized trial of two food interventions, and children with moderate acute malnutrition. Our results indicate that SAM is associated with significant relative microbiota immaturity that is only partially ameliorated following two widely used nutritional interventions. Immaturity is also evident in less severe forms of malnutrition and correlates with anthropometric measurements. Microbiota maturity indices provide a microbial measure of human postnatal development, a way of classifying malnourished states, and a parameter for judging therapeutic efficacy. More prolonged interventions with existing or new therapeutic foods and/or addition of gut microbes may be needed to achieve enduring repair of gut microbiota immaturity in childhood malnutrition and improve clinical outcomes.

Severe acute malnutrition and moderate acute malnutrition (MAM) are typically defined by anthropometric measurements: children are classified as having SAM if their weight-for-height Z-scores (WHZ)4 are below three standard deviations (−3 s.d.) from the median of the World Health Organization (WHO) reference growth standards, whereas those with WHZ between −2 and −3 s.d. are categorized as having MAM. SAM and MAM typically develop between 3 and 24 months after birth4. The relationships between the type of nutritional intervention, the gut microbiota, and therapeutic responses are unclear. In the current study, bacterial species whose proportional representation define a healthy gut microbiota as it assembles during the first two postnatal years were identified by applying a machine-learning-based approach to 16S ribosomal RNA data sets generated from monthly faecal samples obtained from birth onwards in a cohort of children living in an urban slum of Dhaka, Bangladesh, who exhibited consistently healthy growth. These age-discriminatory bacterial species were incorporated into a model that computes a ‘relative microbiota maturity index’ and ‘microbiota-for-age Z-score’ that compare postnatal assembly (defined here as maturation) of a child’s faecal microbiota relative to healthy children of similar chronologic age. The model was applied to twins and triplets (to test for associations of these indices with genetic and environmental factors, including diarrhoea), children with SAM enrolled in a randomized trial of two food interventions, and children with moderate acute malnutrition. Our results indicate that SAM is associated with significant relative microbiota immaturity that is only partially ameliorated following two widely used nutritional interventions. Immaturity is also evident in less severe forms of malnutrition and correlates with anthropometric measurements. Microbiota maturity indices provide a microbial measure of human postnatal development, a way of classifying malnourished states, and a parameter for judging therapeutic efficacy. More prolonged interventions with existing or new therapeutic foods and/or addition of gut microbes may be needed to achieve enduring repair of gut microbiota immaturity in childhood malnutrition and improve clinical outcomes.

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Food is a major factor that shapes the proportional representation of organisms present in the gut microbial community (microbiota), and its gene content (microbiome). The microbiota and microbiome in turn have an important role in extracting and metabolizing dietary ingredients11-14. To investigate the hypothesis that healthy postnatal development (maturation) of the gut microbiota is perturbed in malnutrition12, we monitored 50 healthy Bangladeshi children monthly during the first 2 years after birth (25 singletons, 11 twin pairs, 1 set of triplets; 996 faecal samples collected monthly; see Methods and Supplementary Tables 1–3). By identifying bacterial taxa that discriminate the microbiota of healthy children at different chronologic ages, we were able to test our hypothesis by studying children presenting with SAM, just before, during, and after treatment with two very different types of food intervention, as well as children with MAM. The results provide a different perspective about malnutrition; one involving disruption of a microbial facet of our normal human postnatal development.

To characterize gut microbiota maturation across unrelated healthy Bangladeshi children living in separate households, faecal samples were collected at monthly intervals up to 23.4 ± 0.5 months of age in a training set of 12 children who exhibited consistently healthy anthropometric scores (WHZ, −0.32 ± 0.98 (mean ± s.d.) 22.7 ± 1.5 faecal samples per child; Supplementary Table 4a). The bacterial component of their faecal microbiota samples was characterized by V4-16S rRNA sequencing (Supplementary Table 5) and assigning the resulting reads to operational taxonomic units (OTUs) sharing ≥ 97% nucleotide sequence identity (see Methods; a 97%-identity OTU is commonly construed as representing a species-level taxon). The relative abundances of 1,222 97%-identity OTUs that passed our filtering criterion15 were regressed against the chronologic age of each child at the time of faecal sample collection using the Random Forests machine learning algorithm16. The regression explained 73% of the variance related to chronologic age. The significance of the fit was established by comparing fitted to null models in which age labels of samples were randomly permuted with respect to their 16S rRNA microbiota profiles (P = 0.0001, 9,999 permutations). Ranked lists of all bacterial taxa, in order of ‘age-discriminatory importance’, were determined by considering those taxa, whose relative abundance values when permuted have a larger marginal increase in mean squared error, to be more important (see Methods). Tenfold cross-validation was used to estimate age-discriminatory performance as a function of the number of top-ranking taxa according to their feature importance scores. Minimal improvement in predictive performance was observed when including taxa beyond the top 24 (see Supplementary Table 6 for the top 60). The 24 most age-discriminatory taxa identified by Random Forests are shown in Fig 1a in rank order of their contribution to the predictive accuracy of the model and were selected as inputs to a sparse 24-taxon model.

