Metagenomics Reveals Impact of Geography and Acute Diarrhoeal Disease on the Central Indian Human Gut Microbiome

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28 **Abstract**

29 **Background:** The Central Indian gut microbiome remains grossly understudied. Herein, we

30 sought to investigate the burden of antimicrobial resistance and diarrhoeal diseases, particularly

31 *Clostridioides difficile*, in rural-agricultural and urban populations in Central India, where there

32 is widespread unregulated antibiotic use. We utilised shotgun metagenomics to

33 comprehensively characterise the bacterial and viral fractions of the gut microbiome and their

34 encoded functions in 105 participants.

35 **Results:** We observed distinct rural-urban differences in bacterial and viral populations, with

36 geography exhibiting a greater influence than diarrhoeal status. *Clostridioides difficile* disease

37 was more commonly observed in urban subjects, and their microbiomes were enriched in

38 metabolic pathways relating to the metabolism of industrial compounds and genes encoding

39 resistance to 3rd generation cephalosporins and carbapenems. By linking phages present in the

40 microbiome to their bacterial hosts through CRISPR spacers, phage variation could be directly

41 related to shifts in bacterial populations, with the auxiliary metabolic potential of rural-

42 associated phages enriched for carbon and amino acid energy metabolism.

43 **Conclusions:** We report distinct differences in antimicrobial resistance gene profiles,

44 enrichment of metabolic pathways and phage composition between rural and urban

45 populations, as well as a higher burden of *Clostridioides difficile* disease in the urban

46 population. Our results reveal that geography is the key driver of variation in urban and rural

47 Indian microbiomes, with acute diarrhoeal disease, including *C. difficile* disease exerting a

48 lesser impact. Future studies will be required to understand the potential role of dietary, cultural

49 and genetic factors in contributing to microbiome differences between rural and urban

50 populations.

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Keywords

54   Gut microbiome, antibiotic resistome, virome, diarrhoea, *Clostridioides difficile*, Central India

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Introduction

The human gut houses a complex microbial ecosystem referred to as the microbiome, which includes prokaryotic, eukaryotic and viral components. While the bacterial components of the microbiome have received considerable attention, comparatively little is known about the composition and physiological significance of human gut-associated bacteriophage populations, otherwise known as the phageome.[1] Moreover, despite the growing global burden of antibiotic resistance to modern health care, very few studies have directly[2,3] or indirectly, (through analysing urban sewage)[4] examined the antibiotic resistomes of human faecal metagenomes. Such paucity of data prevents a complete understanding of the global burden and transmission of antimicrobial resistance (AMR), which is essential to support national and global priority setting, public health actions, and treatment decisions. Although recent years have seen an explosion of gut microbiome studies in rural pre-industrialised societies such as hunter-gatherer and other geographically diverse populations,[5-10] little is known about microbial variability and its implications for health and disease in other underrepresented populations in South America, Africa, and regions in Asia, particularly India, where there is a scarcity of microbiome data in diarrhoeal and other populations.[11-14] Diarrhoeal diseases are a major cause of morbidity and mortality in India, making identification of aetiological agents of utmost importance.[15-18]

In India, there is tremendous opportunity to study highly diverse communities with varied geographic distribution, dietary habits and socioeconomic stratification. Some of these communities, including a large tribal population, remain dependent on hunting, agriculture and fishing with their own culture, tradition, dietary habits, language and genetic make-up. Recently, studies have begun to explore the Indian gut microbiome including that of the country's scheduled tribes, principally using 16S rRNA gene amplicon sequencing methods to profile mainly gut bacterial diversity in rural and urban healthy populations[11-14] with only a few

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82   reports employing whole-genome shotgun metagenomic sequencing approaches.[19-20] Whilst

83   the majority of the aforementioned studies have analysed small population cohorts from

84   Northern, Southern and Western Indian territories, there is a dearth of information

85   characterising the gut microbiomes of Central Indian populations. Furthermore, little is known

86   about the burden of *Clostridioides difficile* infection (CDI) in India, the leading worldwide

87   cause of antibiotic-associated diarrhoea in hospitalised and community populations[21-25] and its

