Supplementary Abstract (following CONSORT 2010 guidelines for abstracts)

**Background**
Healthy development of the gut microbiome provides long-term health benefits. Children raised in countries with high infectious disease burdens, like Bangladesh, are frequently exposed to antibiotics and diarrheal pathogens, which perturb gut microbiome assembly. A recent double-blind cluster-randomized controlled in two low-income, densely populated communities in urban Bangladesh found automated water chlorination of shared taps to be an effective strategy for reducing child diarrhea and antibiotic use. Here, we performed exploratory analyses to evaluate the effect of this intervention on children's gut microbiota, including the bacterial pathogens and antibiotic resistance genes (ARGs) they harbored.

**Methods**
The trial was implemented from July 2015 – December 2016 in two low-income communities in urban Bangladesh: Tongi, a community outside Dhaka city, and Dhaka Uddan, a community within Dhaka city. In brief, 100 shared water taps that served as the primary source of drinking water for children younger than five years old were identified in both communities, then randomly assigned (1:1) to have their drinking water automatically chlorinated at the point of collection by a solid tablet chlorine doser (intervention group) or to be treated by a visually identical doser that supplied vitamin C (active control group). Approximately 500 children were enrolled in each group at baseline. Stool samples were collected one year after the start of the intervention. Following a child's stool production, caretakers were instructed to inoculate a small amount of stool in RNALater (a fecal preservative). Field staff then transported samples to the laboratory, where they were frozen at -80C upon arrival and remained frozen during subsequent shipment to the United States. Study staff stratified available RNALater-preserved stool samples by group, study site, and three pre-specified age strata (6-14 months, 15-30 months, 31 months and older) corresponding to distinct phases of gut microbiome development, then randomly selected samples for short-read, paired-end 150 bp sequencing of total stool DNA. The primary outcome was differentially abundant bacterial genera between treatment and control children across different phases of gut microbiome development. This analysis was not pre-specified in the original trial. Both study participants and researchers selecting samples, processing samples, and performing data analysis were unaware of which households were served by chlorinated taps (double-blinded). This trial is registered with ClinicalTrials.gov, number NCT02606981, and is completed.

**Findings**
We examined fecal metagenomes from 130 children from the control (n=64) and treatment groups (n=66). Water chlorination was associated with increased abundance of human enterobacteria, but shifts were small in magnitude. We observed no effects on the overall richness or diversity of taxa, and the prevalence of bacterial pathogens was similar across the two groups. However, several clinically relevant ARGs were relatively more abundant in the gut microbiomes of treatment children.

**Interpretation**
While water chlorination affects the developing gut microbiome of children in urban Bangladesh, its impacts are quite small. Overall, the benefits of automated water chlorination with regards to preventing child diarrhea, reducing antibiotic use, and protecting child health appear to outweigh any potential changes to gut microbiome development in this setting.

**Funding**
The Thrasher Research Fund and The World Bank Strategic Impact Evaluation Fund.

Tables

Supplementary Table 1. Differentially abundant bacterial genera among 130 treatment and control children participating in an automated water chlorination intervention trial in Bangladesh, overall and by three age strata corresponding to distinct phases of gut microbiome development.

