Effect of drinking water chlorination on the intestinal flora and resistomes of Bangladeshi children

Authors: Maya L. Nadimpalli,[1,2] Val F. Lanza,[3] Maria Camila Montealegre,[4] Sonia Sultana,[5] Erica R. Fuhrmeister,[1] Colin J. Worby,[6] Lisa Teichmann,[4] Lea Caduff,[4] Jenna M. Swarthout,[1] Yoshika S. Crider,[7,8] Ashlee M. Earl,[6] Joe Brown,[9] Stephen P. Luby,[10] Mohammad Aminul Islam,[5,11] Timothy R. Julian,[4,12,13#] Amy J. Pickering[2,14,15#*]

[1]Department of Civil and Environmental Engineering, Tufts University, Medford, MA, USA
[2]Stuart B. Levy Center for Integrated Management of Antimicrobial Resistance (Levy CIMAR), Tufts University, Boston, MA, USA
[3]Bioinformatics Unit, Ramón y Cajal Health Research Institute (IRYCIS), Madrid, Spain
[4]Eawag, Swiss Federal Institute of Aquatic Science and Technology, Dübendorf, Switzerland
[5]International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), Dhaka, Bangladesh
[6]Infectious Disease & Microbiome Program, Broad Institute, Cambridge, MA
[7]Energy and Resources Group, University of California Berkeley, Berkeley, CA, USA
[8]King Center on Global Development, Stanford University, Stanford, CA, USA
[9]Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, NC, USA
[10]Infectious Diseases and Geographic Medicine, Stanford University, Stanford, CA, USA
[11]Paul G. Allen School for Global Health, Washington State University, Pullman, WA, USA
[12]Swiss Tropical and Public Health Institute, Basel, Switzerland
[13]University of Basel, Basel, Switzerland
[14]Department of Civil and Environmental Engineering, University of California, Berkeley, CA, USA
[15]Blum Center for Developing Economies, University of California, Berkeley, CA, USA
#Authors contributed equally
*correspondence to: pickering@berkeley.edu; tim.Julian@eawag.ch

This document is the accepted manuscript version of the following article: Nadimpalli, M. L., Lanza, V. F., Montealegre, M. C., Sultana, S., Fuhrmeister, E. R., Worby, C. J., … Pickering, A. J. (2022). Drinking water chlorination has minor effects on the intestinal flora and resistomes of Bangladeshi children. Nature Microbiology, 7(5), 620-629. https://doi.org/10.1038/s41564-022-01101-3

31 **Abstract**

32 Healthy development of the gut microbiome provides long-term health benefits. Children raised
33 in countries with high infectious disease burdens are frequently exposed to antibiotics and
34 diarrheal pathogens, which perturb gut microbiome assembly. A recent cluster-randomized trial
35 in Dhaka, Bangladesh, found that automated water chlorination of shared taps effectively
36 reduced child diarrhea and antibiotic use. Here, we leveraged stool samples collected one year
37 after this intervention to examine differences between treatment and control children's gut
38 microbiota. Water chlorination was associated with increased abundance of several bacterial
39 genera previously linked to improved gut health; however, we observed no effects on the overall
40 richness or diversity of taxa. Several clinically relevant ARGs were relatively more abundant in
41 the gut microbiome of treatment children, possibly due to increases in *Enterobacteriaceae*.
42 While further studies on the long term health impacts of drinking chlorinated water would be
43 valuable, we conclude that access to chlorinated water did not substantially impact child gut
44 microbiome development in this setting, supporting the use of chlorination to increase global
45 access to safe drinking water.

46

47

48 **Main**

49 Normal assembly of the early-life gut microbiome is critical for human health. The gut
50 microbiome is seeded during birth and stabilizes to an adult-like configuration by the third year
51 of life.[1] The progressive, unperturbed colonization of the intestinal tract during this time window
52 is likely essential to the establishment and maturation of multiple developmental pathways
53 related to metabolism, allergy development, weight gain, disease susceptibility, and mental
54 health.[2–5]

55 Children raised in low- and middle-income countries (LMICs) are at high risk of early-life
56 environmental insults that might disrupt optimal gut microbiome development. Due to poor
57 sanitation and lack of access to clean drinking water, children living in poverty are frequently
58 exposed to enteric pathogens.[6] Pathogen establishment and proliferation in the intestinal tract
59 can perturb the normal gut microbiome by triggering local and systemic inflammation.[7,8] In areas
60 with a high infectious disease burden, children also frequently consume antibiotics. In urban
61 Bangladesh, children younger than two years are treated with antibiotics at a rate more than
62 five-times higher than that among similarly aged children in the United States.[9,10] Frequent
63 antibiotic use early in life diminishes gut microbiota diversity, enriches for antibiotic resistance
64 genes (ARGs), and reduces microbiome richness while increasing variability.[11,12] In the United
65 States, these perturbations have been linked to increased risk of multiple childhood-onset health
66 disorders, including asthma, allergy rhinitis, and attention deficit hyperactivity disorder.[5]

67 Water chlorination is a promising strategy for reducing exposure to enteric pathogens
68 and associated antibiotic use among young children in LMICs,[13] with potential benefits for the
69 gut microbiome and long-term health. Chlorination inactivates many microorganisms present in
70 water and reduces recontamination during transport and storage.[14] By reducing children's
71 exposures to pathogens, chlorination could prevent the early establishment and proliferation of
72 pathogens in the gut and the subsequent use of antibiotics. However, water chlorination could
73 indirectly affect the developing gut microbiome in other ways. Chlorination does not inactivate all

74 microorganisms present in drinking water and could systematically impact the types of
75 waterborne organisms that children are exposed to.[15] For example, the introduction of chlorine
76 into drinking water systems has been associated with changes in biofilm communities,[16] the
77 relative abundance of specific bacterial genera in water,[17] and the abundance of ARGs in water
78 systems,[18] with the potential for ARGs that function as efflux pumps to be enriched.[19] Further,
79 ingested trace chlorine residuals or chlorine disinfection by-products could perturb the gut
80 environment in ways that are not fully understood.[20–22]

81 Members of our team recently conducted a blinded, placebo-controlled cluster-
82 randomized trial of passive (automated) water chlorination devices installed at shared taps in
83 urban Bangladesh, which disinfected all water used for domestic purposes including drinking,
84 cooking, personal hygiene, and cleaning.[13] The passive water chlorination intervention reduced
85 caregiver-reported child diarrhea in the past 7 days by 23% and caregiver-reported antibiotic
86 use in the past two months by 7%, relative to controls. The objectives of the present study were
87 to examine the impacts of water chlorination on children's gut microbiomes, including the
88 resistance genes and bacterial pathogens that they harbored, across different phases of gut
89 microbiome development.

90

91 **Results**

92 <u>Child and stool sample characteristics</u>

93 We examined fecal metagenomes from 130 children from the control (n=64) and treatment
94 groups (n=66). Samples included in our final analysis were balanced between two study sites
95 (Dhaka Uddan and Tongi) and three pre-specified age strata (6-14 months, 15-30 months, 31
96 months and older) corresponding to distinct phases of gut microbiome development.[1]

97 Characteristics known to impact the early-life gut microbiome were evenly distributed
98 between the treatment and control groups, including child age,[1] breastfeeding status,[1] recent
99 diarrhea,[23] and recent antibiotic use (**Table 1**).[4,5] Children were exposed to chlorine (treatment)

100 and Vitamin C (active control) doses for an average of 10.5 months (range due to open cohort
101 study design = 1.7 to 14.4 months). Most children (89%) were exposed for at least 6 months.
102 We achieved an average sequencing depth of approximately 6 Gb per sample.
103
104 Water chlorination impacted the relative abundance of several human enterobacteria
105 Exposure to chlorinated water significantly impacted the relative abundance of multiple bacterial
106 genera in children's guts, relative to control children (**Figure 1A**), when adjusting for age and
107 study site. Consistent with previous studies,[1] we observed considerable differences in gut
108 microbiome composition by age group (**Figure 2A**). We estimated treatment coefficients
109 describing the additive change in the logit-transformed relative abundance of bacterial genera
110 between treatment and control children;[24] positive treatment coefficient values indicate elevated
111 levels among children receiving chlorinated water relative to controls. The treatment coefficient
112 generally approximates the log fold change (**Supplementary Figure 1**). Overall, several
113 bacterial genera that are frequently reported to colonize humans were significantly more
114 abundant among treatment children (*fdr*-adjusted *p*-value <0.05), including *Akkermansia* (treat.
115 coef: 2.4, 95% confidence interval (CI): 1.9, 3.0), *Escherichia* (treat. coef: 1.11, 95% CI: 0.7,
116 1.6), *Flavonifractor* (treat. coef: 0.89, 95% CI: 0.5, 1.3), and *Phascolarctobacterium* (treat. coef:
117 2.1, 95% CI: 1.5, 2.7) (**Supplementary Table 1**). Each of these genera comprised at least 0.1%
118 of bacterial reads in children's fecal metagenomes, on average, with the exception of
119 *Escherichia*, which comprised 4% of bacterial reads.
120 The effects of water chlorination on the relative abundance of gut bacteria were modified
121 by age (**Figure 1D-E**). Among the oldest children (age 31-61 months), there were far fewer
122 differentially abundant genera between treatment and controls (*i.e.*, 1 genera compared to 19
123 differentally abundant genera in children aged 15-30 months and 34 among children aged 6-14
124 months) (**Supplementary Table 1**). A substantial portion of metagenomic reads could not be
125 classified to any taxonomy, and the proportion of unclassified reads in each sample increased

