Antibiotic Treatment Leads to Fecal *Escherichia coli* and Coliphage Expansion in Severely Malnourished Diarrhea Patients

Malnutrition predisposes to diarrhea and diarrhea adversely affects the nutritional status creating a vicious cycle. The role of the gut microbiome in malnutrition is an active research area. Parenteral antibiotics are recommended by the World Health Organization in hospitalized pediatric patients with severe acute malnutrition (SAM) presenting signs of infections. Stool microbiota data for such patients are, however, lacking. To fill this gap, we studied the stool microbiota in 19 SAM patients from Bangladesh hospitalized with acute diarrhea (AD) and compared it with that of matched 20 healthy control subjects (HC) (Supplementary Table 1). SAM-AD patients were treated with parenterally administered gentamycin and ampicillin, whereas HC received no antibiotics for at least a month before sample collection.

16S rRNA and metagenome sequencing showed a marked increase of *Escherichia* and *Klebsiella* abundances in SAM-AD over HC (Figures 1A, 1C, and 1A), but not of *Streptococcus* (Figure 1B). Compared with HC, SAM-AD showed a reduced microbiota diversity (Figure 1D) and a decrease in *Prevotella*, *Blautia*, *Ruminococcus*, *Faecalibacterium*, *Megasphaera*, and *Bifidobacterium* (Figures 1A and 2A). SAM-AD patients showed a 10-fold-lower 16S copy number of stool bacteria than HC (Figure 1E), which was partially compensated by a 2-fold higher stool frequency.

Rotavirus was the dominant pathogen (Supplementary Table 2) in SAM-AD, contradicting reports on protection mechanisms could play a role, such as...
Figure 2. Stool microbiome analysis by metagenome sequencing. (A) Taxonomical attribution of sequences from 9 HC subjects and 18 SAM-AD cases to the indicated bacteria and viruses. (B) Attribution of the listed virulence factor genes to cases and control subjects given as counts per million genes. Abundance of sugar and sugar derivate (C, D) and antibiotic-resistance genes (E) in counts per million. (F) Abundance of indicated phage sequences expressed as percentage of total attributed sequences normalized for genome size by MetaPhlAn2. (G) Correlation between the abundance of the reads attributed to Escherichia phage phAPEC8 at hospitalization and change in abundance of Escherichia over a period of 1.5 days estimated by16S rRNA sequencing.

increased accessibility or modified physiology of the bacterial host cells, for example as a consequence of immune system response to bacteria. Among SAM-AD patients, the abundance of sequences attributed to Escherichia phage phAPEC8 was negatively correlated with the abundance of its host (Figure 2A, SparCC [1000 bootstraps]; -0.52; N = 18; P = 0.008). It is unclear, however, whether coliphage expansion could lead to a collapse of E.coli population because high abundance of phage at enrollment was not associated with a greater decrease of E.coli abundance over a period of approximately 1 day (Figure 2G). Longer time series are necessary to determine whether bacteriophages could indeed control the expansion of host bacteria in the gut. Previous attempt to treat E.coli-associated AD with a mixture of T4 bacteriophages had failed to
demonstrate clinical benefit; however, the E coli dominance was much more pronounced in antibiotic-treated SAM-AD patients of the present study than in children with AD.

A marked increase of fecal E coli abundance at the expense of bifidobacteria was also described in European newborns not suffering from diarrhea but treated parenterally with ampicillin and gentamicin for suspected sepsis.

Postantibiotics expansion of E coli and Salmonella typhimurium was also observed in mice model where it was shown to be a consequence of streptomycin-induced production of galactarate and glucarate in host’s cecum. This host-dependent mechanism may have contributed to the observed expansion of E coli, although the main driver was likely the high levels of antibiotic resistance displayed by E coli in Bangladesh. We think that the treatment with antibiotics rather than malnutrition and diarrhea was the main cause of the observed microbiota alteration, because Bangladeshi children with AD showed an increased abundance of commensal streptococci over control subjects, whereas children with SAM displayed a shift to a less mature fecal microbiota composition but not a marked E coli expansion.