| Genera | Treatment Coefficient (95% CI)[a] | fdr-corrected *p-value*[b] |
|---|---|---|
| Overall | | |
| *Akkermansia* | 2.42 (1.87, 2.98) | 4.55E-14 |
| *Escherichia* | 1.11 (0.67, 1.55) | 2.20E-06 |
| *Flavonifractor* | 0.89 (0.5, 1.27) | 1.83E-05 |
| *Phascolarctobacterium* | 2.11 (1.54, 2.69) | 6.44E-11 |
| Age 6-14 months | | |
| *Alysiella* | 0.99 (0.46, 1.52) | 0.00148608 |
| *Aureimonas* | 0.26 (0.13, 0.4) | 0.00097828 |
| *Bombilactobacillus* | 1.31 (0.74, 1.88) | 0.00020148 |
| *Bremerella* | 1.14 (0.63, 1.65) | 0.00025458 |
| *Candidatus Nitrotoga* | 1.22 (0.84, 1.6) | 2.8404E-06 |
| *Candidatus Reidiella* | -1.24 (-1.79, -0.68) | 0.00025753 |
| *Candidatus Vampirococcus* | -1.15 (-1.66, -0.63) | 0.00025989 |
| *Chromohalobacter* | -0.82 (-1.14, -0.51) | 4.3267E-05 |
| *Ferriphaselus* | 1.41 (0.73, 2.09) | 0.00055415 |
| *Furfurilactobacillus* | -1.87 (-2.4, -1.33) | 8.967E-07 |
| *Fusobacterium* | 2.55 (1.37, 3.74) | 0.00037227 |
| *Inhella* | 0.67 (0.42, 0.93) | 4.1354E-05 |
| *Jinshanibacter* | 1.11 (0.65, 1.58) | 0.00013004 |
| *Lactobacillus* | -4.59 (-5.8, -3.39) | 2.3712E-07 |
| *Laribacter* | 1.23 (0.74, 1.73) | 7.5377E-05 |
| *Latilactobacillus* | -0.78 (-1.03, -0.53) | 4.8752E-06 |
| *Leuconostoc* | -3.22 (-4.52, -1.92) | 8.3036E-05 |
| *Mariprofundus* | 0.48 (0.24, 0.71) | 0.00064749 |
| *Mycetohabitans* | 2.12 (1.73, 2.51) | 6.8067E-10 |
| *Natronoglycomyces* | -2.06 (-3.13, -1) | 0.00107337 |
| *Nitrosospira* | -1.14 (-1.73, -0.55) | 0.00104238 |
| *Paenarthrobacter* | -1.16 (-1.77, -0.56) | 0.00109374 |
| *Paraphotobacterium* | 1.79 (0.91, 2.67) | 0.00068419 |
| *Phascolarctobacterium* | 2.68 (1.45, 3.92) | 0.00036026 |
| *Plesiomonas* | 5.04 (3.31, 6.77) | 1.1257E-05 |
| *Propionimicrobium* | 1.15 (0.76, 1.53) | 8.2463E-06 |
| *Rothia* | -1.06 (-1.7, -0.43) | 0.00358964 |