88   impact on Indian metagenomes. Profligate, unregulated antibiotic use and inappropriate

89   prescribing suggest that CDI could be widespread in India, the world's largest consumer of

90   antibiotics. [26]

91   Via a pre-existing research partnership between the University of Nottingham and the Central

92   India Institute of Medical Sciences (CIIMS), we were able to define the gut bacteriome,

93   antibiotic resistome and virome in understudied rural and urban diarrhoeal and control

94   populations in Central India. CIIMS has established multisite links with several hospital

95   laboratories in the surrounding district of Nagpur, as well as a satellite laboratory in the

96   Mahatma Gandhi Tribal hospital, Melghat, home to the Korku tribe of agriculturalists. We also

97   concentrated on the pathogen *Clostridiodies difficile* and assessed its impact on the gut

98   microbiome.

99   Our results indicate that the rural habitants of Melghat show a *Prevotella*-dominant

100   microbiome compared with the urban population of Nagpur, which is enriched with

101   *Bacteroides spp*. Urbanisation is associated with functional enrichment of genes involved in

102   xenobiotic and lipid metabolism. Although a core set of AMR genes are detectable in the Korku

103   population, Nagpurian urbanites display a much higher burden of AMR overall. Viral diversity

104   and composition is more influenced by geography than diarrhoeal status, with urban- and rural-

105   specific phage populations linked to bacterial hosts through CRISPR spacer identification. *C.*

106 *difficile* is principally detected in the urban and peri-urban exposed antibiotic populations,

107 many of which carry AMR genes to virtually every class of antibiotic.

108 **Results**

109 **Cohort Characteristics**

110 For our faecal metagenome study in which we were comparing urban vs rural microbiome

111 profiles and assessing impact of diarrhoea and CDI, we analysed faecal samples collected from

112 105 Central Indian participants comprising 35 rural (12 with diarrhoea) and 70 urban (46 with

113 diarrhoea) participants from Melghat and Nagpur districts, respectively (Supplementary Table

114 1 and Supplementary metadata). We selected an enriched set of faecal DNA samples derived

115 from diarrhoeal samples that had previously tested positive in our aforementioned diagnostic

116 *C. difficile* immunoassays for whole-genome shotgun sequencing (WGS). Of these diarrhoeal

117 samples, 63% (29/46; urban) and 25% (3/12; rural) had tested positive for toxigenic *C. difficile*

118 in the C. DIFF QUIK CHEK assay.

119 Stool samples received centrally by CIIMS were collected at recruitment over 13 months from

120 the 1st of March 2017 to 30th April 2018 from participants resident at 48 sites in Nagpur district

121 (Figure 1) and 19 participating rural villages in Melghat (Supplementary Figure 1), 3 of which

122 were very small villages and are not marked on Google maps. The mean duration of diarrhoea

123 for urban diarrhoeal group (n=34) was 5.2 days (SD 2.7 days). The mean age of participants

124 was greater for urban (42 years) versus rural (35.6 years) participants, *p*=0.01, with a lower

125 percentage of females represented in the urban and rural control groups compared to the

126 diarrhoeal groups which did not reach statistical significance. Mean body mass index (BMI)

127 [weight (kg)/height (m) squared] was also higher in the urban (21.8) compared with rural (19.3)

128 participants group, p<0.0001). It was noteworthy that one third of participants in the urban non-

129 diarrhoeal control group had received antibiotics in the three months prior to recruitment,

130   although none were taking antibiotics when sampled. The vast majority of participant housing

131   in the rural areas was deemed to be of poor quality based on a lack of piped water supply (water

132   tank only), no access to latrines, limited electricity supply (<18 hours/day) and small living

133   space (Supplementary Figure 2), whereas just over half of the urban cohort resided within

134   housing of good quality, as reflected in access to Corporation tap water, longer duration

135   electricity supply (>18 hours/day) and larger living quarters. A higher proportion of rural

136   participants kept domestic animals within their living quarters (cattle, goats, chickens)

137   compared with their urban counterparts.

138   Overall, significant confounding associations were observed between geographic location and

139   several other study variables. Consequently, we focussed our analyses primarily on geographic

140   location, with the understanding this accounts for both subject specific and environmental

141   factors.