Antibiotic-induced Enterobacteriaceae expansion studied in mice has been shown to be involved in the disruption of the symbiosis between colonocytes and obligate anaerobic butyrate producers, resulting in a vicious cycle whereby colonocyte metabolism is subverted to permit the outgrowth of oxygen-tolerant, nitrate-dependent Enterobacteriaceae. It is known that antibiotic treatment in humans may lead to diarrhea even in a presumed absence of obligate pathogens (antibiotic-associated diarrhea), but the microbiota of pediatric antibiotic-associated diarrhea has not been studied.

Currently, there is no evidence from humans that the antibiotic-induced expansion of normally commensal Enterobacteriaceae could be detrimental. However, the observations from animal models suggest that this is a possibility that should be investigated.

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Conflicts of interest
The authors disclose no conflicts.

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Supplementary Information

Patient Characteristics

The study was approved by the Ethical Review Committee of the International Center for Diarrhoeal Diseases Research in Dhaka, Bangladesh (icddr,b) as protocol #PR-14081. A total of 19 children with severe acute malnutrition (SAM) and acute diarrhea (AD) and 20 matched healthy control (HC) children were enrolled in Dhaka, Bangladesh during the winter season 2014–2015 (Supplementary Table 1). SAM-AD showed z scores <−3 indicative of severe underweight, severe stunting, and severe wasting. Because healthy children do not present to the icddr,b hospital, the control children were recruited at a field clinic maintained by icddr,b (Nandipara), whose population corresponds socioeconomically to the children hospitalized at icddr,b.

SAM-AD patients received reduced osmolarity oral rehydration solution supplemented with zinc for diarrhea treatment. SAM-AD is typically associated with a case-fatality rate of 30%–50% because of a high rate of manifest or developing infectious comorbidity (pneumonia, bacteremia, urinary tract infections). Therefore, as recommended by the World Health Organization, all SAM-AD were treated on hospitalization with ampicillin (100 mg/kg/day in 4 doses for 48 hours by intravenous injection) and gentamicin (6 mg/kg/d in 2 doses by intramuscular injection) followed with amoxicillin (50 mg/kg/day in 3 divided doses given orally for 5 days). None of the control subjects had received antibiotics for a month before stool sampling.

No difference was seen for family income (HC, 7:8:5 and SAM, 6:9:4 with very low:low:moderate income; P = .90), maternal education (illiterate: HC, 2; SAM, 1; P = .96; primary school: HC, 12; SAM, 11; P = .57), or numbers of siblings (for 1, 2, 3, >3 children: HC, 10:4:5:1; SAM, 9:9:1:0; P = .13). Vaccination status was comparable in both groups (HC, 10 and SAM, 13 vaccination completed or running; P = .4) and feeding mode at 6 months of age (HC, 15:1:4; SAM, 10:4:5 exclusive breastfeeding vs formula feeding vs partial breastfeeding; P = .24). However, the 2 groups differed for sex (HC, 12:8 and SAM, 3:16 for female: male; P = .01) (Supplementary Table 4) and previous exposure to cow’s milk (HC, 7 and SAM, 18; P = .003).

Stool samples were obtained from the patients at enrollment into the study and the time of transfer to refeeding ward (1.6 + 1.4 days later). Samples were frozen at −80°C immediately after collection.

16S rRNA Sequencing

Total stool DNA was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer’s instructions, except for addition of a series of mechanical disruption steps using a FastPrep apparatus and Lysing Matrix B tubes (MP Biochemicals, Santa Ana, CA).^12^ 16S variable region V3 to V4 were polymerase chain reaction amplified using universal DNA primers with dual indexing^3^ and sequenced with MiSeq reagent kit V3 (Illumina Inc, San Diego, CA) as previously described.^4^ Raw sequence data were analyzed using Mothur V.1.33.0 21 and QIIME V.1.8.2 software packages.^5,6^ Paired-end sequences were demultiplexed and joined as described.^7^ Chimera were identified and removed. Open reference OTUs picking at 97% identity used pick_ottus.py, with options usearch_ref.^7^ Taxonomy assignment used RDP Classifier^8^ on representative sequences. The resulting multiple alignments were used to build a phylogenetic tree with the FastTree method.^7^ Alpha-diversity was reported as the average of 10 rarefactions.