126 from an average of 13% at age 6-14 months to 42% at age 31-61 months (**Supplementary**
127 **Figure 2**).
128 Because of the open cohort study design, study participants were exposed to the
129 intervention for varying durations. To determine whether longer exposure to the intervention,
130 (*i.e.*, ≥6 months) was associated with different impacts on the gut microbiome, we conducted a
131 sub-group analysis among children older than 14 months (since age would be correlated to
132 exposure time for children aged 6-14 months). Among all children older than 14 months
133 (n=103), we identified three genera that were signficantly more abundant among treatment and
134 controls (**Supplementary Table 2**). The direction of the effect was maintained for each of these
135 genera among the subset exposed for at least six months (n=91), although only one effect
136 estimate remained significant.
137
138 Water chlorination impacted gut microbial richness, but only among children in the "transitional
139 phase" of gut microbiome development
140 Estimated genera richness was fairly constant across each age stratum, and we did not observe
141 any association between richness and treatment status among the youngest (6-14 months) or
142 the oldest (31 months and older) children. However, among children aged 15-30 months, water
143 chlorination was associated with lower estimated richness on average (**Figure 1B**). Shannon
144 diversity was lowest among the youngest children (age 6-14 months) and progressively higher
145 among each successive age stratum, a trend that is well-described.[2] We observed no
146 association between Shannon diversity and treatment status within any age stratum (**Figure**
147 **1C**).
148 We identified clusters of similar gut metagenomes by calculating pairwise distances
149 between each sample. Among all children, metagenome clusters were primarily defined by age
150 group (*p*<0.001; two-sided chi-square test), rather than exposure to chlorinated water (p=0.09)

151 (**Figure 2B**). Age group stratified analyses revealed treatment status was associated with
152 clustering only among children aged 15-30 months (p=0.015; **Figure 2C-E**).
153
154 Water chlorination was not associated with the occurrence of specific bacterial pathogens
155 We observed no impacts of automated water chlorination on the occurrence of several bacterial
156 pathogens that can cause enteric infections in children in Bangladesh,[25] after adjusting for
157 child's age and study site (**Table 2**). Using a qualitative multiplex pathogen assay, among the
158 children selected for metagenomic sequencing for whom raw stool aliquots were available for
159 analysis (125/130), we found that 75% of children harbored at least one bacterial pathogen, and
160 children harbored 1.3 bacterial pathogens on average (SD: 1.0 pathogens). *Campylobacter*
161 spp., *Salmonella* spp., Enterotoxigenic *E. coli* [ETEC], and *Shigella* spp. were detected in at
162 least 10% of children's stool, while Shiga toxin-producing *E. coli* [STEC]) and *Clostridioides*
163 *difficile* (<5%) were rarely detected. We repeated this analysis using the full set of children's
164 stool samples from the original trial (n=527) and with a broader set of 14 gastrointestinal
165 pathogens, including viral and protozoan targets. Despite increased power to detect differences,
166 we did not observe significant reductions or increases in any of these pathogens among
167 children receiving chlorinated water (**Supplementary Table 3**).
168
169 ARGs were enriched among children receiving chlorinated water
170 Study children frequently harbored ARGs in their guts, and resistance to the same antibiotic
171 classes was observed in the treatment and control groups (**Supplementary Figure 3A**). We
172 found that resistance to some antibiotic classes was enriched among treatment children
173 (**Supplementary Figure 4**), including sulfonamides and quinolones (all age strata),
174 trimethoprim (children aged 15-30 months), macrolides (15-30 months), and aminoglycosides
175 (31 months and older) (treatment coefficient range for above drug classes: 0.4-1.5). ARGs

176 conferring resistance to beta lactam antibiotics were less abundant among treatment children
177 aged 15-30 months (treat. coeff: -0.84, 95% CI: -1.37, -0.32), relative to controls.
178 We also examined chlorination-induced effects on the occurrence and relative
179 abundance of individual ARGs. Several clinically relevant ARGs were detected among both
180 treatment and control children, including $bla_{CTX\text{-}M}$ alleles (conferring third-generation
181 cephalosporin resistance), *mph(A)* (azithromycin resistance), and *qnrS1 (*low-level
182 fluoroquinolone resistance) (**Supplementary Figure 5)**. We observed no significant difference
183 (using a *fdr*-adjusted *p*-value threshold of 0.05) in the presence/absence of any ARG between
184 treatment and control children when controlling for age and study site. We did, however,
185 observe differences in the relative abundance of several ARGs (**Figure 3A**). Among all children
186 receiving chlorinated water we observed a higher relative abundance of *mdf(A) and tet(A)*,
187 (multidrug and tetracycline resistance-conferring efflux pumps, respectively), *sul2 (*sulfonamide
188 resistance) and *aadA5* (streptomycin and spectinomycin resistance). Among specific age strata
189 we observed increases in additional ARGs, including *tet(X)* (tetracycline resistance) among
190 children aged 6-14 months; *erm(X)* (cross-resistance to macrolides, lincosamides, and
191 streptogramins), *mph(A)* and *nimE* (nitroimidazole resistance) among children aged 15-30
192 months; *dfrA17* (trimethoprim resistance) among children aged 31-61 months; and bla_{TEM}
193 (penicillin resistance) among children aged 15 months and older (treatment coefficient range for
194 above ARGs: 0.9-7.3). ARGs that encode efflux pumps were slightly enriched in the treatment
195 group (treat. coef.: 0.3, 95% CI: 0, 0.5) but the direction and significance of this effect was not
196 consistent across age groups. The *ant(6')-Ia* gene (aminoglycoside resistance) was less
197 abundant among treatment children aged 15-30 months relative to control children (treat. coeff.:
198 -1.3, 95% CI: -2.2, -0.37).
199 Exposure to chlorinated water was also associated with estimated ARG richness in the
200 gut (*p*=0.019),[26,27] when controlling for age and study site. Specifically, we observed higher ARG
201 richness among treatment relative to control children in the 15-30 months and 31-61 months

202 age strata (*p*=0.015 and p=0.05, respectively) (**Figure 3B**). We also identified a marginal

203 increase in the Shannon diversity of ARGs among treatment children aged 31-61 months

204 relative to controls (*p*=0.07), but not among other age strata (**Figure 3C**).

205 Antibiotic use in the two months prior to stool collection was most frequently reported

206 among the youngest children (17/27) and least commonly reported among children aged 31-61

207 months (10/52) (**Supplementary Figure 3B**). Caretakers rarely reported the types of antibiotics

208 used. Reported antibiotic use was not associated with ARG richness or ARG Shannon diversity

209 in children's stool (*p*=0.30 and *p*=0.14, respectively, by Wilcoxon signed-rank test).

210

211 <u>Differentially abundant resistance genes were significantly correlated with specific bacterial taxa</u>

212 We observed statistically significant Spearman's correlations (fdr-adjusted *p*-value <0.05)

213 between the relative abundance of several bacterial families and the *ant(6)-1a*, bla_{TEM}, *erm(X)*,

214 *mdf(A)*, *mph(A)*, *sul2*, and *tet(A)* (**Figure 3D**) genes, indicating potential origins for these

215 differentially abundant ARGs. In particular, we observed very strong correlations (*rho*>0.9)

216 between *mdf(A)* and *Enterobacteriaceae*[28,29] and *erm(X)* and *Bifidobacteriaceae*,[30,31]

217 associations which are well-described. We also observed a strong correlation (*rho*=0.66)

218 between the relative abundance of *mph(A)* and *Enterobacteriaceae*. Of note, *Escherichia* spp.,

219 which belong to the family *Enterobacteriaceae*, were enriched among children receiving

220 chlorinated water. Other significant correlations were observed between bacterial families and

221 ARGs that were differentially abundant between treatment and control children, but were

222 moderate to weak in strength (*i.e.*, *rho*<0.6).

223

224 **Discussion**

225 In this analysis of fecal metagenomes from 130 children who participated in a cluster-

226 randomized water chlorination trial in urban Bangladesh, automated water chlorination at the

227 point of collection impacted children's gut microbiomes, including the resistance genes they

228 harbored, though shifts in taxa abundance were generally small in magnitude. Children
229 receiving chlorinated water harbored a higher abundance of bacterial genera that are often
230 detected in the human gut, including *Akkermansia* spp., *Flavonifractor* spp.,
231 *Phascolarctobacterium* spp., and *Escherichia* spp. Despite these changes, water chlorination
232 did not impact the overall richness or diversity of children's gut microbiomes and was not
233 associated with differences between children's gut microbiome communities, except among
234 children aged 15-30 months. These findings suggest that automated water chlorination, an
235 effective strategy for reducing child diarrhea and associated antibiotic demand,[13] does not
236 substantially impact children's developing gut microbiomes.