| | | |
|---|---|---|
| *Sphingosinithalassobacter* | -0.97 (-1.29, -0.65) | 6.1356E-06 |
| *Streptococcus* | -1.66 (-2.55, -0.77) | 0.00144412 |
| *Telmatocola* | 1.68 (1.11, 2.24) | 9.4924E-06 |
| *Terricaulis* | -2.13 (-3.06, -1.21) | 0.00019193 |
| *Thermanaerovibrio* | 2.46 (1.3, 3.63) | 0.0004472 |
| *Thermodesulfatator* | -2.02 (-2.95, -1.09) | 0.00035531 |
| *Usitatibacter* | 0.39 (0.21, 0.58) | 0.0004252 |
| Age 15-30 months | | |
| *Bergeyella* | -1.11 (-1.62, -0.59) | 1.22E-04 |
| *Casimicrobium* | -0.95 (-1.5, -0.4) | 1.40E-03 |
| *Ciceribacter* | -1.46 (-2.29, -0.63) | 1.26E-03 |
| *Comamonas* | -1.49 (-2.2, -0.78) | 1.54E-04 |
| *Erysipelatoclostridium* | 0.95 (0.49, 1.41) | 2.19E-04 |
| *Flavonifractor* | 1.76 (1.27, 2.25) | 9.23E-09 |
| *Fusobacterium* | 1.07 (0.46, 1.69) | 1.36E-03 |
| *Lancefieldella* | 1.15 (0.61, 1.7) | 1.46E-04 |
| *Leuconostoc* | -2.95 (-4.02, -1.89) | 2.24E-06 |
| *Methylacidimicrobium* | -0.84 (-1.28, -0.39) | 6.42E-04 |
| *Microcystis* | -0.85 (-1.33, -0.37) | 1.24E-03 |
| *Parabacteroides* | 1.19 (0.52, 1.85) | 1.05E-03 |
| *Pasteurella* | -0.65 (-0.96, -0.34) | 1.55E-04 |
| *Phascolarctobacterium* | 2.3 (1.44, 3.16) | 4.12E-06 |
| *Piscirickettsia* | -0.95 (-1.45, -0.44) | 6.16E-04 |
| *Psychrobacter* | -0.61 (-0.94, -0.28) | 7.69E-04 |
| *Tuwongella* | -0.9 (-1.4, -0.4) | 1.06E-03 |
| *Verrucomicrobium* | -0.75 (-1.18, -0.32) | 1.24E-03 |
| *Weissella* | -1.76 (-2.77, -0.76) | 1.28E-03 |
| Age 31-61 months | | |
| *Akkermansia* | 3.03 (2.09, 3.98) | 1.01E-07 |

[a]Treatment coefficients generated by the R package *corncob*, representing the additive change in the logit-transformed relative abundance of bacterial genera between treatment and control children.
[b]Benjamini-Hochberg method was used to correct for multiple comparisons.