Metagenomics

Stool DNA was extracted using MoBio PowerMag Microbiome DNA Isolation Kit (QIAGEN, Hilden, Germany) on an epMotion M5073 (Vaudaux-Eppendorf AG, Basel, Switzerland) followed by Zymo DNA Clean & Concentrator Kit (Zymo Research, Irvine, CA). Library preparation was done according to the Nextera XT protocol from Illumina. The quality and quantity check was based on LabChip GX Touch HT (Perkin Elmer, Waltham, MA) results. Sequencing was performed on HiSeq 2500 using chemistry HighOutput v4 PE125 (Illumina). The paired-end reads were filtered using KneadData v0.5.1 (https://bitbucket.org/biobakery/kneaddata), which included quality filtering based on Trimmomatic and excluded reads mapping to the human genome. A median number of 3.2 × 10^7^ reads and a minimum of 2.2 × 10^7^ reads per sample were evaluated.

Taxonomic profiles were generated with MetaPhlAn2 2.5.0.10 Functional annotation was performed with HUMAnn2 v0.7.1 and integrated into pathways from the MetaCyc database.^11,12^ The number of reads are first normalized by the length of the reference genome and then by million reads (counts per million). Functional annotation was used to calculate the abundance of antibiotic resistance genes from CARD database.^13^

ShortBRED^14^ was used to profile the metagenomics samples for virulence factors from the VFDB database.^15^ The mapped reads were first normalized by million reads and then by the length of reference sequence (RPKM).

The crucial step of bead-beating was included in both protocols of DNA extraction, ensuring an equal efficiency of DNA extraction from Firmicutes, Actinobacteria, and Bacteroidetes. We found a good correlation between MetaPhlAn and 16S rRNA analysis excluding major differences introduced by the 2 DNA extraction methods.

Pathogen Detection

Pathogens were identified by TaqMan Array Card (Thermo Fisher Scientific, Waltham, MA) detecting 19 enteropathogens,^16^ providing semi-quantitative cycle threshold values for each target. We normalized the values with respect to the total bacteria by quantitative polymerase chain reaction using universal primers.^17^ We considered pathogens as detected when the cycle threshold value was lower than in HC children. For the pathogens that were not detected in HC (Salmonella, Vibrio cholera, Ascaris, Cryptosporidium, and Trichuris) a threshold of 35 was imputed. This was complemented by screening of the metagenome sequences for pathogen taxa, defined as taxa targeted by
TaqMan Array Card, including pathotypes of *Escherichia coli* and associated virulence factors.

**Data Availability**
16S rDNA and metagenome reads are available under the Bio Project accession numbers SRP100410 and SRP100895.

**Statistics**
Because microbiota abundance have a nonnormal distribution, nonparametric tests were used. The data met all assumptions of nonparametric tests. Nonparametrical tests do not require homoscedasticity for group comparison. If not otherwise mentioned the 2-sided Mann-Whitney test was used for continuous variables and a chi-square test for categorical variables. Data are shown as individual data points or as boxplots. In the boxplots the box represent the quintiles of the dataset, whereas the whisker extend to 1.5 times the interquartile range.

Because the HC and SAM-AD differed in female/male ratio, we explored potential impact of sex on the major findings of the study. However, the children of the 2 sexes displayed similar findings (Supplementary Table 4).