237 Children in the treatment group harbored a higher abundance of several bacterial genera
238 previously linked to improved gut health. *Akkermansia* was more abundant among treatment
239 children overall, relative to controls, and comprised 0.13% of bacterial reads in children's fecal
240 metagenomes. *A. muciniphila* is a mucin-degrading gut commensal that improves intestinal
241 barrier integrity[32] and reduces gut inflammation.[33] In addition, *Flavonifractor* was approximately
242 twice as abundant among treatment compared to control children and comprised 0.19% of
243 bacterial reads in children's fecal metagenomes. *F. plautii* metabolize flavonoids, which are
244 naturally-occurring compounds common in fruit and vegetables, and the by-products of this
245 metabolic activity modulate gut inflammation and weight gain.[34,35] However, *F. plautii* have been
246 associated with some disease states (*e.g.*, colorectal cancer, bipolar disorder).[36,37] Both *A.*
247 *muciniphila* and *F. plautii* are being explored as probiotic targets.[38–40] *Phascolarctobacterium*
248 spp. are commonly detected in the human gut[41] and were four times as abundant among
249 treatment compared to control children. *Phascolarctobacterium* spp. solely metabolize luminal
250 succinate, which is a key growth substate for enteric pathogens like *Clostridioides difficile* and
251 *Salmonella* serovar Typhimurium.[42,43] By reducing bioavailable succinate,
252 *Phascolarctobacterium* spp. may strengthen gut colonization resistance.[42] Finally, *Escherichia*
253 spp. were more abundant in treatment compared to control children. *Escherichia* spp. include

254 commensal strains that contribute to colonization resistance against bacterial pathogens,[44] as
255 well as strains that cause diarrhea or other infections.[45,46] Notably, pathogenic *E. coli* (*e.g.*,
256 ETEC, STEC) were no more common among treatment relative to control children by the highly
257 sensitive (>90%) and specific (≥99%) qualitative multiplex assay we used,[47] suggesting that the
258 higher relative abundance of *Escherichia* in the treatment group was not driven by increases in
259 pathogenic *E. coli*. Overall, the higher relative abundance of several human enterobacteria
260 among treatment children compared to controls suggests that chlorination-mediated impacts on
261 the gut microbiome could support engraftment of commensal strains.
262 Water chlorination was associated with microbiota clustering and diminished genera
263 richness among children aged 15-30 months, but this effect were not observed in younger or
264 older children. In a longitudinal study of children born in Europe and the United States, the 15-
265 30 month age range was associated with the "transitional phase" of gut microbiome
266 development, characterized by significant shifts in dominant phyla and Shannon diversity.[1]
267 Further studies are needed to examine why children's gut microbiomes may be affected by
268 water chlorination specifically during this age range and what impacts (if any) this may have on
269 future gut composition. Of note, the impacts of water chlorination on the differential abundance
270 of gut taxa were weakest among children in the oldest age stratum (ages 31 months and older).
271 This could suggest that the microbial gut community is less perturbable by water chlorination
272 later on in life. Longitudinal analyses are needed to capture if the subtle impacts of water
273 chlorination on children's developing gut microbiomes that we describe here have any long-term
274 health effects.
275 Findings from the parent trial that water chlorination reduced child diarrhea could be
276 related to the microbiome shifts we observed here. The original trial (in which this study was
277 nested) leveraged >4,000 child observations to detect a 23% relative reduction in diarrhea
278 prevalence over a 1-year time period.[13] Among the 130 children included in this study, we
279 observed no differences in the presence of eight diarrheal pathogens between treatment and

control children. This could be due to our small sample size, seasonal variability in diarrhea prevalence, or because the presence of enteric pathogens does not necessarily imply a disease state. Instead, growing evidence from high child mortality settings suggests that many enteric pathogens are just as commonly detected among children with no clinical manifestations of diarrhea,[48] suggesting that other factors like immunity, exposure dose, inflammation, or intestinal barrier functionality likely play a role in determining disease onset.[49] Here, we observed that several gut commensal bacteria were enriched among children receiving chlorinated water; it is possible that the metabolic by-products of these bacteria, their interactions with other gut microbes, or other unmeasured changes to the gut microbiome environment could have improved gut colonization resistance and immunity more broadly.[50] Longitudinal stool and environmental sampling in future water chlorination intervention trials could help elucidate whether reductions in child diarrhea from access to chlorinated drinking water are due to disrupted exposures to human, animal, and environmental-origin pathogens, and/or due to improved gut health conferred by shifts in microbiota.[50,51]

Because children randomized to the water chlorination intervention in the parent trial experienced a 7% reduction in recent antibiotic use, we hypothesized that water chlorination could indirectly reduce the relative abundance of ARGs in children's guts.[11] The water chlorination intervention instead increased the relative abundance of several ARGs in children's guts, which was likely the result of a higher relative abundance of *Enterobacteriaceae* spp. among treatment children versus controls. *Enterobacteriaceae* spp., like *E. coli*, frequently harbor mobile ARGs,[52] especially in Bangladesh.[28,29,53,54] Our findings suggest that in settings like urban Bangladesh, where mobile ARGs are exceptionally common among commensal bacteria,[28,29,53] water chlorination alone may not be sufficient to reduce the burden of ARGs circulating in the community. Instead, other interventions might be necessary to curb the selection and spread of antibiotic resistance,[55] not just in human communities but also in the food animal production sector, where many of the same antibiotics are used.[56] Notably, because

306 we did not use functional genomics to characterize all resistance mechanisms present in
307 children's fecal microbiomes, we may have missed effects on novel ARGs.

308 Our findings should be interpreted in the context of this study's limitations. First, due to
309 the open cohort study design children were exposed to the intervention for varying durations,
310 though most children were exposed for at least six months. Duration of exposure was strongly
311 correlated with age, so we did not control for this covariate in our analyses. Second, stool
312 samples were not collected at the time of enrollment, which could have helped inform whether
313 there were preexisting differences in gut community composition between the selected subset of
314 treatment and control children, and the extent to which the intervention shifted gut composition.
315 Baseline survey data in the parent trial indicated that the treatment and control groups were well
316 balanced across a range of socioeconomic and child health variables that could plausibly
317 influence gut community composition.[13] Third, a substantial proportion of children's fecal
318 metagenomic reads could not be classified to any known taxonomy, comprising an average of
319 42% of reads among the oldest children. This may have affected our ability to assess the
320 relative effect of chlorination of children's gut microbiomes, given that our analyses were based
321 solely on the classifiable fraction. The unclassified portion that we observed is comparable to
322 other studies that have included fecal metagenome data from LMICs (42%-68%),[57,58] providing
323 further evidence of biases in existing taxonomy databases towards high-income, Western
324 countries.[59] Finally, an important caveat of our results is that control children in the parent trial
325 received water dosed with trace amounts of Vitamin C, which could have conferred a nutritional
326 benefit. The impacts of Vitamin C on the gut microbiome are not well-known.[60] One pilot trial of
327 healthy adults in Europe[61] found that high doses (500 mg/day) administered directly to the colon
328 increased genera richness and the relative abundance of some bacterial taxa, including
329 *Akkermansia*, but the setting, study population, and administration route are not comparable to
330 the present study. Given that Vitamin C was dosed at very low levels, we anticipate that any

331 subclinical changes to the gut microbiome would not have been distinguishable from natural
332 variation in gut microbiome development among children in this setting.
333

334 **Conclusion**

335 To our knowledge, this is the first study to experimentally evaluate the impact of water
336 chlorination, the most common form of drinking water disinfection worldwide, on children's
337 developing gut microbiomes. Because we leveraged stool samples from a double-blinded,
338 cluster-randomized placebo-controlled automated water chlorination trial, water chlorination can
339 be causally attributed to the outcomes measured here. Our findings suggest that water
340 chlorination does not substantially affect the developing gut microbiomes of children in urban
341 Bangladesh. Specifically, while we observed chlorination-induced shifts in the relative
342 abundance of some bacteria taxa, including beneficial gut commensals, we observed no effects
343 on overall gut genera richness or diversity. However, long-term studies may be needed to
344 confirm that these types of subtle changes do not affect health later in life. Overall, the benefits
345 of automated water chlorination with regards to preventing child diarrhea, reducing antibiotic
346 use, and protecting child health appear to outweigh any potential changes to gut microbiome
347 development in this setting.
348

349 **Materials and Methods**

350 Stool collection

351 Stool samples were collected from children participating in a double-blinded, cluster-randomized
352 automated water chlorination trial implemented from July 2015-December 2016 in two low-
353 income communities in urban Bangladesh: Tongi, a community outside Dhaka city, and Dhaka
354 Uddan, a community within Dhaka city.[13] In brief, shared water taps that served as the primary
355 source of drinking water for children younger than five years old were identified in each
356 community, then randomized to either the treatment (n=50) or control (n=50) arms. Although

357 water taps were selected based on reliance as a primary drinking water source, water from
358 these taps was used for all purposes, including bathing, cleaning, and washing clothes. Over 14
359 months, treatment water points were automatically dosed with chlorine using a passive water
360 chlorination device, achieving a mean chlorine residual of 0.37 ppm, while control water points
361 were dosed with trace amounts of Vitamin C (active control).[13] Vitamin supplementation was
362 included as an active control in order to improve acceptability to study participants and the local
363 human subjects protection board. Vitamin C was specifically chosen by study investigators
364 because tablets compatible with the dosing devices could be locally acquired.