To test the extent to which this sparse model could be applied, we applied it, with no further parameter optimization, to additional monthly faecal samples collected from two other healthy groups of children: 13 singletons (WHZ, −0.4 ± 0.8 (mean ± s.d.)) and 25 children from a birth-cohort study of twins and triplets (WHZ, −0.5 ± 0.7 (mean ± s.d.)), all born and raised in Mirpur, Bangladesh (Supplementary Table 4b, c). We...
Figure 1 | Bacterial taxonomic biomarkers for defining gut-microbiota maturation in healthy Bangladeshi children during the first 2 years of life. a. Twenty-four age-discriminatory bacterial taxa were identified by applying Random Forests regression of their relative abundances in faecal samples against chronologic age in 12 healthy children (n = 272 faecal samples). Shown are 97%-identity OTUs with their deepest level of confident taxonomic annotation (also see Supplementary Table 6), ranked in descending order of their importance to the accuracy of the model. Importance was determined based on the percentage increase in mean-squared error of microbiota age prediction when the relative abundance values of each taxon were randomly permuted (mean importance ± s.d., n = 100 replicates). The insert shows tenfold cross-validation error as a function of the number of input 97%-identity OTUs used to regress against the chronologic age of children in the training set, in order of variable importance (blue line). b. Microbiota age predictions in healthy singletons and triplets used to train the Random Forests model were compared to another birth cohort of healthy singletons used to train the 24 bacterial taxon model (brown, each circle represents an individual faecal sample). The trained model was subsequently applied to two sets of healthy children: 13 singletons set aside for model testing (green circles, n = 276 faecal samples) and another birth cohort of 25 twins and triplets (blue circles, n = 448 faecal samples). The curve is a smoothed spline fit between microbiota age and chronologic age in the validation sets (right two panels of b), accounting for the observed sigmoidal relationship (see Methods). c. Heatmap of mean relative abundances of the 24 age-predictive bacterial taxa plotted against the chronologic age of healthy singletons used to train the Random Forests model, and correspondingly in the healthy singletons, and twins and triplets used to validate the model (hierarchical clustering performed using the Spearman rank correlation distance metric).

a birth cohort of healthy singletons used to train the 24 bacterial taxon model (brown, each circle represents an individual faecal sample). The trained model was subsequently applied to two sets of healthy children: 13 singletons set aside for model testing (green circles, n = 276 faecal samples) and another birth cohort of 25 twins and triplets (blue circles, n = 448 faecal samples). The curve is a smoothed spline fit between microbiota age and chronologic age in the validation sets (right two panels of b), accounting for the observed sigmoidal relationship (see Methods). c. Heatmap of mean relative abundances of the 24 age-predictive bacterial taxa plotted against the chronologic age of healthy singletons used to train the Random Forests model, and correspondingly in the healthy singletons, and twins and triplets used to validate the model (hierarchical clustering performed using the Spearman rank correlation distance metric).
followed by randomization to either an imported peanut-based RUTF intervention or an intervention with locally produced, rice- and lentil-based therapeutic foods (Khichuri and Halwa; see Methods and Supplementary Table 13 for compositions of all foods used during nutritional rehabilitation). During this second ‘nutritional rehabilitation phase’ (1.3 ± 0.7 weeks long) children received 150–250 kcal kg⁻¹ body weight per day of RUTF or Khichuri–Halwa (3–5 g protein kg⁻¹ per day), plus micronutrients including iron. Children were discharged from the hospital after the completion of this second phase; during the ‘post-intervention phase’, periodic follow-up examinations were performed to monitor health status. Faecal samples were obtained during the acute phase before treatment with Khichuri–Halwa or RUTF, then every 3 days during the nutritional rehabilitation phase, and monthly thereafter during the post-intervention follow-up period.

There was no significant difference in the rate of weight gain between the RUTF and Khichuri–Halwa groups (10.9 ± 4.6 versus 10.4 ± 5.4 g kg⁻¹ body weight per day (mean ± s.d.); Student’s t-test, $P = 0.7$). The mean WHZ at the completion of nutritional rehabilitation was significantly improved in both treatment groups ($-3.1 ± 0.7$ (mean ± s.d.) RUTF, $P < 0.001$; and $-2.7 ± 1.6$ Khichuri–Halwa, $P < 0.0001$), but not significantly different between groups ($P = 0.15$). During follow-up, WHZ remained significantly lower compared to healthy children ($-2.1 ± 1.2$, Khichuri–Halwa; $-2.4 ± 0.8$ RUTF versus $-0.5 ± 1.1$ for healthy, $P < 0.0001$; Extended Data Fig. 4a). Children in both treatment arms also remained markedly below normal height and severely underweight throughout the follow-up period (Extended Data Fig. 4b, c).

The Random Forests model derived from healthy children was used to define relative microbiota maturity for children with SAM at the time of enrollment, during treatment, at the end of either nutritional intervention, and during the months of follow-up. The results revealed that compared to healthy children, children with SAM had significant microbiota immaturity at the time that nutritional rehabilitation was initiated and at cessation of treatment (Dunnett’s post-hoc test, $P < 0.0001$ for both groups; Fig. 2b). Within 1 month of follow-up, both groups had improved significantly. However, improvement in this metric was short-lived for the RUTF and Khichuri–Halwa groups, with regression to significant immaturity relative to healthy children beyond 4 months after treatment was stopped (Fig. 2b and Supplementary Table 14). MAZ, like relative microbiota maturity, indicated a transient improvement after RUTF intervention that was not durable beyond 4 months. In the Khichuri–Halwa group, relative microbiota maturity and MAZ improved following treatment, but subsequently regressed, exhibiting significant differences relative to healthy children at 2–3 months, and >4 months after cessation of treatment (Fig. 2b and Supplementary Table 14).