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144   **Rural subjects have a distinct microbiome when compared with urban subjects**

145   Principal coordinates analysis was performed on a Bray-Curtis Dissimilarity matrix of the

146   species-level taxonomic profiles (n=105), excluding viral taxa. Urban (n=70) and rural (n=35)

147   subjects separated well along the 1st principal component (Figure 2A) but diarrhoeal status

148   (control n=47 vs. diarrhoeal n=58) did not appear to have as much influence on sample

149   clustering. This observation was confirmed by PERMANOVA which indicated that geographic

150   location (urban vs rural) accounted for 7.7% of the variation between samples (F=8.67,

151   p=0.001) while diarrhoeal status accounted for a further 1.7% (F=1.94, p=0.028). Including *C.*

152   *difficile* toxin status and recent antibiotic exposure in the model accounted for an additional

153   2.1% (F=2.48, p=0.005) and 1.4% (F=1.62, p=0.09) of variation respectively. Considering

154   other demographic variables of interest, including age, gender, BMI, housing quality and

155   animal ownership when combined with geography, only age (2.1%, F=2.41, p=0.008)

156   contributed significantly to the residual variation explained, reflecting the strong association of

157   these variables with study location.

158   Sample alpha diversity was calculated using the Inverse Simpson Index for the taxonomic

159   abundances at species level and compared between control and diarrhoeal subjects from either

160   an urban or rural location (Figure 2B). Rural diarrhoeal subjects had the lowest diversity (n=12,

161   mean 3.66 +/- 2.5) which was significantly lower than urban control subjects who had the

162   highest diversity (n=24, 6.75 +/- 3.5, p.corr=0.05).

163   Individual taxonomic profiles showed a high level of heterogeneity at genus level both within

164   and between study groups (Figure 2C). Overall, profiles from urban areas tended to be

165   dominated by *Bacteroides spp.* with 25/70 urban subjects having a relative abundance of

166   greater than 30 % compared to only 3/35 rural subjects (Chi-squared test; p=0.006). Conversely

167   in rural subjects, *Prevotella spp.* were predominant, particularly in control subjects (15/35 rural

168   subjects with > 30 % *Prevotella spp.* compared to 9/70 urban subjects, Chi-squared test;

169   p=0.001).

170   Analysing the species-level taxonomic abundances using generalized linear models yielded 26

171   taxa which differed significantly between rural and urban control subjects, and 16 taxa which

172   differed significantly between control and diarrhoeal subjects (Figure 2D, Supplementary

173   Tables 2& 3). A direct comparison was also made between diarrhoeal subjects testing positive

174   and negative for *C. difficile* toxin, yielding 18 taxa which differed significantly (Supplementary

175   Table 4).

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Antimicrobial resistance is more prevalent in urban areas

Antimicrobial resistance gene profiles were compiled from the faecal metagenomes of all subjects in the study using ARIBA. Individual gene counts were aggregated by antibiotic class to identify broad trends between subjects according to geographic location and antibiotic exposure (Figure 3A). Genes conferring resistance to beta-lactam antibiotics, tetracyclines and macrolides, lincosamides and streptogramins (MLS) were identified in virtually all subjects. Average resistance gene counts aggregated by class were compared between subjects from rural and urban areas, regardless of diarrhoeal status or antibiotic exposure, indicating that counts for 13 of the 18 classes were significantly higher in urban subjects (Mann Whitney U test, FDR corrected, Figure 3A). Grouping subjects by geography, diarrhoeal status and antibiotic exposure revealed a subset of rural subjects whose faecal metagenomes had resistance to the least number of different antibiotic classes, while some of the urban subjects were carrying antibiotic resistance genes to virtually every class of antibiotic (Figure 3A). This included resistance to glycopeptides (predominantly *vanA* genes) and two classes from the World Health Organisation essential medicines reserve group; fosfomycin and lipopeptides (daptomycin). Compared with other antibiotic classes, metronidazole resistance was rare and only detected in a single subject.