365 Survey data were collected approximately every two months from an open cohort of
366 children younger than five years old, of which over 1000 were enrolled at baseline. The trial
367 used an open cohort study design given high migration rates in both communities. Thus, new
368 children living in compounds served by either a treatment or control water tap were continuously
369 enrolled at every follow-up survey round. Field workers confirmed at each study visit that the
370 primary source of drinking water for each enrolled child was either a control or treatment pump.
371 Across all survey rounds in the parent trial, less than 4% of households reported using a
372 secondary water source for drinking.[13] Both study participants and researchers processing the
373 samples and performing data analysis were unaware of which households were served by
374 chlorinated taps (double-blinded). This study and the cluster randomized trial in Bangladesh[13]
375 followed the CONSORT checklist for cluster randomized trials (**Supplementary Material).** The
376 study protocol for the original trial was approved by the International Centre for Diarrhoeal
377 Diseases Research, Bangladesh (icddr,b) scientific and ethical review committees (protocol
378 number 14022) and the human subjects institutional review board at Stanford University
379 (protocol number 30456). Field staff obtained informed written consent from the owner of each
380 water point enrolled and all study participants, including consent for biospecimens to be used for
381 future unplanned analyses.

382 Children's stool samples were collected approximately one year into the trial.
383 Households were provided stool collection kits that included latex gloves and sterile scoops, and
384 were instructed on safe collection procedures prior to handling stool. Following stool production,
385 the child's mother or other caretaker were instructed to immediately transfer a small amount of
386 feces into pre-prepared vials containing 1 mL of RNAlater, which has been demonstrated to be
387 a suitable preservative for fecal samples,[62] and then invert the tube a few times. Field staff
388 transported children's stool samples to the laboratory within 8 hours. Field staff conducted up to
389 three follow-up visits per household to retrieve specimens. Stool aliquots stored in RNAlater
390 were frozen at -80°C upon arrival at the International Centre for Diarrheal Disease Research,
391 Bangladesh (icddr,b) in Dhaka and remained frozen during storage and transport on dry ice to
392 Tufts University in the United States for DNA extraction.
393
394 Sample selection for metagenomic sequencing
395 We performed short-read metagenomic sequencing on RNAlater-preserved stool specimens
396 collected from children older than 6 months in the control and treatment groups. Sample
397 selection was done by stratified random sampling of archived child stool samples to balance
398 across treatment and control groups, study community (*i.e.*, Dhaka Uddan versus Tongi), and
399 across three age groups that others have demonstrated to correspond to distinct phases of gut
400 microbiome development (*i.e.*, 6 -14 months, 15-30 months, 31 months and older).[1] Total DNA
401 was extracted from approximately 0.25 g of frozen feces at Tufts University using the QIAamp
402 PowerFecal DNA Kit (Qiagen) according to manufacturer's instructions and quantified using a
403 Qubit 4 fluorometer (Invitrogen). Extraction blanks were included with each batch of extractions;
404 DNA concentrations were below the Qubit level of detection for all. We performed duplicate
405 extractions on two stool samples. DNA extracts were sent to Novogene (UK) Company Limited
406 for short-read, paired-end 150 bp sequencing on an Illumina Novaseq 6000 System using SP4
407 flow cells to achieve 10 Gb per sample.

408

409 Processing metagenomic data

410 Fastq sequences were trimmed and filtered to remove sequencing adaptors and low quality
411 reads using Trimmomatic v0.36. We tabulated the number of raw reads for each sample and
412 excluded from further analysis any sample with fewer reads than two standard deviations below
413 the mean. Human contaminant sequences were removed from each sample by discarding
414 reads that mapped to a non-redundant version of the Genome Reference Consortium Human
415 Build 38 (GRCh38; www.ncbi.nlm.nih.gov) using Bowtie2 v2.2.3. Human sequence-filtered raw
416 reads were deposited in the Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra)
417 under the project number PRJNA726052. We randomly chose one extraction duplicate per
418 sample to include in subsequent metagenomic analyses.

419

420 Profiling taxonomy and classifying source

421 Taxonomic assignment of short reads was performed using Kraken2, using its standard built-in
422 database comprising all complete bacterial, archeal, and viral genomes in NCBI's RefSeq at the
423 time of build (03 June 2021).[63] We used the Bracken species-level sequence abundance
424 estimation algorithm to estimate organism abundance at every taxonomic level.[64] For all
425 analyses, we considered organisms classified as bacteria in NCBI's taxonomy database
426 (https://ftp.ncbi.nlm.nih.gov/pub/taxonomy/), downloaded 23 November 2021.

427

428 Identifying antibiotic resistance genes and calculating abundance

429 We screened for antibiotic resistance genes (ARGs) by mapping short reads to the Resfinder
430 database (v. 3.1.1) using the KMA tool.[65] We considered matches with >90% coverage and
431 >95% identity to be true hits. To examine the abundance of ARGs across samples, we
432 calculated the RPKM (Reads Per Kilobase Million) for each ARG in a sample as the number of
433 hits divided by the total number of matched paired-end bacterial reads per million for that

434 sample, then divided by the ARG length in kilobases. To visualize the relative abundance of
435 ARG classes across samples, we summed the RPKM for all ARGs belonging to a class.
436
437 Clustering similar metagenomes
438 All-against-all genomic distances were estimated between each metagenome using Mash.[66] We
439 reduced the dimensionality of the resulting matrix using UMAP from the *uwot* package
440 (https://github.com/jlmelville/uwot) and used the *MClust* R package[67] to classify samples into
441 clusters. The MClust algorithm chooses the number of clusters and the specific clustering model
442 (of eight models considered) to maximize the BIC; a minimum of one cluster is considered by
443 default and we did not assign a maximum.[67,68] We repeated this same process within each age
444 stratum after subsetting the Mash distance matrix. We used chi-square tests to examine
445 associations between treatment status and cluster classification, both within each age group
446 and overall. All *p*-values are reported in **Figure 2**.
447
448 Identifying gastrointestinal pathogens
449 Given that rare taxa like gastrointestinal pathogens are difficult to capture using metagenomic
450 sequencing approaches, and that pathogenic *E. coli* (*e.g.*, ETEC, STEC) may be impossible to
451 distinguish from commensal strains using short-read sequencing data, we used a qualitative
452 multiplex assay with >90% sensitivity and ≥99% specificity to determine pathogen occurrence,
453 the Luminex xTAG® Gastrointestinal Pathogen Panel.[47] For children whose stool samples were
454 analyzed using short-read metagenomic sequencing, we examined additional, raw aliquots of
455 their stool specimens for the presence of 8 bacterial pathogens, including
456 *Campylobacter*; *Clostridioides difficile*; Enterotoxigenic *E. coli* (ETEC) LT/ST; Shiga-like toxin-
457 producing *E. coli* (STEC) stx1/stx2; *Salmonella* spp.; *Shigella* spp.; *Yersinia enterocolitica*, and
458 *Vibrio cholerae*. We also examined all stool specimens collected during the parent trial for a
459 broader set of 14 pathogens, including the aforementioned bacterial pathogens as well as

460 adenovirus 40/41; norovirus GI/GII; rotavirus A; *Giardia* spp.; *Cryptosporidium* spp.; and
461 *Entamoeba histolytica*. Methods, including DNA and RNA extraction protocols, have been
462 described in detail elsewhere.[69]
463
464 Comparing extraction duplicates
465 We compared the taxonomic profiles of both sets of extraction duplicates (**Supplementary**
466 **Figure 9**) and observed no clear differences in the relative abundance of any bacterial family or
467 genera that comprised at least 1% of bacterial reads, on average, across all of the samples we
468 sequenced (n=132). We observed some discordance in the genera that were identified within
469 each extraction pair (3 discordant genera versus 1552 concordant genera among extraction
470 duplicates for Sample A; 85 discordant genera versus 1063 concordant genera among
471 extraction duplicates for Sample B); however, all discordant genera were of exceptionally low
472 abundance (<0.007%). We randomly chose one extraction duplicate per sample to include in
473 subsequent metagenomic analyses.
474
475 Data Analysis
476 We examined differences in bacterial taxa (primary outcome), ARGs (secondary outcome), and
477 the occurrence of gastrointestinal pathogens (secondary outcome) between treatment and
478 control children using several approaches. Analysis of differences in bacteria taxa and ARGs
479 were not planned as part of the original trial, though differences in the occurrence of
480 gastrointestinal pathogens was pre-specified as a secondary outcome in the original trial's
481 registration and statistical analysis plan (available at: https://osf.io/t98bv/). The original trial was
482 registered with ClinicalTrials.gov, number NCT02606981. Because nearly all children (126/130)
483 lived in different households, each child's fecal metagenome was considered as an independent
484 data point for these analyses.