Supplementary Table 2. Effect of extended exposure to a water chlorination intervention (greater than or equal to 6 months) on differentially abundant genera among treatment and control children aged 15 months and older.

| | 15-61 months (n=103) | | 15-61 months and exposed to the intervention for at least 6 months (n=91) | |
|---|---|---|---|---|
| Genera | Treat. Coef. (95% CI)[a] | fdr-corrected *p-value*[b] | Treat. Coef. (95% CI)[a] | fdr-corrected *p-value*[b] |
| *Akkermansia* | 2.44 (1.84, 3.03) | 1.90E-12 | 0.64 (0.22, 1.07) | 0.179423723 |
| *Flavonifractor* | 0.92 (0.5, 1.33) | 0.00777148 | 0.83 (0.38, 1.28) | 0.097962071 |
| *Phascolarctobacterium* | 1.97 (1.34, 2.59) | 5.79E-07 | 1.95 (1.25, 2.64) | 5.60E-05 |

[a]Treatment coefficients generated by the R package *corncob*, representing the additive change in the logit-transformed relative abundance of bacterial genera between treatment and control children.

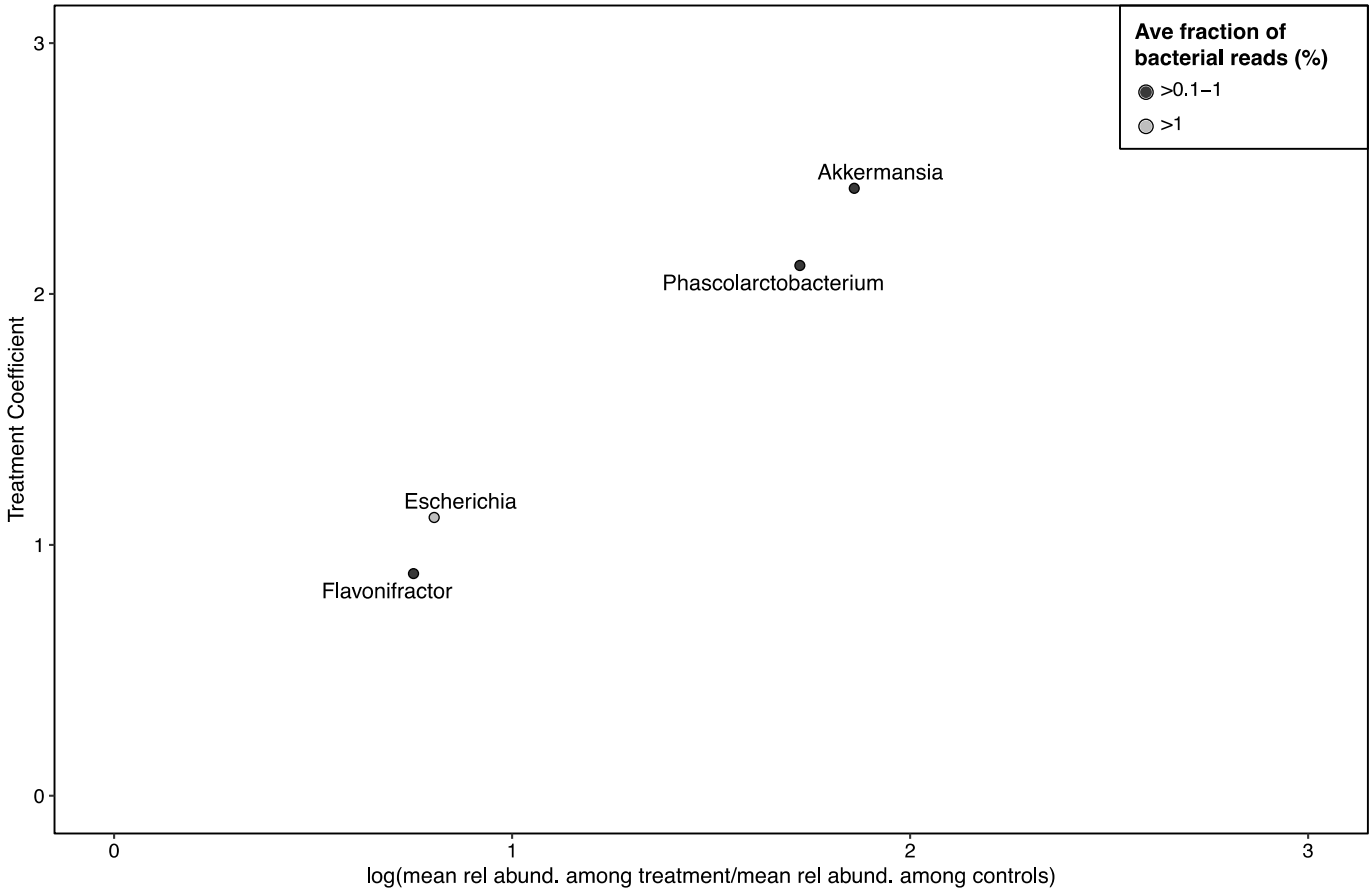
[b]Benjamini-Hochberg method was used to correct for multiple comparisons.