Both food interventions had non-durable effects on other microbiota parameters. The reduced bacterial diversity associated with SAM persisted after Khichuri–Halwa and only transiently improved with RUTF (Extended Data Fig. 5 and Supplementary Table 14). We identified a total of 220 bacterial taxa that were significantly different in their proportional representation in the faecal microbiota of children with SAM compared to healthy children; 165 of these 220 97%-identity OTUs were significantly diminished in the microbiota of children with SAM during the longer term follow-up period in both treatment groups (Extended Data Figs 6 and 7, and Supplementary Table 15).

Although the majority of children in both treatment arms of the SAM study were unable to provide faecal samples before the initiation of antibiotic treatment due to the severity of their illness, a subset of nine children each provided one or two faecal samples ($n = 12$) before administration of parenteral ampicillin and gentamicin, and oral amoxicillin. Microbiota immaturity was manifest at this early time-point before antibiotics in these nine children (relative microbiota maturity: $-5.15 ± 0.9$ months versus $-0.03 ± 0.1$ for the 38 reference healthy controls; Mann–Whitney, $P < 0.0001$). Sampling these nine children after treatment with parenteral and oral antibiotics but before initiation of RUTF or Khichuri–Halwa (6 ± 3.6 days after hospital admission) showed that there was no significant effect on microbiota maturity (Wilcoxon matched-pairs rank test, $P = 1$). When pre-antibiotic faecal samples from these nine children were compared to samples collected at the end of all treatment interventions (dietary and antibiotic, 20 ± 9 days after admission), no significant differences in relative microbiota maturity (Wilcoxon, $P = 0.7$), MAZ, bacterial diversity (or WHZ) were found (Extended Data Fig. 8a–d). This is not to say that these interventions were without effects on overall community composition: opposing changes in the relative abundance of Streptococcaceae and Enterobacteriaceae were readily apparent.
and malnourished populations living in different locales, representing need to be clarified since they themselves may be therapeutic candidates in addition to food-based interventions. The functional roles (niches) of Next-generation probiotics using gut-derived taxa may also be required for an extended period of administration of existing or new types of food supplements that can help recondition for generations on a diet will respond more favourably to nutrition interventions than those who were already malnourished. The results show that the model generalizes to a cohort of 47 Malawian twins and triplets, aged 0.4–25.1 months, and that the model has higher predictive accuracy compared to the random forests model, using analysis of variance (ANOVA) of linear mixed models followed by Dunnett’s post-hoc comparisons. The threshold for statistical significance was set at p < 0.05. In conclusion, definition of microbiota maturity using bacterial taxonomic biomarkers that are highly discriminatory for age in healthy children has provided a way to characterize malnourished states, including whether responses to food interventions endure for prolonged periods of time beyond the immediate period of treatment. RUTF and Khichuri– Halwa produced improvements in microbiota maturity indices that were not significant. Addressing the question of how to achieve durable responses in children with varying degrees of malnutrition may involve extending the period of administration of existing or new types of food interventions. One testable hypothesis is that a population’s microbiota conditioned on a diet will respond more favourably to nutrient supplementation based on food groups represented in that diet. Next-generation probiotics using gut-derived taxa may also be required in addition to food-based interventions. The functional roles (niches) of the age-discriminatory taxa identified by our Random Forests model need to be clarified since they themselves may be therapeutic candidates and/or form the basis for low cost field-based diagnostic assessments. Systematic analyses of microbiota maturation in different healthy and malnourished populations living in different locales, representing different lifestyles and cultural traditions, may yield a taxonomy-based model that is generally applicable to many countries and types of diagnostic and therapeutic assessments. Alternatively, these analyses may demonstrate a need for geographic specificity when constructing such models (and diagnostic tests or therapeutic regimens). Two observations are notable in this regard. First, expansion of our sparse model from 24 to 60 taxa yielded similar results regarding the effects of diarrhea in healthy individuals, MAM and SAM (and its treatment with RUTF and Khichuri–Halwa) on microbiota maturity (see Supplementary Notes). Second, we applied the model that we used for Bangladeshi children to healthy children in another population at high risk for malnutrition. The results show that the model generalizes to a cohort of 47 Malawian twins and triplets, aged 0.4–25.1 months, who were concordant for healthy status in a previous study (WHZ, −0.23 ± 0.97 (mean ± s.d.); Supplementary Table 18). Age-discriminatory taxa identified in healthy Bangladeshi children show similar age-dependent changes in their representation in the microbiota of healthy Malawian children, as assessed by the Spearman rank correlation metric (Extended Data Fig. 10c, d).