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Supplemental Graphical Summary.
### Supplementary Table 1. Baseline Characteristics of SAM-AD Case and Matched HC

|                          | HC          | SAM-AD      | P value |
|--------------------------|-------------|-------------|---------|
| N                        | 20          | 19          |         |
| Age child, mo            | 13.0 (10.8 to 16.0) | 13.0 (9.5 to 18.5) | .989    |
| Age mother, y            | 25.5 (22.0 to 30.5) | 24.0 (22.0 to 27.5) | .411    |
| Weight, kg               | 8 (8 to 9)  | 5.9 (5.1 to 6.7) | 2.93e-07|
| Height, cm               | 73.5 (69.8 to 77.4) | 68.0 (64.5 to 70.7) | .0064   |
| Mid arm circumference, cm| 13 (13 to 14) | 12 (11 to 12) | 3.27e-05|
| Weight for age z score   | -1 (-1 to -1) | -4 (-5 to -4) | 1.01e-07|
| Height for age z score   | -1 (-2 to -1) | -3 (-4 to -3) | 4.35e-06|
| Body mass index          | 16 (15 to 16) | 13 (12 to 13) | 1.18e-07|
| Weight for height z score| -1 (-1 to -0) | -3 (-4 to -3) | 1.01e-07|
| Body mass index z score  | -0 (-1 to 0)  | -3.4 (-4.2 to -2.8) | 1.37e-07|
| Mid arm circumference z score | -1 (-1 to -1) | -3 (-3 to -3) | 2.34e-05|
| Rectal temperature, °C   | 36.5 (36.0 to 36.7) | 37.2 (37.0 to 37.2) | 1.08e-07|
| Pulse, min⁻¹             | 110.0 (100.0 to 120.0) | 132.0 (130.0 to 136.0) | 7.23e-08|
| Respiration rate, min⁻¹  | 30.0 (30.0 to 32.0) | 36.0 (35.0 to 36.0) | 4.46e-07|
| Vomiting, d⁻¹            | 0.0 (0.0 to 0.0)  | 0.0 (0.0 to 2.0) | .00345  |
| Duration of diarrhea, d  | 4 (4 to 4)    | 4 (4 to 4)  |         |
| Stool frequency, d⁻¹     | 2.5 (2.5 to 2.5) | 5.0 (3.5 to 7.0) | 6.15e-06|
| Systolic blood pressure, mm Hg | 90.0 (90.0 to 90.0) | 90.0 (90.0 to 90.0) | .0166   |
| Diastolic blood pressure, mm Hg | 60.0 (60.0 to 60.0) | 60.0 (60.0 to 60.0) | .101    |
| Exclusive breastfeeding, mo | 6.0 (5.8 to 6.0)  | 6.0 (2.5 to 6.0) | .0578   |
| Number of siblings       | 1.5 (1.0 to 3.0) | 2.0 (1.0 to 2.0) | .541    |

NOTE. Values are medians (interquartile range: first, third quartile). P values are calculated by a 2-sided Mann-Whitney test. Categorical variables were compared by chi-square test.
### Supplementary Table 2. Pathogen Detection in Stools of SAM-AD Patients

| Patient ID | TaqMan Pathogen taxa | Escherichia coli pathogens | Virulence factors |
|------------|-----------------------|----------------------------|------------------|
| 506        | Adenovirus (8)        | neg                        | neg              |
| 511        |Cryptosporidium (3)    | neg                        | neg              |
| 503        | Ascaris (3)           | neg                        | EAEC-aaiC (0.01) |
| 516        | neg                   | Shigella (1)               | neg              |
| 518        |Vibrio cholerae (7)    | neg                        | EPEC-bfp (36)    |
| 517        | Cryptosporidium (15)  | neg                        | neg              |
| 513        | NA                    | neg                        | EAECA-aaiC (0.01) |
| 515        | Norovirus (5)         | neg                        | EAECA-aaiC (120) |
| 501        | Rotavirus (10)        | neg                        | neg              |
| 502        |Rotavirus (5)          | neg                        | EAECA-aaiC (275) |
| 504        |Rotavirus (12)         | neg                        | neg              |
| 505        |Rotavirus (8)          | neg                        | EAECA-aaiC (202) |
| 507        |Rotavirus (8)          | Adenovirus (3)             | neg              |
| 508        | Rotavirus (6)         | neg                        | neg              |
| 510        | Rotavirus (6)         | neg                        | ETEC-lt (346)   |
| 514        | Rotavirus (11)        | neg                        | neg              |
| 519        | Rotavirus (10)        | NA                         | NA               |
| 509        | neg                   | Shigella (0.5)             | neg              |
| 512        | Cryptosporidium (9)   | Shigella (2)               | neg              |