Supplementary Table 3. Detection of 14 gastrointestinal pathogens in the stool of 527 children participating in a cluster-randomized automated water chlorination trial.

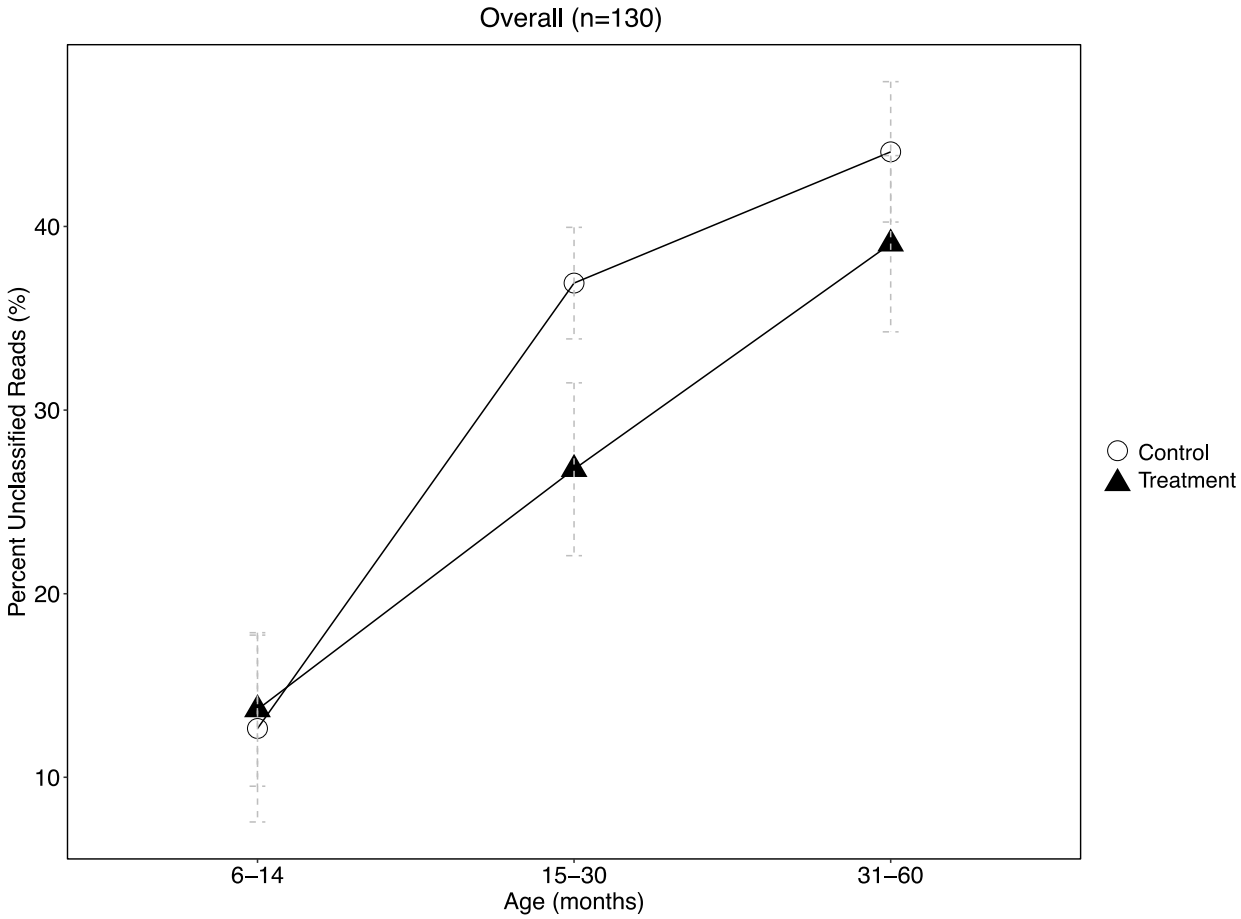
| | Control n=278 (%) | Treatment n=249 (%) | RR (95% CI) | Adjusted *p*-value[a] |
|---|---|---|---|---|
| Norovirus GI/GII | 51 (18) | 31 (12) | 0.65 (0.41, 1.02) | 0.56 |
| *Campylobacter* | 55 (20) | 49 (20) | 0.93 (0.63, 1.37) | 0.78 |
| *Salmonella* | 94 (34) | 79 (32) | 0.93 (0.69, 1.26) | 0.78 |
| Enterotoxigenic *E. coli* (ETEC) LT/ST | 77 (28) | 66 (27) | 0.93 (0.67, 1.30) | 0.78 |
| *Shigella* | 71 (26) | 73 (29) | 1.15 (0.83, 1.60) | 0.78 |
| *Giardia* | 105 (38) | 102 (41) | 1.05 (0.80, 1.39) | 0.78 |
| Pathogenic *E. coli*[b] | 132 (48) | 123 (49) | 1.05 (0.82, 1.34) | 0.78 |
| *Cryptosporidium* | 12 (4) | 8 (3) | 0.70 (0.27, 1.72) | 0.78 |
| *C. difficile* | 12 (4) | 9 (4) | 0.94 (0.38, 2.23) | 0.89 |
| Shiga-like toxin-producing *E. coli* (STEC) stx1/stx2 | 21 (8) | 9 (4) | 0.52 (0.22, 1.10) | 0.56 |
| Adenovirus 40/41 | 8 (3) | 5 (2) | 0.69 (0.21, 2.11) | 0.78 |
| Rotavirus A | 1 (0) | 3 (1) | -- | |
| *Yersinia enterocolitica* | -- | -- | -- | |
| *Vibrio cholerae* | 1 (0) | -- | -- | |
| *Entamoeba histolytica* | 2 (1) | 1 (0) | -- | |