The question of whether microbiota immaturity associated with SAM and MAM is maintained during and beyond childhood also underscores the need to determine the physiologic, metabolic and immunologic consequences of this immaturity, and how they might contribute to the associated morbidities and sequelae of malnutrition, including increased risk for diarrhoea disease, stunting, impaired vaccine responses, and cognitive abnormalities. Our study tests a testable hypothesis: namely, that assessments of microbiota maturation, including in the context of the maternal–infant dyad, will provide a more comprehensive view of normal human development and of developmental disorders, and generate new directions for preventive medicine. Testing this hypothesis will require many additional clinical studies but answers may also arise from analyses of gut microbiota samples that have already been stored from previous studies.

**METHODS SUMMARY**

All subjects lived in Dhaka, Bangladesh (see Methods and Supplementary Notes for anthropologic assessment of Mirpur, an urban slum in Bangladesh, where most subjects resided). Informed consent was obtained and studies were conducted using protocols approved by the ICDDR,B, Washington University, and University of Virginia institutional review boards (IRBs). Linear mixed models were applied to test hypotheses in repeated measurements of relative abundance of 97%–identity OTUs and maturation metrics in time-series profiling of faecal microbiota. To account for similarity between observations from repeated sampling of the same individual and family, we fit random intercepts for each subject in the case of adults and singletons, nested these intercepts within each family in the case of twins and triplets, and included age as a fixed-effect covariate, while testing the significance of associations between the microbiota and specified host and environmental factors. Differences between microbiota maturation metrics in each treatment phase of SAM were compared to values at enrollment in each treatment group, and to healthy children within the same age range (excluding samples from children used to train the Random Forests model), using analysis of variance (ANOVA) of linear mixed models followed by Dunnett’s post-hoc comparisons.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions S.S. and J.I.G. designed the metagenomic study, S.H., T.A., R.H., M.A.A., M.M., W.A.P. Jr designed and implemented the clinical monitoring and sampling for the SAM trial, participated in patient recruitment, sample collection, sample preservation and/or clinical evaluations; S.S. generated the 16S rRNA data with assistance from M.F.M. and B.D.M.; A.B. and J.D. performed the anthropology study; S.S., T.Y., Q.Z., L.G.V., M.J.B., M.A.P. and J.I.G. analysed the data; S.S. and J.I.G. wrote the paper.

Author Information 16S rRNA sequences, generated from faecal samples in raw format prior to post-processing and data analysis, have been deposited at the European Nucleotide Archive (accession number PRJEB5482). Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.I.G. (jgordon@wustl.edu).
METHODS

Singleton birth cohort. Full details of the design of this now-complete birth cohort study have been described previously.11 Faecal microbiota samples were profiled from 25 children who had consistently healthy anthropometric measures based on quarterly (every 3 months) measurements (Supplementary Table 1). The WHZ threshold used for ‘healthy’ (on average above −2 s.d.) was based on median weight and height measurements obtained from age- and gender-matched infants and children by the Multi-Centre Growth Reference study of the World Health Organization. Clinical parameters, including diarrhea and antibiotic consumption associated with each of their faecal samples are provided in Supplementary Table 2.

A second group studied from this singleton cohort consisted of 33 children sampled cross-sectionally at 18 months, including those who were incorporated as healthy reference controls, and those with a WHZ < −2 who were classified with MAM (Supplementary Table 17).

Twins and triplets birth cohort. Mothers with multiple pregnancy, identified by routine clinical and sonographic assessment at the Radda Maternal Child Health and Family Planning (MCH-FP) Clinic in Dhaka, were enrolled in a prospective longitudinal study (n = 11 mothers with twins, 1 mother with triplets). The zygosity of twin pairs and triplets was determined using plasma DNA and a panel of 96 polymorphic single-nucleotide polymorphisms (SNPs) (Center for Inherited Disease Research, Johns Hopkins University). Four twin pairs were monzygotic; six were dizygotic, and the set of triplets consisted of a monzygotic pair plus one fraternal sibling (Supplementary Table 1; note that one of the 11 twin pairs could not be tested for zygosity because plasma samples were not available). Information about samples from healthy twins, triplets and their parents, including clinical parameters associated with each faecal sample, is provided in Supplementary Tables 2 and 3.

The three healthy Bangladeshi groups used for model training and validation had the following WHZ scores: −0.22 ± 1 (mean ± s.d.; 12 singletons randomized to the training set), −0.44 ± 0.8 (13 singletons randomized to one of the two validation sets), and −0.46 ± 0.7 (twins and triplets in the other validation set) (Supplementary Table 4). The average number of diarrheal episodes in the singleton training set, the singleton validation set, and the twin and triplet validation set (4.4, 4.6 and 1.7, respectively) was comparable to values reported in previous surveys of another cohort of 0–2-year-old Bangladeshi children (4.25 per child per year).

There were no significant differences in the number of diarrheal episodes per year per child and the number of diarrheal days per year per child between the singleton training and validation sets (Student’s t-test, P = 0.5). Moreover, across all training and validation sets, neither of these diarrheal parameters correlated with mean age-adjusted Shannon diversity indices (Spearman’s Rho, −0.18 and −0.12, P = 0.22 and 0.4, respectively). The fraction of faecal samples collected from each child where oral antibiotics had been consumed within the prior 7 days was not significantly different between the training and two validation sets (one-way ANOVA, P = 0.14; see Supplementary Table 4).