Beta lactam antibiotics are widely used in clinical practice and resistance to broad spectrum beta lactam antibiotics, particularly carbapenems, is of significant public health concern. Individual beta lactam gene clusters derived from the MegaRes antibiotic database were analysed in more detail by subject to identify differences in average gene counts between rural and urban subjects (Figure 3B) with those differing significantly shown in more detail in Figure 4C (Mann Whitney U test, FDR corrected). Resistance mechanisms included production of beta-lactamases (Ambler class A to D), alteration of penicillin binding proteins (PBPs) and mutation of outer membrane porins in Gram negative bacteria.

203 Of the gene clusters with increased counts in urban subjects, many encoded clinically relevant

204 beta-lactamases, including extended spectrum beta-lactamases (CTX) and carbapenemases

205 (NDM). Prevalence of key beta-lactamase genes was analysed by comparing the number of

206 subjects in which the gene cluster was detected in their metagenome. The CFX gene cluster,

207 encoding an Ambler class A beta-lactamase, was the most prevalent cluster detected, identified

208 in 94 of 105 subjects. The prevalence of several clinically relevant beta lactam gene clusters

209 was higher in urban subjects when compared to rural subjects, including CTX, NDM and OXA

210 (Supplementary Table 5). Gene clusters encoding the other clinically important

211 carbapenemases, KPC, VIM and IMP, were not detected in any of the subjects.

212

213 **Microbiota variations between groups are predicted to drive functional shifts in**

214 **metabolic pathways**

215 Differentially abundant metabolic pathways between urban and rural subjects and their

216 predicted taxonomic contributions were identified with FishTaco (Figure 4). A total of 28

217 pathways were enriched in urban subjects, with the majority (24/28) in the following

218 categories; xenobiotics biodegradation and metabolism (16/28), lipid metabolism (6/28) and

219 amino acid metabolism (2/28). Several *Bacteroides spp.*, *Parabacteroides distasonis,*

220 *Klebsiella pneumoniae* and *E. coli* were identified as potential contributors to the enrichment

221 of these pathways in urban subjects.

222 Of the 33 pathways enriched in rural subjects, 13/33 related to metabolism of amino acids, 4/33

223 to carbohydrate metabolism and 4/33 to metabolism of cofactors and vitamins. *Prevotella*

224 *copri*, *Prevotella stercorea* and several members of the *Firmicutes* phylum, including

225 *Ruminococcus bromii*, *Eubacterium rectale* and *Faecalibacterium prausnitzii*, were identified

226 as potentially important contributors to the enrichment of these pathways in rural subjects,

227 counterbalanced by the presence of *Parabacteroides distasonis* in urban subjects.

228 As the contribution of each taxa to the functional shifts had been inferred based on a

229 comparison of taxonomic abundance to gene abundance across all samples, we sought further

230 evidence based on the genomic content of related reference genomes to corroborate these

231 findings. KEGG orthology copy number data for the top 10 urban and rural enriched metabolic

232 pathways were obtained for 4 representative rural and urban genomes (Supplementary Tables

233 6 & 7. Several pathways relating to xenobiotics biodegradation and metabolism enriched in

234 urban subjects were encoded at high copy number by the *Klebsiella pneumoniae* and *E. coli*

235 reference genomes but were absent or encoded at low copy number by representative rural

236 species, particularly *Prevotella copri*. For the rural enriched pathways, most were encoded at

237 high copy number across all 8 representative rural and urban species, consistent with the more

238 balanced FishTaco profiles for these pathways. Although copy number by species for rural

239 enriched pathways tended to be slightly higher for the urban representative species, their

240 overall contribution may be offset by their relative abundance as a proportion of the total

241 microbiota per subject.

242 Although no differences were identified in pathway enrichment between *C. difficile* positive

243 and negative diarrhoeal subjects, 54 pathways were enriched in control non-diarrhoeal subjects

244 when compared with diarrheal subjects. These included multiple pathway categories relating

245 to amino acid metabolism (14/54), carbohydrate metabolism (10/54), cofactors and vitamins

246 (8/54) and energy metabolism (6/54).