485 For bacterial taxa, we used beta-binomial regression models that account for variable
486 sequencing depth to identify taxa that were differentially abundant between treatment and
487 control children with the R package *corncob*,[24] while controlling for the child's age and study
488 site. This was the primary outcome we examined. The Benjamini-Hochberg method was used to
489 correct for multiple comparisons (default method for *corncob*).[70] Only taxa that were present in
490 at least 20% of samples were included in these analyses. Given frequent discordance in
491 species-level taxonomy assignments among commonly used taxonomic labeling tools for short-
492 read sequencing data,[71] we only examined differences at the genera level and above. We
493 filtered differentially abundant taxa that were exclusively driven by influential points from further
494 analyses. Differentially abundant genera and families (as identified by *corncob*) were visualized
495 using the *metacoder* package.[72] We also calculated two estimates of bacterial alpha diversity for
496 each sample: richness, which describes the number of bacterial genera, and the Shannon
497 index. Genera richness was estimated using the *breakaway* package.[27] Shannon diversity was
498 calculated using the *phyloseq* package.[73] Differences between treatment and control children for
499 each of these metrics were examined within each age stratum using Wilcoxon signed-rank
500 tests.
501 We used logistic regression models to examine differences in the presence/absence of
502 ARGs between treatment and control children, while controlling for age and study site. Because
503 allelic variants of beta lactamase genes can differ by as few as one single nucleotide
504 polymorphism, we considered these genes as groups (*e.g.*, $\textit{bla}_{\text{CTX}}$, $\textit{bla}_{\text{OXA}}$, $\textit{bla}_{\text{TEM}}$) rather than as
505 individual variants. Resulting *p*-values were adjusted for multiple comparisons using the
506 Benjamini–Hochberg method. For ARGs that were common among children in this setting
507 (present in at least 20% of samples), we used the R package *corncob* to identify differentially
508 abundant ARGs between treatment and control children, while controlling for age and study site
509 (secondary outcome). We separately used *corncob* to evaluate whether ARGs that function as
510 efflux pumps were differentially abundant between treatment and control children, by creating a

511 composite indicator variable for all efflux pump ARGs in our dataset. We determined the
512 functional annotation of detected ARGs by cross-referencing with the Comprehensive Antibiotic
513 Resistance Database (CARD).[74] For any ARGs not listed in CARD (*i.e.*, *nimE*, *nimJ*, *mefA*,
514 *tet(O/32/O)*, *tet(O/W)*, *tet(W/32/O)*, we determined their function by searching the literature. We
515 used *breakaway*'s betta function[26] to examine the effect of chlorination on estimated ARG
516 richness while controlling for study site, both overall (analysis also controlled for age) and within
517 each age stratum (*i.e.*, 6 -14 months, 15-30 months, 31 months and older). We examined
518 differences in ARG diversity between treatment and control children using Wilcoxon signed-rank
519 tests.

520 To examine differences in gastrointestinal pathogen burden between treatment and
521 control children, we used Poisson regression models to examine associations between
522 treatment status and the presence of any pathogen that was harbored by at least 5% of
523 children, while controlling for child's age and study site (secondary outcome). The resulting *p*-
524 values were adjusted for multiple comparisons using the Benjamini–Hochberg method.

525 We conducted a Spearman's correlation analysis to investigate how changes in gut
526 taxonomic structure might be driving changes in ARG relative abundance between treatment
527 and control children. Only correlations between ARGs and bacterial families that occurred in at
528 least half of samples were examined. The *rho* and *p*-values were calculated using the rcorr
529 function of the *Hmisc* package (https://github.com/harrelfe/Hmisc/) and resulting *p*-values were
530 adjusted for multiple comparisons using the Benjamini–Hochberg method.

531 All analyses were conducted in R v. 3.5.0 and R Studio v. 1.1.463. All p-values were
532 two-sided and considered to be statistically significant at the 0.05 level.

533

534 Correspondence and requests for materials should be addressed to Dr. Amy Pickering
535 (pickering@berkeley.edu) and Dr. Tim Julian (tim.Julian@eawag.ch)

536

537 Data Availability

538 All raw reads (human sequences removed) were deposited in the Sequence Read Archive
539 (SRA; https://www.ncbi.nlm.nih.gov/sra) under the project number PRJNA726052. Metadata are
540 publicly available at the following link: https://osf.io/wb3pv/.

541

542 Acknowledgements

543 We thank Nazrin Akter for excellent field management. This work was funded by the Thrasher
544 Research Fund (#14205) and The World Bank Strategic Impact Evaluation Fund. M.L.N. was
545 supported by NIH award KL2TR002545 and the Stuart B. Levy Center for Integrated
546 Management of Antimicrobial Resistance at Tufts (Levy CIMAR), a collaboration of Tufts
547 Medical Center and the Tufts University Office of the Vice Provost for Research (OVPR)
548 Research and Scholarship Strategic Plan (RSSP). C.J.W. and A.M.E were supported by the
549 National Institute of Allergy and Infectious Diseases of the National Institutes of Health (NIH)
550 under award no. U19AI110818 to the Broad Institute. E.R.F. was supported by the NSF
551 Postdoctoral Research Fellowships in Biology Program under Grant No. 1906957. Any opinions,
552 findings, and conclusions or recommendations expressed in this material are those of the
553 author(s) and do not necessarily reflect the views of any of the aforementioned funding
554 organizations.

555

556 Role of the funding source

557 The funder had no role in data collection, data analysis, data interpretation, or writing of this
558 report.

559

560 Author contribution statement

561 AJP and TRJ conceptualized this follow-up study and obtained funding. AJP, SS, and SPL
562 designed the original trial. AJP, SS, YSC, and JS contributed to data collection in the original

563 trial. MCM, LT, LC, and ERF performed stool DNA extractions. YSC and JB contributed to the
564 enteric pathogen analysis. CW, AME, MAI, and VFL provided input on methods and data
565 interpretation. MLN and VFL contributed to metagenomic data analysis. MLN wrote the first
566 draft. All co-authors contributed to writing and editing the manuscript.
567

568 Tables

569 Table 1. Characteristics of 130 Bangladeshi children participating in a cluster-randomized
570 automated water chlorination trial at the time of stool sample collection.
571

	Control	Treatment
	n=64 (%)	n=66 (%)
Female	40 (63)	37 (57)
Age		
6-14 months	13 (20)	14 (21)
15-30 months	25 (41)	26 (39)
31-61 months	26 (41)	26 (39)
Study site		
Dhaka Uddan	28 (44)	28 (42)
Tongi	36 (56)	38 (58)
Currently receiving any human milk[a,b]	32 (50)	29 (45)
Experienced fever in past seven days[a]	15 (23)	9 (14)
Experienced diarrhea in past seven days[a]	3 (5)	3 (5)
Received antibiotics in past two months[a]	25 (36)	23 (35)
Months enrolled in trial (mean, SD)	11.5 (3.0)	10.8 (3.1)

572 [a]Caregiver reported at time of stool collection.
573 [b]Exclusive breastfeeding was rare at the time of stool sample collection; no children in the
574 control group and only one child in the treatment group (age 14 months) was exclusively
575 breastfed.
576
577 Table 2. Detection of 8 bacterial pathogens in the stool of 125 children participating in a cluster-
578 randomized automated water chlorination trial following 10 months of exposure, on average.[a]

	Control n=61 (%)	Treatment n=64 (%)	RR (95% CI)	Adjusted *p*-value[b]
Campylobacter spp.	10 (16)	16 (25)	1.42 (0.65, 3.25)	0.81
Salmonella spp.	15 (25)	25 (39)	1.56 (0.83, 3.02)	0.81
Enterotoxigenic *E. coli* (ETEC) LT/ST	22 (36)	20 (31)	0.87 (0.47, 1.60)	0.81
Shigella spp.	19 (31)	23 (36)	1.17 (0.64, 2.18)	0.81
C. difficile	3 (5)	3 (5)	0.90 (0.17, 4.90)	0.90
Shiga-like toxin-producing *E. coli* (STEC) stx1/stx2	4 (7)	0	--	--
Vibrio cholerae	0	0	--	--

579 [a]Raw stool aliquots from 125 of 130 children were available for pathogen analysis.
580 [b]Adjusted for multiple comparisons using the Benjamini–Hochberg method.
581 *Note*: Relative risk ratios rates (RR) were calculated using Poisson regression models adjusted
582 for child's age and study site. RRs, associated 95% CIs, and adjusted p-values are only
583 presented for pathogens that were detected among at least 5% of samples. 75% of children
584 harbored at least one bacterial pathogen; 67% of children harbored at least one non-
585 *Escherichia*-related bacterial pathogen.
586
587
588
589
590
591
592