**NOTE.** TaqMan, results of detection of 19 pathogens with TaqMan array card (difference to threshold expressed as cycle threshold), *E. coli* pathotypes and the detected virulence factors are indicated, neg, no pathogen detection; pathogen taxa, percentage of taxons determined in metagenome sequencing of the indicated pathogen, neg, <0.1% of taxa; *E. coli* pathogens, the indicated virulence genes of the specified *E. coli* pathotype with % of identified *E. coli* genes, neg, <0.01 of genes; virulence factors, gene read number corrected per million reads and length of target gene coverage.

NA, the corresponding sample was not investigated by metagenome sequencing.
### Supplementary Table 3. Top 10 Pathways Significantly Enriched in SAM-AD Over HC in Stool Metagenome Data

| Description                                                                 | HC | SAM-AD | Q   |
|-----------------------------------------------------------------------------|----|--------|-----|
| Superpathway of L-arginine and L-ornithine degradation                      | 13 | 105    | 0.0002 |
| Superpathway of L-arginine, putrescine, and 4-aminobutanoate degradation   | 13 | 105    | 0.0002 |
| D-glutarate degradation II                                                  | 21 | 161    | 0.0004 |
| Phytol degradation                                                          | 45 | 391    | 0.0004 |
| D-galacturate degradation II                                                | 20 | 120    | 0.0007 |
| Superpathway of D-glutarate and D-galacturate degradation                   | 20 | 120    | 0.0007 |
| Methylphosphonate degradation I                                            | 11 | 94     | 0.0007 |
| Superpathway of fermentation                                                | 48 | 179    | 0.0012 |
| Phytate degradation I                                                       | 28 | 218    | 0.0012 |
| NAD/NADP-NADH/NADPH mitochondrial interconversion                           | 32 | 177    | 0.0012 |

NOTE. Median counts per million reads as assessed by 1-sided Mann-Whitney U test corrected for multiple testing by the Benjamini-Hochberg procedure. NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

### Supplementary Table 4. The Main Variables That Were Evaluated in the Study, Stratified By Sex

|                           | HC     | SAM-AD |
|---------------------------|--------|--------|
|                           | Female | Male   | Female | Male   |
| 16S rRNA sequencing       |        |        |
| N                         | 12     | 8      | 2      | 16     |
| *Bifidobacterium* (proportion of reads) | 25.0 (6.62–47.1) | 24.9 (18.8–55.6) | 0.024 (0.02–0.02) | 0.405 (0.05–6.07) |
| *Escherichia* (proportion of reads) | 1.46 (0.71–9.76) | 6.32 (1.95–24.1) | 62.6 (46.6–78.6) | 57.0 (20.8–73.8) |
| Diversity (Faith index)   | 4.007 (2.86–4.37) | 3.308 (3.08–3.51) | 1.360 (1.28–1.44) | 1.306 (1.06–2.19) |
| Shotgun metagenomics      |        |        |
| N                         | 12     | 3      | 16     |
| Antibiotic resistance genes (counts per million reads)                      | 955 (767–1213) | 608 (530–1326) | 4117 (3063–4140) | 3978 (2115–6495) |
| D-galactarate degradation pathway (counts per million reads)               | 13.6 (6.94–30.50) | 20.3 (5.65–25.30) | 85.7 (78.80–119.01) | 127 (70.90–186) |
| Phage sequences (percentage of total sequences, normalized for genome size)| 0.469 (0.00–2.30) | 0.000 (0.00–0.00) | 73.0 (49.3–85.4) | 0.794 (0.04–15.8) |

NOTE. Median and interquartile range are displayed.