[a]Adjusted for multiple comparisons using the Benjamini–Hochberg method.
[b]Defined as any of the following: ETEC, STEC, or *Shigella.*
*Note*: Relative risk ratios rates (RR) were calculated using Poisson regression models adjusted for child's age and study site. RRs, associated 95% CIs, and adjusted p-values are only presented for pathogens that were detected among at least 1% of samples; models failed to converge below this threshold.

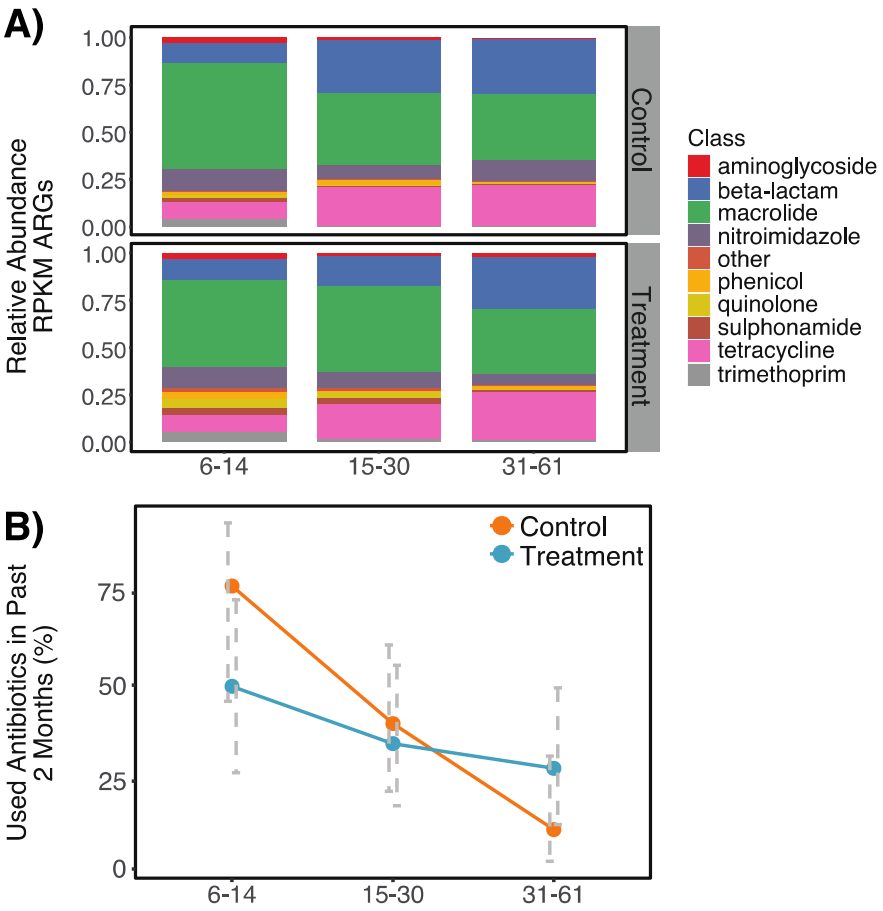
Figures



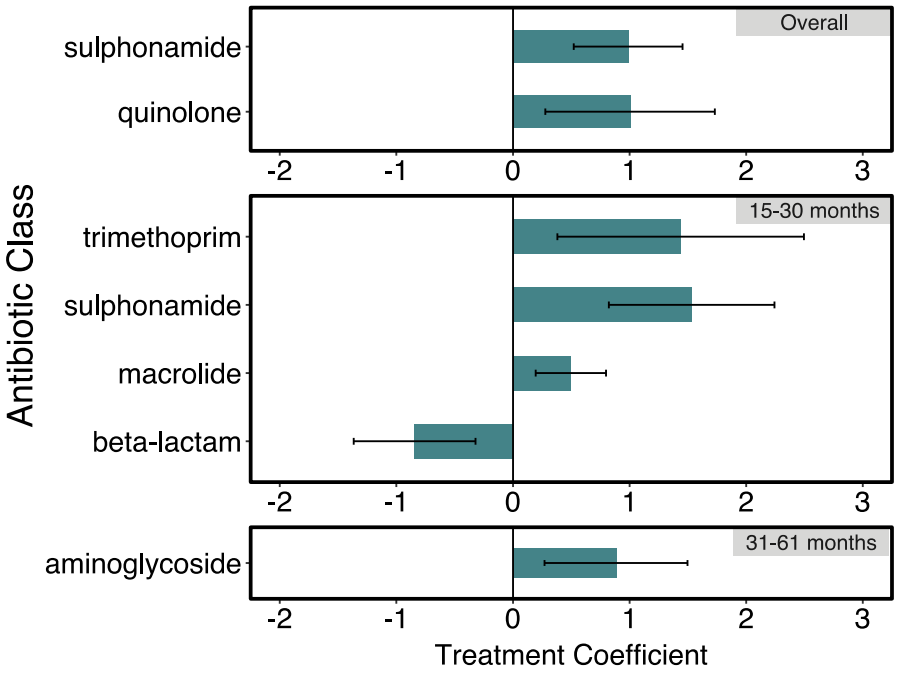

**Supplementary Figure 1.** Treatment coefficients generated by the R package *corncob*, representing the additive change in the logit-transformed relative abundance of bacterial genera between treatment and control children, compared to the log of the ratio of the mean relative abundance among treatment children to the mean relative abundance among control children, *i.e.*, the log fold change. Treatment coefficients generated by *corncob* generally approximate the log fold change.