Severe acute malnutrition study. Sixty-four children in the Nutritional Rehabilitation Unit of ICDDR,B, Dhaka Hospital suffering from SAM (defined as having a WHZ lower than −3 s.d. and/or bilateral pedal oedema) were enrolled in a randomized intervention trial to compare an imported peanut-based RUTF, Plumpy’Nut (Nutriset Plumpifyield, India) and locally produced Khichuri–Halwa (clinical trial NCT01331044). Initially, children were stabilized by rehydration and feeding ‘suji’, which contains whole bovine milk powder, rice powder, sugar and soybean oil (approximately 100 kcal kg⁻¹ body weight per day, including 1.5 g protein kg⁻¹ per day). Children were then randomized to the Khichuri–Halwa or RUTF groups. Khichuri consists of rice, lentils, green leafy vegetables and soybean oil; Halwa consists of wheat flour (atta), lentils, molasses and soybean oil. Children randomized to the Khichuri–Halwa treatment arm also received milk suji ‘100’ during their nutritional rehabilitation phase (a form of suji with a higher contribution of calories from milk powder compared to suji provided during the acute phase). RUTF is a ready-to-use paste that does not need to be mixed with water; it contains of pea nut paste mixed with dried skimmed milk, vitamins and minerals (energy density 5.4 kcal g⁻¹). Khichuri and Halwa are less energy-dense than RUTF (1.45 kcal g⁻¹ and 2.4 kcal g⁻¹, respectively, see Supplementary Table 13 for a list of ingredients for all foods used during nutritional rehabilitation).

The primary outcome measurement, rate of weight gain (kg kg⁻¹ per day), along with improvement in WHZ after nutritional rehabilitation are reported by child in Supplementary Table 10. Faecal samples were collected before randomization to the RUTF and Khichuri–Halwa treatment arms, every 3 days during nutritional rehabilitation and once a month during the follow-up period (information associated with each faecal sample is provided in Supplementary Table 1).

Anthropologic study. To obtain additional information about household practices in the Mirpur slum of Dhaka, in-depth semi-structured interviews and observations were conducted over the course of 1 month in nine households (n = 30 individuals). This survey, approved by the Washington University and ICDDR,B IRBs, involved three ICDDR,B field research assistants, and three senior scientific staff in the ICDDR,B Centre for Nutrition and Food Security, plus two anthropologists affiliated with Washington University in St. Louis. Parameters that might affect interpretation of metagenomic analyses of gut microbial-community structure were noted, including information about daily food preparation, food storage, personal hygiene and childcare practices.

Characterization of the bacterial component of the gut microbiota by V4-16S rRNA sequencing. Faecal samples were frozen at −20 °C within 30 min of their collection. A subset of samples were subsequently stored at −80 °C. Total DNA was isolated by bead-beating in phenol and chloroform, purified further (QIAquick column), quantified (Qubit) and subjected to polymerase chain reaction (PCR) using primers directed at variable region 4 (V4) of bacterial 16S rRNA genes. Bacterial V4-16S rRNA data sets were generated by multiplex sequencing of amplimers prepared from 1,897 faecal DNA samples (26,580 ± 26,312 (mean ± s.d.) reads per sample, paired-end 162- or 250-nucleotide reads; Illumina MiSeq platform; Supplementary Table 5). Reads of 250 nucleotides in length were trimmed to 162 nucleotides, then all reads were processed using previously described custom scripts, and overlapped to 253-nucleotide fragments spanning the entire V4 amplicon.

Mock communities, consisting of mixtures of DNAs isolated from 48 sequenced bacterial members of the human gut microbiota combined in one equivalent and two intentionally varied combinations, were included as internal controls in the Illumina MiSeq runs. Data from the mock communities were used for diversity and precision-sensitivity analyses employing methods described previously.14,21,22

Reads with ≥ 97% nucleotide sequence identity (97%-identity) across all studies were binned into operational taxonomic units (OTUs) using QIIME (v 1.5.0), and matched to entries in the Greengenes reference database (version 4feb2011).21,22 Reads that did not map to the Greengenes database were clustered de novo with UCLUST at 97%-identity and retained in further analysis. A total of 1,222 97%-identity OTUs were found to be present at or above a level of confident detection (0.1% relative abundance) in at least two faecal samples from all studies. Taxonomy was assigned based on the naive Bayesian RDP classifier version 2.4 using 0.8 as the minimum confidence threshold for assigning a level of taxonomic classification to each 97%-identity OTU.

Definition of gut-microbiota maturation in healthy children using Random Forests. Random Forests regression was used to regress relative abundances of OTUs in the time-series profiling of the microbiota of healthy singletons against their chronologic age using default parameters of the R implementation of the algorithm (R package ‘randomForest’, ntree = 10,000, using default miny of p/5 where p is the number of input 97%-identity OTUs (features))23. The Random Forests algorithm, due to its non-parametric assumptions, was applied and used to detect both linear and nonlinear relationships between OTUs and chronologic age, thereby identifying taxa that discriminate different periods of postnatal life in healthy children. A rarefied OTU table at 2,000 sequences per sample served as input data. Ranked lists of taxa in order of Random Forests reported ‘importance’ were determined over 100 iterations of the algorithm. To estimate the minimal number of top ranking age-discriminatory taxa required for prediction, the rfcv function implemented in the ‘randomForest’ package was applied over 100 iterations. A sparse model consisting of the top 24 taxa was then trained on a single collection set of 12 healthy singletons (372 faecal samples). Without any further parameter optimization, this model was validated in other healthy children (13 singletons, 25 twins and triplets) and then applied to samples from children with SAM and MAM. A smoothing spline function was fit between microbiota age and chronologic age of the host (at the time of faecal sample collection) for healthy children in the validation sets to which the sparse model was applied.