247

248 **Indian faecal viromes differ by geographic location**

249   A total of 8,746 non-redundant viral sequences were detected in the whole community

250   metagenomic sequencing data for 105 Indian faecal samples. These viruses group into 1,344

251   Viral Clusters (VCs), which are concordant with viral genera.[22] Network visualisation of the

252   shared protein clusters between VCs shows the majority of Indian faecal viruses identified are

253   connected to previously described *Caudovirales* (Figure 5A). Several *Microviridae*,

254   *Inoviridae*, and archaeal viruses of the *Rudiviridae* and *Bicaudaviridae* families, were also

255   detected. Unknown viruses were observed which did not share protein clusters with previously

256   characterised viruses.

257   As viruses were identified in whole community metagenomic data, and not specifically targeted

258   using viral isolation and sequencing protocols, it is expected that rare viruses are poorly

259   represented in the final Indian faecal virome. Therefore, for diversity comparisons between

260   cohorts, the Inverse Simpson's index was employed as it is less sensitive to rare taxa. No

261   difference in viral diversity was observed between diarrhoeal and control subjects within

262   specific residence locations. However, a difference in the Inverse Simpson's index was

263   detected between the rural and urban cohorts (rural mean 58.00 +/- 37.53 versus urban mean

264   46.01 +/- 25.36, p adj=0.002; Figure 5B).

265   The unique composition of Indian faecal viromes were assessed through PCoA. The greatest

266   variance is attributable to geographical residence, with 7.8% of the data explained by urban or

267   rural location (F=8.67, p=0.001; Figure 5C). The interaction of geographical residence and the

268   diarrhoeal status of subjects accounts for a further 2.1% of the observed viral differences

269   (F=2.36, p=0.012). Amongst the urban and rural Indian cohorts that were suffering from

270   diarrhoea, the *C. difficile* status of individuals only accounted for an additional 0.6% of the

271   PCoA variation (F=0.63, p=0.897). The impact of antibiotic usage with the geographical

272   residence or diarrhoeal status of subject explains 1.0% and 1.4% of the calculated differences,

273   respectively (F=1.13, p=0.315 and F=1.64, p=0.071, respectively). Additional recorded

274 variables were tested for their effect on the Indian faecal virome. However, in combination,

275 age, gender, BMI, and housing condition did not make a significant contribution to the variance

276 explained, only accounting for 1.3% of the Indian faecal virome dissimilarities (F=1.50,

277 p=0.09).

278 Specific VCs were strongly associated with distinct geographical locations and diarrhoeal

279 status. The relative abundance differences observed for the 50 VCs that had the greatest fold

280 change by geographical location demonstrates that specific VCs are also associated with

281 controls (Figure 5D). Particular VCs associated with urban residing subjects were also clearly

282 associated with diarrhoea. Amongst individuals experiencing diarrhoea, differences in the

283 virome composition were noted between CDT positive and negative faecal samples

284 (Supplementary Figure 3).

285 CRISPR spacers were used to link VCs to their potential bacterial hosts. The relative abundance

286 of VCs and the number of CRISPR spacers against specific VCs demonstrates that urban

287 subjects contain a greater abundance of phages targeting *Bacteroides*, *Parabacteroides*,

288 *Bifidobacterium* and *Escherichia spp*., while there are trends towards more *Eubacterium* and

289 *Prevotella*-infecting VCs amongst rural-residing individuals (Figure 5E). The enterotypes of

290 Indian microbiomes (n=105) are dominated by *Bacteroides* (n=50), *Prevotella* (n=34), and

291 *Escherichia* (n=21). When Indian faecal viromes are analysed in the context of microbiome

292 enterotypes, *Bacteroides*-, *Prevotella*-, and *Escherichia*-infecting phages are prevalent in the

293 corresponding microbial enterotypes (Supplementary Figures 4A & B). Similarly, a trend

294 towards more crAss-like phages predicted to infect *Prevotella* spp. are observed in rural

295 samples (Supplementary Figure 4B; Kruskal-Wallis test, p-value 0.059).