Figures

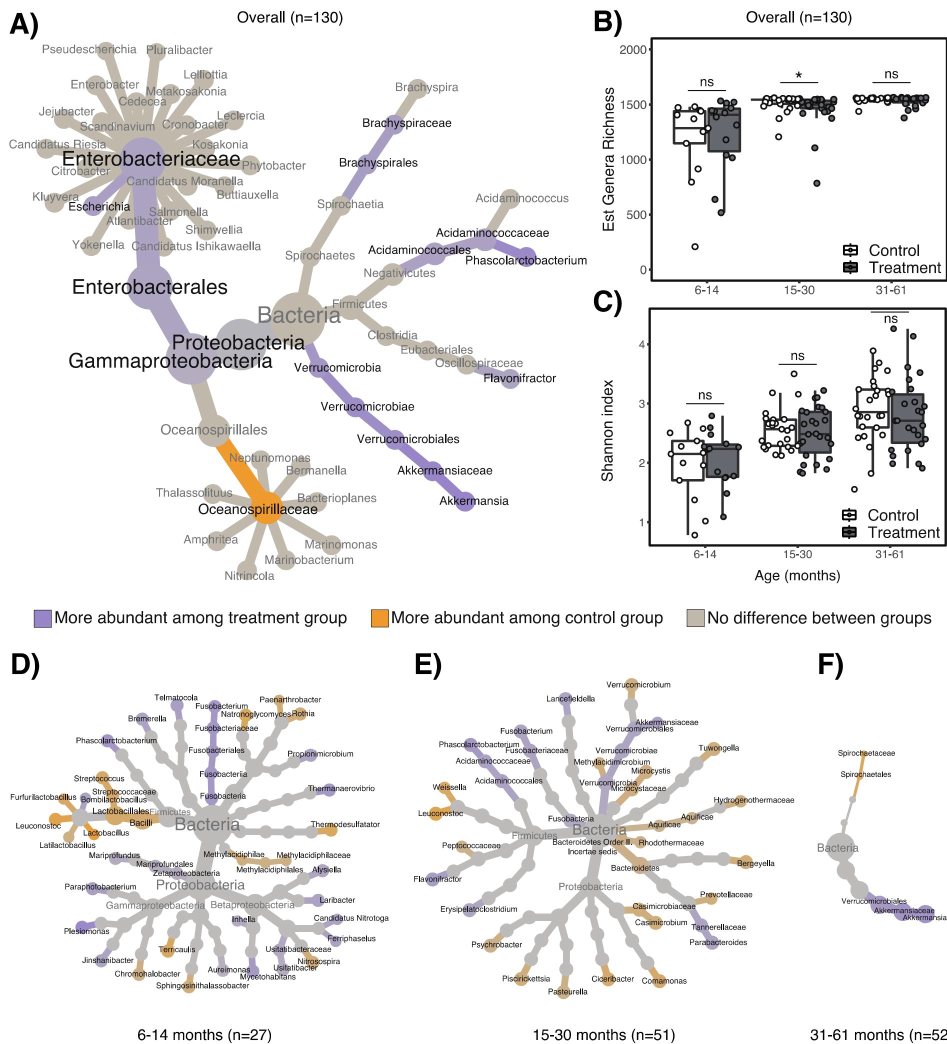
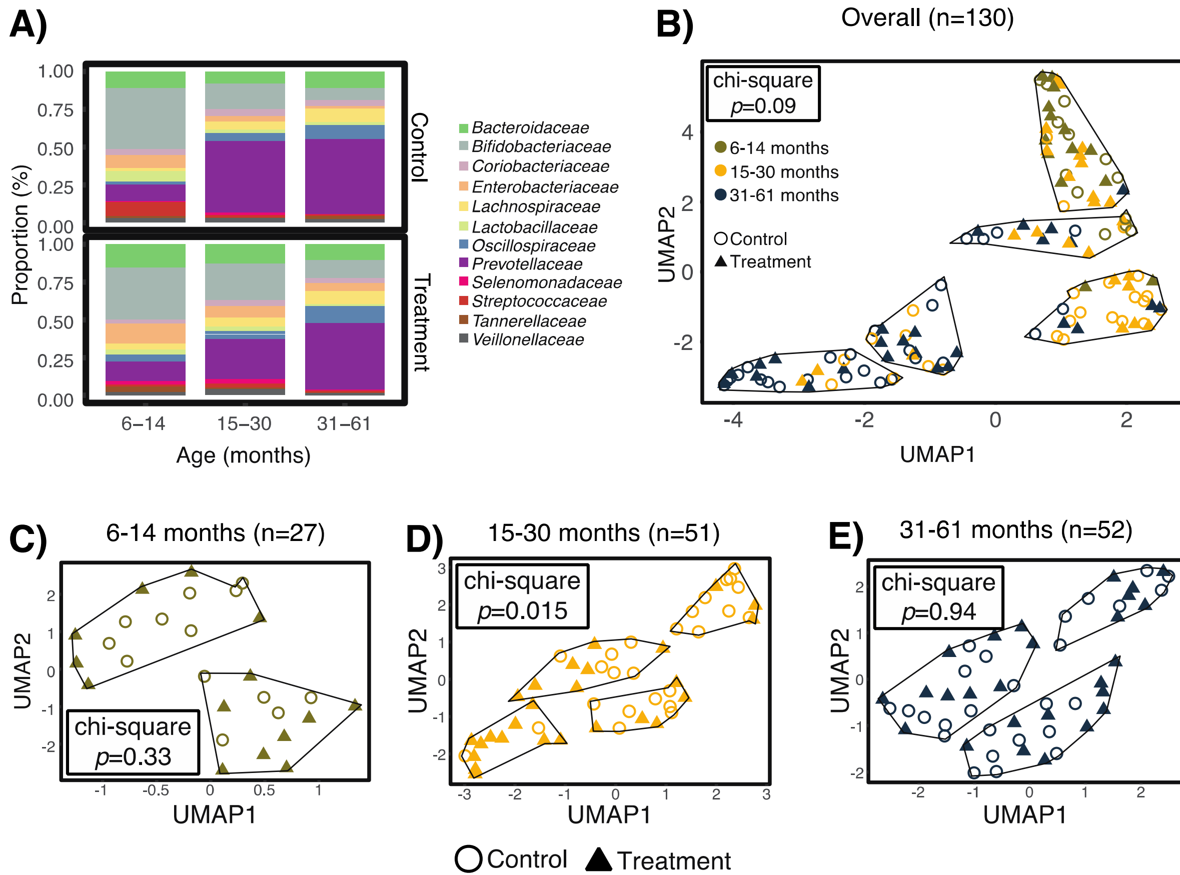


Figure 1. Differentially abundant gut taxa among children aged 6-61 months who were cluster randomized to an automated chlorinated water intervention in urban Bangladesh and effects on overall richness and diversity. Panel A) is a differential heat tree depicting the taxonomies of bacterial genera and families that significantly differed in their relative abundance between treatment and control children. Genera within differentially abundant families are also depicted. For any given taxonomic level, only taxa that were significantly less abundant (orange) or more abundant (purple) among treatment relative to control children are depicted in color; non-significant taxa are depicted in gray. Panel B) depicts estimated genera richness and Panel C) depicts Shannon diversity indices for treatment and control children, stratified by child age. Estimated genera richness differed between treatment and control children aged 15-30 months by the Wilcoxon signed-rank test; Shannon diversity did not significantly differ for any age stratum. For all box plots, center line indicates the median; box limits indicate the upper and lower quartiles; and whiskers indicate 1.5x interquartile range. Panels D) – E) are differential heat trees depicting the taxonomies of bacterial genera and families that significantly differed in their relative abundance between treatment and control children aged D) 6-14 months, E) 15-30 months, and F) 31-61 months, controlling for study site.

Note: ns=non-significant. *indicates p<0.05 by Wilcoxon signed-rank test.



612
613 **Figure 2. Effects of automated water chlorination on the taxonomic structure of**
614 **children's gut microbiomes in urban Bangladesh.** Water chlorination impacted the structure
615 of the gut microbiomes of children aged 15-30 months, but not younger or older children. Panel
616 A) depicts the average relative abundance of bacterial families with ≥1% mean relative
617 abundance across all samples. Panel B) is a two-dimensional representation of the pairwise
618 genomic distances between each sample, as identified by Mash. We observed a marginal
619 association between treatment status and cluster classification by the chi-square test. Panels C-
620 E) depict pairwise distances and resulting clusters when stratified by child age. Treatment status
621 was only associated with cluster classifications among children aged 15-30 months.
622