Alpha diversity comparisons. Estimates of within-sample diversity were made at a rarefaction depth of 2,000 reads per sample. A linear regression was fit between the Shannon diversity index (SDI) and postnatal age in the 50 healthy children using a mixed model (see the additional details regarding statistical methods, below). A set of 100 surrogate samples of the coefficient of variation of SDIs within each sample set was extracted, residuals of this regression were defined as a ASDI metric, and associations of this metric with clinical parameters were tested in the cohort of healthy twins and triplets. To test for differences in SDI as a function of health status and chronologic age in malnourished children, we compared the distribution of age-adjusted ASDIs in children with SAM between treatment phases.

Detection of associations of bacterial taxa with nutritional status and other parameters. Relative abundances of 97%-identity OTUs were used in linear mixed models as response variables to test for associations with clinical metadata as predictors. For each comparison, we restricted our analysis to 97%-identity OTUs and bacterial families whose variable values had a level of confidence detection (0.1%) in a minimum of 1% of samples in each comparison. Pseudocounts of 1 were added to 97%-identity OTUs to account for variable depth of sequencing between samples, and relative abundances were arcsin-square-root-transformed to approximate homoscedasticity when applying linear models. P values of associations
of factors with the relative abundance of bacterial taxa were computed using ANOVA type III (tests of fixed effects), subjected to Benjamini–Hochberg false discovery rate (FDR) correction.

**Enteropathogen testing.** Clinical microscopy was performed for all faecal samples collected at monthly intervals from the singleton birth cohort and from healthy twins and triplets, and screened for *Entamoeba histolytica*, *Entamoeba dispar*, *Escherichia coli*, *Blastocystis hominis*, *Trichomonas hominis*, Coccidian-like bodies, *Giardia lamblia*, *Ascaris lumbricoides*, *Trichuris Tricuria*, *Ancylostoma duodenale*/Necator americanus, *Hymenolepsis nana*, *Endolimax nana*, *Isospora belli* and *Chilomastix mesnili*. The effects of enteropathogens, detected by microscopy on relative microbiota maturity, MAZ and SDI were included in our analysis of multiple environmental factors in Extended Data Fig. 2 and Supplementary Table 7. In cases in which children presented with SAM plus diarrhoea, faecal samples collected before nutritional rehabilitation were cultured for *Vibrio cholerae*, *Shigella flexneri*, *Shigella boydi*, *Shigella sonnei*, *Salmonella enterica*, *Aeromonas hydrophila* and *Hafnia alvei*. See Supplementary Tables 10 and 19 for results of enteropathogen testing.

**Additional details regarding statistical methods.** Linear mixed models were applied to test for associations of microbiota metrics (relative microbiota maturity, MAZ and SDI) with genetic and environmental factors in twins and triplets. Log-likelihood ratio tests and F-tests were used to perform backward elimination of non-significant random and fixed effects27. Relative microbiota maturity, MAZ and SDI were defined at different phases of treatment and at defined periods of follow-up (<1 month, 1–2, 3–4 and >4 months after completion of the RUTF or Khichuri–Halwa nutritional intervention) in children with SAM relative to healthy children. ‘Treatment phase’ was specified as a categorical multi-level factor in a univariate mixed model with random by-child intercepts. Dunnett’s post-hoc comparison procedure was performed to compare each treatment phase relative to healthy controls and relative to samples collected at enrollment in each food intervention group.

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Extended Data Figure 1 | Illustration of the equations used to calculate 'relative microbiota maturity' and 'microbiota-for-age Z-score'. a, b, The procedure to calculate both microbiota maturation metrics are shown for a single faecal sample from a focal child (pink circle) relative to microbiota age values calculated in healthy reference controls. These reference values are computed in samples collected from children used to validate the Random-Forests-based sparse 24-taxon model and are shown in a, as a broken line of the interpolated spline fit and in b, as median ± s.d. values for each monthly chronologic age bin from months 1 to 24.