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297 **Virome-associated auxiliary metabolic functions**

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298   While the Indian faecal virome composition analysis was conducted on VCs present in 2 or

299   more individuals, all viral-associated auxiliary metabolic functions were assessed on VCs

300   present in 10 or more individuals. These criteria were implemented in order to focus on the

301   functions associated with the most abundant Indian faecal viruses. There were 723 VCs shared

302   by 10 or more individuals. Of these VCs, the majority (419/723 VCs, 57.95%) are detectable

303   amongst both rural and urban habiting individuals (Figure 6A). However, urban and rural-

304   specific VCs were also observed (240 and 64 VCs, respectively).

305   The functions associated with the largest representative sequence of each VC was predicted.

306   As expected for virome analyses, the most abundant functional predictions corresponded to

307   eggNOG category S: 'Function unknown' and category L: 'Replication, recombination, and

308   repair' (Figure 6C). The presence/absence similarity between VC-encoded functions associated

309   with an individual's virome were compared using PCoA. The variation of the virome-

310   associated auxiliary metabolic functions were better explained by geography than diarrheal

311   status (7.1% versus 2.2%, p=0.001 and 0.025, respectively; Figure 6B).

312   In order to assess the energy harvesting metabolic potential or urban and rural viral

313   communities, eggNOG categories E and G ('Amino acid transport and metabolism', and

314   'Carbohydrate transport and metabolism', respectively) were investigated. The rurally

315   abundant VCs encode at statistically higher frequency genes involved in amino acid and

316   carbohydrate transport and metabolism (Figure 6D-E).

317

318   **Discussion**

319   The composition of the gut microbiome in the context of health and to a much lesser extent,

320   disease, in Indian populations is not well understood. This study is the first to utilise shotgun

321   metagenomics sequencing to comprehensively characterise the gut bacteriome, resistome and

322   virome of rural and urban diarrhoeal and control populations without diarrhoea living in two

323   geographically and culturally distinct regions of Central India, Nagpur and Melghat. Although

324   there is very limited data on the incidence and epidemiology of CDI in India as a whole, a

325   handful of reports mainly conducted in hospitalised patients, indicate detection rates in the

326   range of 6-15.7%. [19-21] In our faecal metagenome study in which we also sought to characterise

327   the impact of *C. difficile*, we selected an enriched set of faecal DNA samples derived from

328   diarrhoeal samples testing positive in diagnostic *C. difficile* immunoassays for whole-genome

329   shotgun sequencing (WGS). As such, the *C. difficile* toxin positivity rates presented herein,

330   may not reflect true prevalence rates in the selected study populations. Nevertheless, our results

331   suggest that CDI is an emerging but as yet under-recognised healthcare-associated infection

332   and is associated mainly with urbanisation and antibiotic exposure. These findings highlight

333   the need to enhance awareness of and testing of subjects with diarrhoea for *C. difficile* in India,

334   particularly in high-risk individuals with recent or ongoing antibiotic exposure or

335   hospitalisation.

336   The taxonomic profiles revealed geographically distinct gut microbiota signatures. As

337   compared with the urban population of Nagpur district, the rural villagers of the Korku tribe in

338   Melghat were observed to have a significantly higher abundance of *Prevotella spp*, particularly

339   in the control subjects, and an underrepresentation of common members of urban-industrial gut

340   microbiomes (e.g., *Bacteroides spp*.). *Prevotella* has been reported as the most prevalent genus

341   associated with the healthy Indian population in previous microbiome studies[11,14,19-20] and has

342   also been observed as the dominant genus in Mongolian, Amerindian and Malawian groups,[11]

343   indicating the occurrence of Enterotype 2 as proposed by Arumugam et al., 2011.[28] *Prevotella*

344   predominance may reflect the diet of the Korku tribe, which is rich in carbohydrates and dietary

345   fibres. In contrast, Nagpur samples were associated with enterotype-1, which were driven by

346   *Bacteroides* and may be again explained by this population's dietary habits, which typically

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347 consists of rice, with some meat and fish. Interestingly, multivariate analysis revealed that

348 geographic location actually accounted for most of the variation in gut microbial communities

349 with diarrhoeal status, including *C. difficile* toxin positivity and antibiotics contributing to a

350 lesser extent. Consistent with recent findings from a large-scale clinical microbiome study

351 which surveyed over 7000 individuals across 14 districts within the Guangdong province in