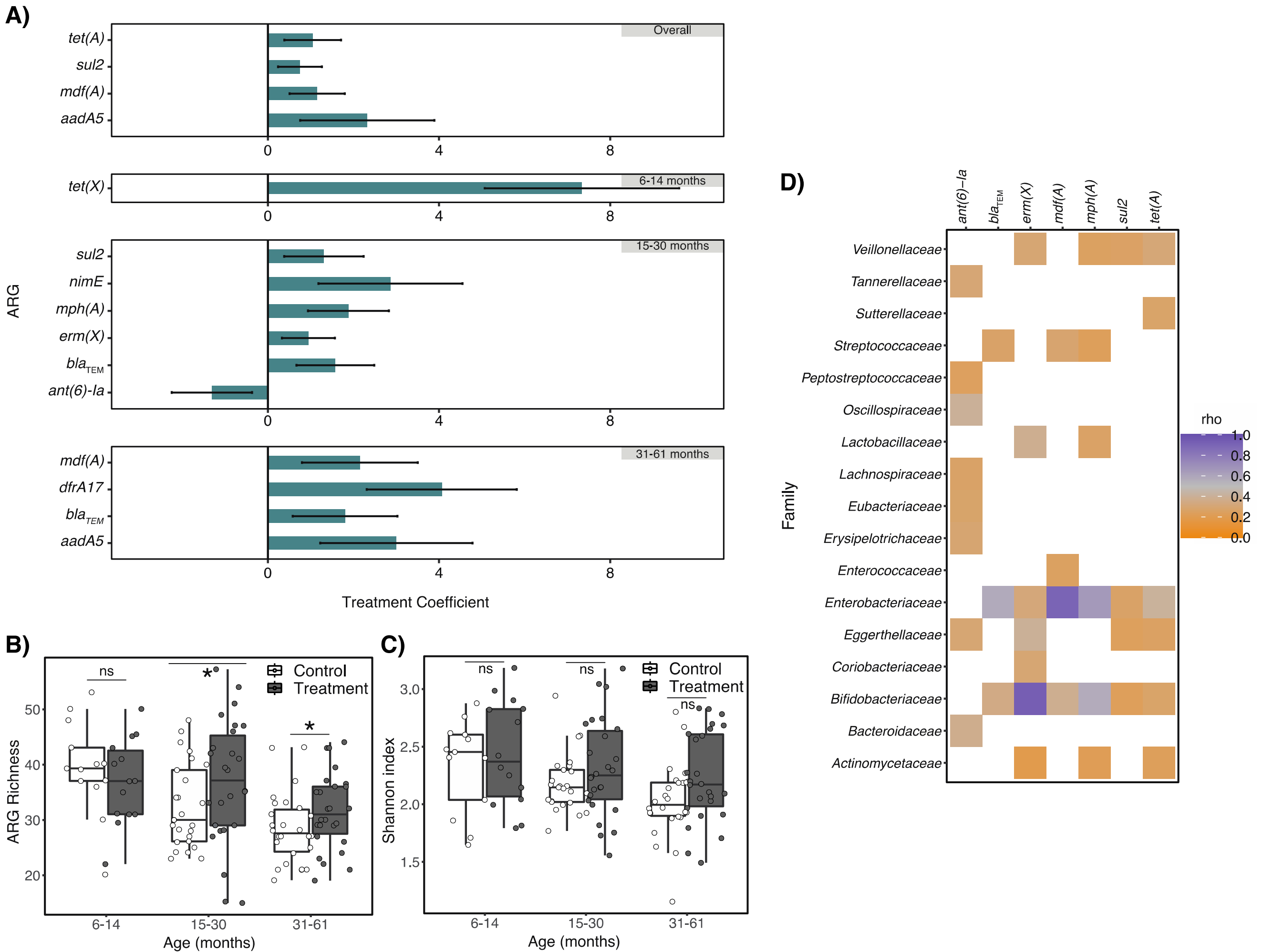


Figure 3. Effects of automated water chlorination on the richness, diversity, and relative abundance of antibiotic resistance genes (ARGs) harbored by children's intestinal flora in urban Bangladesh. Panel A) describes ARGs that were differentially abundant between treatment and control children, controlling for study site (and child age in the "Overall" panel only). Error bars depict the 95% confidence interval. Positive treatment coefficient values indicate ARGs were more abundant among treatment children relative to controls; negative values indicate ARGs were less abundant. Panel B) depicts the estimated number of unique ARGs (*i.e.*, ARG richness) detected in children's fecal metagenomes, stratified by child age. Estimated ARG richness significantly differed between treatment and control children aged 15-30 months and 31-61 months using the *betta* function of the R package *breakaway*. Panel C) depicts the Shannon diversity indices for treatment and control children, stratified by child age. There was no statistical association between treatment status and ARG diversity for any of the age stratum by the Wilcoxon signed-rank test. For all box plots, center line indicates the median; box limits indicate the upper and lower quartiles; and whiskers indicate 1.5x interquartile range. Panel D) is a heatmap depicting Spearman correlations between ARGs listed in Panel A) that occurred in at least half of samples and bacterial families that occurred in at least half of samples. *Rho* values are only depicted for statistically significant correlations (p<0.05 after adjustment for multiple testing).

Note: ns=non-significant. *indicates p≤0.05.

References

645 1. Stewart, C. J. *et al.* Temporal development of the gut microbiome in early childhood from
646 the TEDDY study. *Nature* **562**, 583–588 (2018).
647 2. Robertson, R. C., Manges, A. R., Finlay, B. B. & Prendergast, A. J. The Human Microbiome
648 and Child Growth – First 1000 Days and Beyond. *Trends in Microbiology* **27**, 131–147 (2019).
649 3. Bisgaard, H. *et al.* Reduced diversity of the intestinal microbiota during infancy is associated
650 with increased risk of allergic disease at school age. *Journal of Allergy and Clinical*
651 *Immunology* **128**, 646-652.e5 (2011).
652 4. Cox, L. M. *et al.* Altering the Intestinal Microbiota during a Critical Developmental Window
653 Has Lasting Metabolic Consequences. *Cell* **158**, 705–721 (2014).
654 5. Aversa, Z. *et al.* Association of Infant Antibiotic Exposure With Childhood Health Outcomes.
655 *Mayo Clinic Proceedings* **96**, 66–77 (2021).
656 6. Platts-Mills, J. A. *et al.* Use of quantitative molecular diagnostic methods to assess the
657 aetiology, burden, and clinical characteristics of diarrhoea in children in low-resource
658 settings: a reanalysis of the MAL-ED cohort study. *Lancet Glob Health* **6**, e1309–e1318
659 (2018).
660 7. Carding, S., Verbeke, K., Vipond, D. T., Corfe, B. M. & Owen, L. J. Dysbiosis of the gut
661 microbiota in disease. *Microb Ecol Health Dis* **26**, (2015).
662 8. Borton, M. A. *et al.* Chemical and pathogen-induced inflammation disrupt the murine
663 intestinal microbiome. *Microbiome* **5**, 47 (2017).
664 9. Vaz, L. E. *et al.* Recent trends in outpatient antibiotic use in children. *Pediatrics* **133**, 375–
665 385 (2014).

666 10. Rogawski, E. T. *et al.* Use of antibiotics in children younger than two years in eight
667 countries: a prospective cohort study. *Bull. World Health Organ.* **95**, 49–61 (2017).
668 11. Schwartz, D. J., Langdon, A. E. & Dantas, G. Understanding the impact of antibiotic
669 perturbation on the human microbiome. *Genome Medicine* **12**, 82 (2020).
670 12. McDonnell, L. *et al.* Association between antibiotics and gut microbiome dysbiosis in
671 children: systematic review and meta-analysis. *Gut Microbes* **13**, 1–18 (2021).
672 13. Pickering, A. J. *et al.* Effect of in-line drinking water chlorination at the point of collection on
673 child diarrhoea in urban Bangladesh: a double-blind, cluster-randomised controlled trial.
674 *The Lancet Global Health* **7**, e1247–e1256 (2019).
675 14. World Health Organization. *Guidelines for drinking-water quality: fourth edition*
676 *incorporating the first addendum*. https://www.who.int/publications-detail-
677 redirect/9789241549950.
678 15. Dai, Z. *et al.* Disinfection exhibits systematic impacts on the drinking water microbiome.
679 *Microbiome* **8**, 42 (2020).
680 16. Waak, M. B., Hozalski, R. M., Hallé, C. & LaPara, T. M. Comparison of the microbiomes of
681 two drinking water distribution systems-with and without residual chloramine disinfection.
682 *Microbiome* **7**, 87 (2019).
683 17. Chiao, T.-H., Clancy, T. M., Pinto, A., Xi, C. & Raskin, L. Differential resistance of drinking
684 water bacterial populations to monochloramine disinfection. *Environ Sci Technol* **48**, 4038–
685 4047 (2014).
686 18. Shi, P. *et al.* Metagenomic insights into chlorination effects on microbial antibiotic
687 resistance in drinking water. *Water Res* **47**, 111–120 (2013).

688 19. Hou, A.-M. *et al.* Chlorine injury enhances antibiotic resistance in Pseudomonas aeruginosa
689 through over expression of drug efflux pumps. *Water Res* **156**, 366–371 (2019).
690 20. Li, D. & Gu, A. Z. Antimicrobial resistance: A new threat from disinfection byproducts and
691 disinfection of drinking water? *Current Opinion in Environmental Science & Health* **7**, 83–91
692 (2019).
693 21. Martino, D. The Effects of Chlorinated Drinking Water on the Assembly of the Intestinal
694 Microbiome. *Challenges* **10**, (2019).
695 22. Sasada, T. *et al.* Chlorinated Water Modulates the Development of Colorectal Tumors with
696 Chromosomal Instability and Gut Microbiota in Apc-Deficient Mice. *PLoS One* **10**, e0132435
697 (2015).
698 23. Pop, M. *et al.* Diarrhea in young children from low-income countries leads to large-scale
699 alterations in intestinal microbiota composition. *Genome Biol* **15**, R76 (2014).
700 24. Martin, B. D., Witten, D. & Willis, A. D. Modeling microbial abundances and dysbiosis with
701 beta-binomial regression. *Ann Appl Stat* **14**, 94–115 (2020).
702 25. Sharif, N. *et al.* Molecular and Epidemiologic Analysis of Diarrheal Pathogens in Children
703 With Acute Gastroenteritis in Bangladesh During 2014-2019. *Pediatr Infect Dis J* **39**, 580–
704 585 (2020).
705 26. Willis, A., Bunge, J. & Whitman, T. Improved detection of changes in species richness in high
706 diversity microbial communities. *Journal of the Royal Statistical Society: Series C (Applied
707 Statistics)* **66**, 963–977 (2017).
708 27. Willis, A. & Bunge, J. Estimating diversity via frequency ratios. *Biometrics* **71**, 1042–1049
709 (2015).

710 28. Montealegre, M. C. *et al.* High Genomic Diversity and Heterogenous Origins of Pathogenic
711 and Antibiotic-Resistant Escherichia coli in Household Settings Represent a Challenge to
712 Reducing Transmission in Low-Income Settings. *mSphere* **5**, (2020).
713 29. Ahmed, S. *et al.* High prevalence of mcr-1 -encoded colistin resistance in commensal
714 Escherichia coli from broiler chicken in Bangladesh. *Scientific Reports* **10**, 18637 (2020).
715 30. Wang, N., Hang, X., Zhang, M., Peng, X. & Yang, H. New genetic environments of the
716 macrolide-lincosamide-streptogramin resistance determinant erm(X) and their influence on
717 potential horizontal transferability in bifidobacteria. *International Journal of Antimicrobial*
718 *Agents* **50**, 572–580 (2017).
719 31. Cao, L. *et al.* Literature-Based Phenotype Survey and In Silico Genotype Investigation of
720 Antibiotic Resistance in the Genus Bifidobacterium. *Curr Microbiol* **77**, 4104–4113 (2020).
721 32. Chelakkot, C. *et al.* Akkermansia muciniphila-derived extracellular vesicles influence gut
722 permeability through the regulation of tight junctions. *Experimental & Molecular Medicine*
723 **50**, e450–e450 (2018).
724 33. Zhao, S. *et al.* Akkermansia muciniphila improves metabolic profiles by reducing
725 inflammation in chow diet-fed mice. *J Mol Endocrinol* **58**, 1–14 (2017).
726 34. Borgo, F. *et al.* Body Mass Index and Sex Affect Diverse Microbial Niches within the Gut.
727 *Front. Microbiol.* **9**, (2018).
728 35. Mikami, A. *et al.* Oral Administration of Flavonifractor plautii, a Bacteria Increased With
729 Green Tea Consumption, Promotes Recovery From Acute Colitis in Mice via Suppression of
730 IL-17. *Front. Nutr.* **7**, (2021).