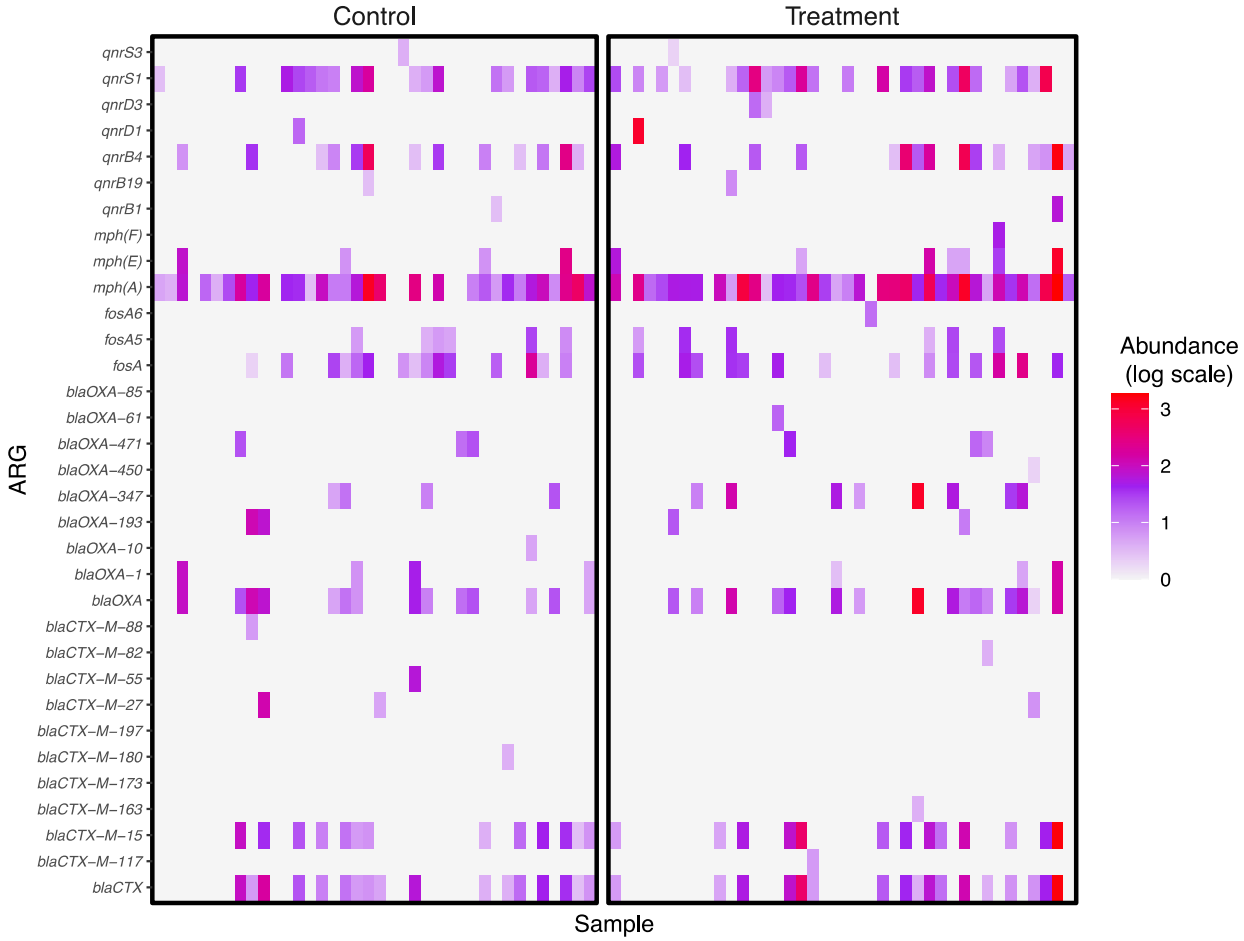

**Supplementary Figure 2. Average fraction of reads from 130 Bangladeshi children's gut metagenomes that were not classified to any taxonomy by Kraken2, stratified by age.** Error bars represent the 95% confidence interval around the mean. The proportion of unclassified reads significantly differed at the p=0.05 level between treatment and control children aged 15-30 months by a two-sided, two-sample t-test, but not among other age strata.