\[ \text{Relative microbiota maturity (units in mo)} = \frac{\text{Microbiota age of fecal sample from focal child}}{\text{Microbiota age of interpolated spline fit from healthy reference controls}} \]

\[ \text{Microbiota-for-Age Z score (MAZ)} = \frac{\text{Microbiota age of fecal sample from focal child}}{\text{Median microbiota age of healthy reference controls within same monthly bin as focal child}} - \text{Standard deviation of microbiota ages of healthy reference controls within same monthly bin as focal child} \]
Extended Data Figure 2 | Transient microbiota immaturity and reduction in diversity associated with diarrhoea in healthy twins and triplets. a, The transient effect of diarrhoea in healthy children. Seventeen children from 10 families with healthy twins or triplets had a total of 36 diarrhoeal illnesses where faecal samples were collected. Faecal samples collected in the months immediately before and following diarrhoea in these children were examined in an analysis that included multiple environmental factors in the ‘healthy twins and triplets’ birth cohort. Linear mixed models of these specified environmental factors indicated that ‘diarrhoea’, ‘month following diarrhoea’ and ‘presence of formula in diet’ have significant effects on relative microbiota maturity, while accounting for random effects arising from within-family and within-child dependence in measurements of this maturity metric. The factors ‘postnatal age’, ‘presence or absence of solid foods’, ‘exclusive breastfeeding’, ‘enteropathogen detected by microscopy’, ‘antibiotics’ as well as ‘other periods relative to diarrhoea’ had no significant effect. The numbers of faecal samples (n) are shown in parenthesis. Mean values ± s.e.m. are plotted. *P < 0.05, ***P < 0.001. See Supplementary Table 7 for the effects of dietary and environmental covariates. b, Effect of diarrhoea and recovery on age-adjusted Shannon diversity index (SDI). Mean values of effect on SDI ± s.e.m. are plotted. *P < 0.05, **P < 0.01.
Between cohabiting spouses
Between non-cohabiting adults
Mother-to-child
Father-to-child

Within each father
Within each mother

Less similar
More similar

Unweighted UniFrac distance
Hellinger distance

Between unrelated age-matched children
Between co-twins/co-triplets

OTU ID
Taxonomic annotation
Bacteroides thetaiotaomicron
Bacteroides sp.
Bifidobacterium longum
Bifidobacterium sp.
Bifidobacterium sp.
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Extended Data Figure 3 | Gut microbiota variation in families with twins and triplets during the first year of life. a, Maternal influence. Heatmap of the mean relative abundances of 13 bacterial taxa (97%-identity OTUs) found to be statistically significantly enriched in the first month post-partum in the faecal microbiota of mothers (see column labelled 1) compared to microbiota sampled between the second and twelfth months post-partum (FDR-corrected $P < 0.05$; ANOVA of linear mixed-effects model with random by-mother intercepts). An analogous heatmap of the relative abundance of these taxa in their twin or triplet offspring is shown. Three of these 97%-identity OTUs are members of the top 24 age-discriminatory taxa (blue) and belong to the genus *Bifidobacterium*. b–e, comparisons of maternal, paternal and infant microbiota. Mean values ± s.e.m. of Hellinger and unweighted UniFrac distances between the faecal microbiota of family members sampled over time were computed. Samples obtained at postnatal months 1, 4, 10 and 12 from twins and triplets, mothers and fathers were analysed ($n = 12$ fathers; 12 mothers; 25 children). b, Intrapersonal variation in the bacterial component of the maternal microbiota is greater between the first and fourth months after childbirth than variation in fathers. c, Distances between the faecal microbiota of spouses (each mother–father pair) compared to distances between all unrelated adults (male–female pairs). The microbial signature of co-habitation is only evident 10 months following childbirth. d, e, The degree of similarity between mother and infant during the first postpartum month is significantly greater than the similarity between microbiota of fathers and infants (d) while the faecal microbiota of co-twins are significantly more similar to one another than to age-matched unrelated children during the first year of life (e). For all distance analyses, Hellinger and unweighted UniFrac distance matrices were permuted 1,000 times between the groups tested. $P$ values represent the fraction of times permuted differences between tested groups were greater than real differences between groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 
Extended Data Figure 4 | Anthropometric measures of nutritional status in children with SAM before, during and after both food interventions.

a–c, Weight-for-height Z-scores (WHZ) (a) height-for-age Z-scores (HAZ) (b) and weight-for-age Z-scores (WAZ) (c). Mean values ± s.e.m. are plotted and referenced to national average anthropometric values for children surveyed between the ages of 6 and 24 months during the 2011 Bangladeshi Demographic Health Survey (BDHS).
Extended Data Figure 5 | Persistent reduction of diversity in the gut microbiota of children with SAM. Age-adjusted Shannon diversity index for faecal microbiota samples collected from healthy children (n = 50), and from children with SAM at various phases of the clinical trial (mean values ± s.e.m. are plotted). The significance of differences between SDI at various stages of the clinical trial is indicated relative to healthy controls (above the bars) and versus the time of enrollment before treatment (below the bars). *P < 0.05, **P < 0.01, ***P < 0.001 (post-hoc Dunnett’s multiple comparison procedure of linear mixed models). See Supplementary Table 14.
Extended Data Figure 6 | Heatmap of bacterial taxa significantly altered during the acute phase of treatment and nutritional rehabilitation in the microbiota of children with SAM compared to similar-age healthy children. Bacterial taxa (97%-identity OTUs) significantly altered (FDR-corrected $P < 0.05$) in children with SAM are shown (see Supplementary Table 15 for $P$ values and effect size for individual taxa). Three groups of bacterial taxa are shown: those enriched before the food intervention (a); those enriched during the follow-up phase compared to healthy controls (b); and those that are initially depleted but return to healthy levels (c). Members of the top 24 age-discriminatory taxa are highlighted in blue. Note that there were no children represented in the Khichuri–Halwa arm under the age of 12 months during the ‘follow-up after 3 months’ period.