731 36. Gupta, A. *et al.* Association of Flavonifractor plautii, a Flavonoid-Degrading Bacterium, with
732 the Gut Microbiome of Colorectal Cancer Patients in India. *mSystems* **4**, e00438-19 (2019).
733 37. Coello, K. *et al.* Gut microbiota composition in patients with newly diagnosed bipolar
734 disorder and their unaffected first-degree relatives. *Brain Behav Immun* **75**, 112–118
735 (2019).
736 38. Depommier, C. *et al.* Supplementation with Akkermansia muciniphila in overweight and
737 obese human volunteers: a proof-of-concept exploratory study. *Nature Medicine* **25**, 1096–
738 1103 (2019).
739 39. Hodges, J. K. *et al.* Intestinal-level anti-inflammatory bioactivities of catechin-rich green tea:
740 Rationale, design, and methods of a double-blind, randomized, placebo-controlled
741 crossover trial in metabolic syndrome and healthy adults. *Contemporary Clinical Trials*
742 *Communications* **17**, 100495 (2020).
743 40. Nagao, T., Hase, T. & Tokimitsu, I. A Green Tea Extract High in Catechins Reduces Body Fat
744 and Cardiovascular Risks in Humans. *Obesity* **15**, 1473–1483 (2007).
745 41. Wu, F. *et al.* Phascolarctobacterium faecium abundant colonization in human
746 gastrointestinal tract. *Exp Ther Med* **14**, 3122–3126 (2017).
747 42. Nagao-Kitamoto, H. *et al.* Interleukin-22-mediated host glycosylation prevents
748 Clostridioides difficile infection by modulating the metabolic activity of the gut microbiota.
749 *Nat Med* **26**, 608–617 (2020).
750 43. Spiga, L. *et al.* An Oxidative Central Metabolism Enables Salmonella to Utilize Microbiota-
751 Derived Succinate. *Cell Host & Microbe* **22**, 291-301.e6 (2017).

752 44. Eberl, C. *et al.* E. coli enhance colonization resistance against Salmonella Typhimurium by
753 competing for galactitol, a context-dependent limiting carbon source. *Cell Host & Microbe*
754 **29**, 1680-1692.e7 (2021).
755 45. Westerman, L. J. *et al.* Brachyspira Species and Gastroenteritis in Humans. *J Clin Microbiol*
756 **51**, 2411–2413 (2013).
757 46. Kaper, J. B., Nataro, J. P. & Mobley, H. L. T. Pathogenic Escherichia coli. *Nature Reviews*
758 *Microbiology* **2**, 123–140 (2004).
759 47. Navidad, J. F., Griswold, D. J., Gradus, M. S. & Bhattacharyya, S. Evaluation of Luminex xTAG
760 gastrointestinal pathogen analyte-specific reagents for high-throughput, simultaneous
761 detection of bacteria, viruses, and parasites of clinical and public health importance. *J Clin*
762 *Microbiol* **51**, 3018–3024 (2013).
763 48. Rogawski, E. T. *et al.* Use of quantitative molecular diagnostic methods to investigate the
764 effect of enteropathogen infections on linear growth in children in low-resource settings:
765 longitudinal analysis of results from the MAL-ED cohort study. *The Lancet Global Health* **6**,
766 e1319–e1328 (2018).
767 49. Baker, J. M. *et al.* Association of enteropathogen detection with diarrhoea by age and high
768 versus low child mortality settings: a systematic review and meta-analysis. *The Lancet*
769 *Global Health* **9**, e1402–e1410 (2021).
770 50. Sassone-Corsi, M. & Raffatellu, M. No Vacancy: How beneficial microbes cooperate with
771 immunity to provide colonization resistance to pathogens. *J Immunol* **194**, 4081–4087
772 (2015).

773 51. Buffie, C. G. & Pamer, E. G. Microbiota-mediated colonization resistance against intestinal
774 pathogens. *Nat. Rev. Immunol.* **13**, 790–801 (2013).
775 52. Hu, Y. *et al.* The Bacterial Mobile Resistome Transfer Network Connecting the Animal and
776 Human Microbiomes. *Appl Environ Microbiol* **82**, 6672–6681 (2016).
777 53. Rousham Emily K. *et al.* Human Colonization with Extended-Spectrum Beta-Lactamase-
778 Producing E. coli in Relation to Animal and Environmental Exposures in Bangladesh: An
779 Observational One Health Study. *Environmental Health Perspectives* **129**, 037001.
780 54. Montealegre, M. C. *et al.* Risk Factors for Detection, Survival, and Growth of Antibiotic-
781 Resistant and Pathogenic Escherichia coli in Household Soils in Rural Bangladesh. *Appl.*
782 *Environ. Microbiol.* **84**, (2018).
783 55. Nadimpalli, M. L. *et al.* Urban informal settlements as hotspots of antimicrobial resistance
784 and the need to curb environmental transmission. *Nature Microbiology* **5**, 787–795 (2020).
785 56. Masud, A. A. *et al.* Drivers of Antibiotic Use in Poultry Production in Bangladesh:
786 Dependencies and Dynamics of a Patron-Client Relationship. *Front. Vet. Sci.* **7**, (2020).
787 57. Nordahl Petersen, T. *et al.* Meta-genomic analysis of toilet waste from long distance flights;
788 a step towards global surveillance of infectious diseases and antimicrobial resistance. *Sci*
789 *Rep* **5**, 11444 (2015).
790 58. Hendriksen, R. S. *et al.* Global monitoring of antimicrobial resistance based on
791 metagenomics analyses of urban sewage. *Nature Communications* **10**, 1124 (2019).
792 59. Porras, A. M. & Brito, I. L. The internationalization of human microbiome research. *Curr.*
793 *Opin. Microbiol.* **50**, 50–55 (2019).

794 60. Yang, Q. *et al.* Role of Dietary Nutrients in the Modulation of Gut Microbiota: A Narrative
795 Review. *Nutrients* **12**, 381 (2020).
796 61. Pham, V. T. *et al.* Effects of colon-targeted vitamins on the composition and metabolic
797 activity of the human gut microbiome– a pilot study. *Gut Microbes* **13**, 1875774 (2021).
798 62. Tang, Q. *et al.* Current Sampling Methods for Gut Microbiota: A Call for More Precise
799 Devices. *Front. Cell. Infect. Microbiol.* **10**, (2020).
800 63. Wood, D. E., Lu, J. & Langmead, B. Improved metagenomic analysis with Kraken 2. *Genome*
801 *Biol.* **20**, 257 (2019).
802 64. Lu, J., Breitwieser, F. P., Thielen, P. & Salzberg, S. L. Bracken: estimating species abundance
803 in metagenomics data. *PeerJ Computer Science* **3**, e104 (2017).
804 65. Clausen, P. T. L. C., Aarestrup, F. M. & Lund, O. Rapid and precise alignment of raw reads
805 against redundant databases with KMA. *BMC Bioinformatics* **19**, 307 (2018).
806 66. Ondov, B. D. *et al.* Mash: fast genome and metagenome distance estimation using
807 MinHash. *Genome Biol* **17**, 132 (2016).
808 67. Fraley, C. & Raftery, A. E. *MCLUST: Software for rvlodel-Based Clustering, Density Estimation*
809 *and Discriminant Analysis*. 50 (2002).
810 68. Fraley, C. & Raftery, A. E. Model-Based Clustering, Discriminant Analysis, and Density
811 Estimation. *Journal of the American Statistical Association* **97**, 611–631 (2002).
812 69. Knee, J. *et al.* Effects of an urban sanitation intervention on childhood enteric infection and
813 diarrhea in Maputo, Mozambique: A controlled before-and-after trial. *eLife* **10**, e62278
814 (2021).

815 70. Benjamini, Y. & Hochberg, Y. Controlling the False Discovery Rate: A Practical and Powerful
816 Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B*
817 *(Methodological)* **57**, 289–300 (1995).
818 71. Konstantinidis, K. T. & Tiedje, J. M. Genomic insights that advance the species definition for
819 prokaryotes. *Proc Natl Acad Sci U S A* **102**, 2567–2572 (2005).
820 72. Foster, Z. S. L., Sharpton, T. J. & Grünwald, N. J. Metacoder: An R package for visualization
821 and manipulation of community taxonomic diversity data. *PLOS Computational Biology* **13**,
822 e1005404 (2017).
823 73. McMurdie, P. J. & Holmes, S. phyloseq: An R Package for Reproducible Interactive Analysis
824 and Graphics of Microbiome Census Data. *PLOS ONE* **8**, e61217 (2013).
825 74. Alcock, B. P. *et al.* CARD 2020: antibiotic resistome surveillance with the comprehensive
826 antibiotic resistance database. *Nucleic Acids Res* **48**, D517–D525 (2020).
827