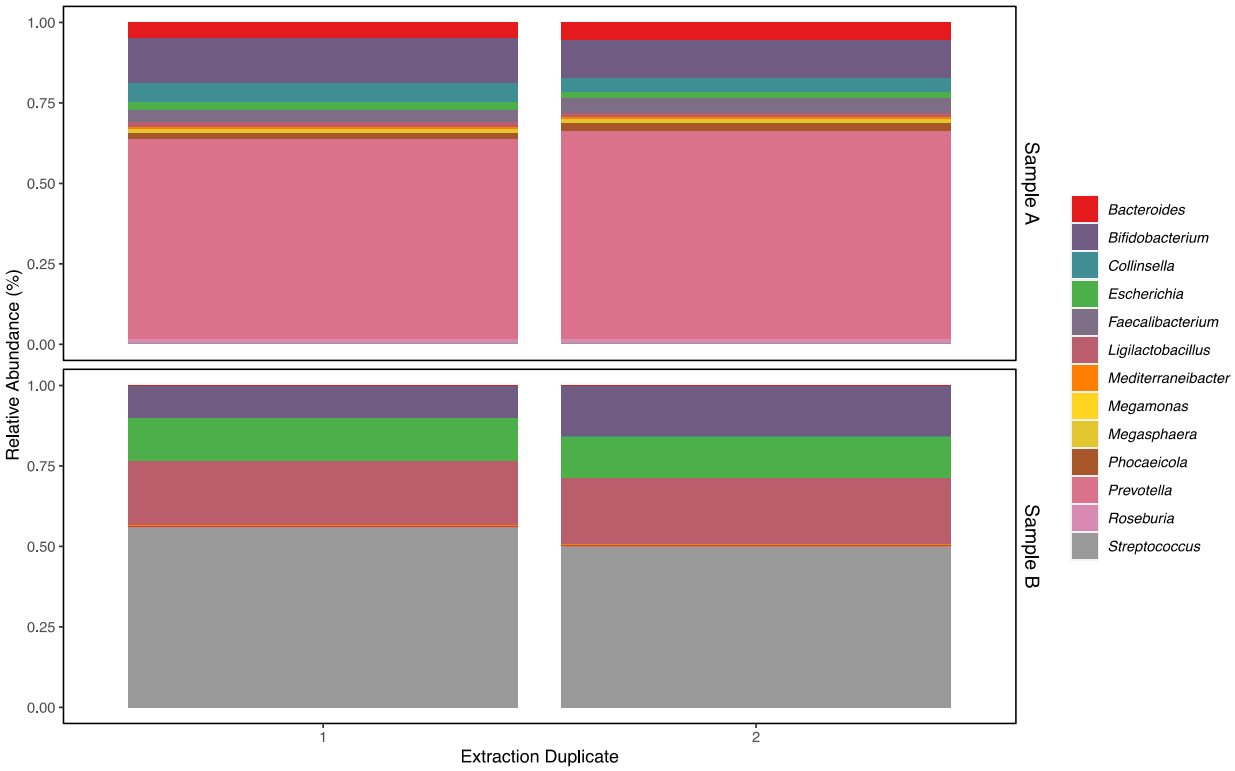

**Supplementary Figure 3. Gut resistomes and antibiotic consumption patterns among 130 children participating in an automated water chlorination trial in urban Bangladesh.** Panel A) depicts relative abundance of antibiotic resistance genes (ARGs) belonging to each ARG class harbored by the fecal metagenomes of control and treatment children, expressed as reads per kilobase of read per million (RPKM). Panel B) depicts the average proportion of control and treatment children whose caretakers reported they had consumed antibiotics in the two months prior to stool collection, stratified by age. Error bars represent the 95% confidence interval around the mean. Antibiotic use was significantly associated with age strata ($p<0.001$ by chi-square) but was not associated with treatment status in this subset of children from the parent trial.




**Supplementary Figure 4. Differences in resistance to specific antibiotic classes as detected in the fecal metagenomes of 130 children participating in an automated water chlorination trial in urban Bangladesh.** Treatment coefficients were generated by the R package *corncob*. Positive treatment coefficients indicate that ARGs belonging to the given antibiotic class were relatively more abundant among treatment children relative to controls; negative treatment coefficients indicate ARGs belonging to the given antibiotic class were relatively less abundant. Error bars depict the 95% confidence interval.




**Supplementary Figure 5**. **Relative abundance of genes conferring resistance to medically important antibiotics in the fecal metagenomes of 130 children participating in an automated chlorinated water intervention trial in urban Bangladesh.** Genes conferring resistance to fluoroquinolones (*qnr*), azithromycin (*mph*), fosfomycin (*fos*), beta lactams ($bla_{OXA}$), and third-generation cephalosporins ($bla_{CTX}$) were detected. Genes conferring resistance to colistin and carbapenems, which are considered “last-resort” antibiotics, were not detected.




**Supplementary Figure 6. Comparison of two sets of extraction controls, extracted from the stool of a child aged 6-14 months (Sample A) and 31-61 months (Sample B).** Within each set of duplicates, we observed a similar relative abundance of bacterial families and genera that comprised at least 1% of bacterial reads among all fecal metagenomes sequenced for this study. We observed some discordance in the genera that were identified within each extraction pair (3 discordant genera versus 1552 concordant genera among extraction duplicates for Sample A; 85 discordant genera versus 1063 concordant genera among extraction duplicates for Sample B); however, all discordant genera were of exceptionally low abundance (<0.007%).