| OTU ID | Taxonomic annotation |
|--------|----------------------|
| 142054 | Enterobacteriaceae sp. |
| 210289 | Enterobacteriaceae sp. |
| 9715  | Enterobacteriaceae sp. |
| 563485 | Enterobacteriaceae sp. |
| 436723 | Enterobacteriaceae sp. |
| 512514 | Enterobacteriaceae sp. |
| 310585 | Enterobacteriaceae sp. |
| 307581 | Enterobacteriaceae sp. |
| 307582 | Enterobacteriaceae sp. |
| 505780 | Escherichia coli |
| 113558 | Enterobacteriaceae sp. |
| 250706 | Enterobacteriaceae sp. |
| 540230 | Enterococcus faecalis |
| 15362  | Streptococcus genticulatus |
| 249155 | Streptococcus sp. |
| 316587 | Streptococcus genticulatus |

**a** Taxa enriched at enrollment in children with SAM relative to healthy

**b** Taxa enriched during follow-up in children with SAM relative to healthy

**c** Taxa depleted before but not after intervention in SAM relative to healthy

Relative abundance of a bacterial taxon

| Minimum | Maximum |
|---------|---------|

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Extended Data Figure 7 | Heatmap of bacterial taxa altered during long-term follow-up in the faecal microbiota of children with SAM compared to similar-age healthy children. a, b. Bacterial taxa (97%-identity OTUs) significantly altered (FDR-corrected \( P < 0.05 \)) in children with SAM are shown (see Supplementary Table 15 for \( P \) values and effect sizes for individual taxa).

a. Taxa depleted across all phases of SAM relative to healthy. b. Those depleted during the follow-up phase. Members of the top 24 age-discriminatory taxa are highlighted in blue. Note that there were no children under the age of 12 months represented in the Khichuri–Halwa treatment arm during the ‘follow-up after 3 months’ period.
Extended Data Figure 8 | Effects of antibiotics on the microbiota of children with SAM. Plots of microbiota and anthropometric parameters in nine children sampled before antibiotics (abx), after oral amoxicillin plus parenteral gentamicin and ampicillin, and at the end of the antibiotic and dietary interventions administered over the course of nutritional rehabilitation in the hospital. All comparisons were made relative to the pre-antibiotic sample using the non-parametric Wilcoxon matched-pairs rank test, in which each child served as his or her own control. a–c, Microbiota parameters, plotted as mean values ± s.e.m., include relative microbiota maturity, microbiota-for-age Z-score (MAZ), and SDI. WHZ scores are provided in d. e, f, The two predominant bacterial family-level taxa showing significant changes following antibiotic treatment. ns, not significant; **P < 0.01.
Extended Data Figure 9 | Relative microbiota maturity and MAZ correlate with WHZ in children with MAM.   a–c, WHZ are significantly inversely correlated with relative microbiota maturity (a) and MAZ (b) in a cross-sectional analysis of 33 children at 18 months of age who were above and below the anthropometric threshold for MAM (Spearman’s Rho = 0.62 and 0.63, respectively; ***P < 0.001). In contrast, there is no significant correlation between WHZ and microbiota diversity (c). d–l, Relative abundances of age-discriminatory 97%-identity OTUs that are inputs to the Random Forests model that are significantly different in the faecal microbiota of children with MAM compared to age-matched 18-month-old healthy controls (Mann-Whitney U-test, P < 0.05). Box plots represent the upper and lower quartiles (boxes), the median (middle horizontal line), and measurements that are beyond 1.5 times the interquartile range (whiskers) and above or below the 75th and 25th percentiles, respectively (points) (Tukey’s method, PRISM software v6.0d). Taxa are presented in descending order of their importance to the Random Forests model. See Extended Data Fig. 10a, b.
Extended Data Figure 10 | Cross-sectional assessment of microbiota maturity at 18 months of age in Bangladeshi children with and without MAM, plus extension of the Bangladeshi-based model of microbiota maturity to Malawi. **a**, Children with MAM (WHZ lower than $-2$ s.d.; grey) have significantly lower relative microbiota maturity (a) and MAZ (b) compared to healthy individuals (blue). Mean values $\pm$ s.e.m. are plotted with $**P < 0.01$ (Mann–Whitney U-test). See Extended Data Fig. 9 for correlations of metrics of microbiota maturation with WHZ and box-plots of age-discriminatory taxa whose relative abundances are significantly different in children with MAM relative to healthy reference controls. **c**, Microbiota age predictions resulting from application of the Bangladeshi 24-taxon model to 47 faecal samples (brown circles) obtained from concordant healthy Malawian twins and triplets are plotted versus the chronologic age of the Malawian donor (collection occurred in individuals ranging from 0.4 to 25.1 months old). The results show the Bangladeshi model generalizes to this population, which is also at high risk for malnutrition (each circle represents an individual faecal sample collected during the course of a previous study\(^\text{11}\)). **d**, Spearman rho and significance of rank order correlations between the relative abundances of age-discriminatory taxa, and the chronologic age of all healthy Bangladeshi children described in the present study as well as concordant healthy Malawian twins and triplets. *$P < 0.05$.  

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