352 China,[29] inter-individual differences in the composition of the gut microbiome could be

353 overwhelmingly explained by an individual's geographic location. Nevertheless, it is also now

354 accepted that ethnicity strongly selects for specific taxa, although it is unclear what aspects of

355 ethnicity, whether culturally related activities or genetics, underlie its observed association with

356 the microbiota.[29,30]

357 The misuse and overuse of antibiotics in veterinary, agricultural and clinical applications is

358 rampant in India, fuelling antimicrobial resistance. Inadequate public health infrastructure,

359 poor sanitation, and infection control practices in the primary healthcare system increase

360 demand for parallel markets and further contribute to the overuse of antibiotics. Antibiotic

361 resistance is also being driven environmentally by untreated urban waste, sewage effluent from

362 Indian hospitals,[31] and pharmaceutical pollution of waterways.[32] Indiscriminate use of beta-

363 lactam antibiotics in both the community setting and hospitals has given rise to the presence of

364 antibiotic-resistant *Enterobacteriaceae* in healthy human faecal samples in North India.[33] Our

365 faecal resistome data has corroborated recent shotgun metagenomics data indicating the

366 widespread presence of AMR genes in virtually all subjects irrespective of geographic location

367 and is consistent with that reported in Chinese, Hazda hunter-gatherer and resource-limited

368 Latin American faecal microbiotas.[2,3,7] However, although genes conferring resistance to beta-

369 lactam antibiotics, tetracyclines and macrolides, lincosamides and streptogramins (MLS)

370 appeared to be common throughput Nagpur district and Melghat habitats, rural subjects from

371 the Korku tribe generally reported lower exposure to antibiotics and thus displayed a lower

372 abundance of other AMR genes compared with the urban Nagpur participants. In this latter

373 group, those individuals with *C. difficile* infection on antibiotics were carrying AMR genes to

374 virtually every antibiotic class.

375 The co-occurrence of pathogens and AMR genes for critically important antibiotics offers

376 increased opportunities for unwanted horizontal gene transfer events.[31] Perhaps of most

377 concern, the Ambler class B metallo-beta-lactamase NDM, which was detected in only 1 of 35

378 rural subjects but was found in 32/70 urban subjects, and also supports clinical data detecting

379 carbapenemase producing pathogens from Mumbai,[34] and another recent study showing that

380 NDM-1 is also common in hospital effluent from Delhi.[35] Our findings suggest that improving

381 sanitation, health, and education as part of the UN Sustainable Development Goals as well as

382 the consideration of new legislative measures for curtailing environmental pollution may be

383 effective strategies for limiting the burden of AMR in India and globally.

384 Analysis of taxon-level shift contribution profiles in the Nagpurian population suggested that

385 distinct bacteria such as *Bacteroides spp., Parabacteorides distasonis, Klebsiella pneumonia*

386 and *E. coli* may potentially possess xenobiotic, lipid and amino acid metabolising capabilities.

387 In support of these observations, *Parabacteroides distasonis* has recently been shown to

388 transform bile acids which have lipid-digestive and absorptive functions, and enhances the

389 level of succinate in the gut. *Bacteroides spp.* are also dominant in amino acid metabolism in

390 the large intestine.[36] In addition, different species of *Klebsiella* appear to have substantial

391 potential for the biodegradation of diverse pollutants, such as halogenated aromatic and

392 nitroaromatic compounds.[37] This result is in line with previous evidence, which suggest that

393 individuals belonging to different geographies have microbiota with distinct xenobiotic

394 metabolising capacities.[38] Our analysis of taxa associated shifts in metabolic function could

395 also reflect diet and/or the higher exposure of these urban habitants to industrial/agricultural

396 chemicals such as pesticides, fertilisers, antibiotics and other pharmaceuticals.

17

397　Rural subjects tended to have a higher abundance of *Prevotella spp.* (and certain members of

398　the Firmicutes phylum including *Roseuburia spp.* and *Eubacterium spp.*) and showed

399　enrichment in pathways comprising amino acid and carbohydrate metabolism and metabolism

400　of cofactors and vitamins. The FishTaco analysis indicated a potential association between

401　these. These observations are consistent with previous evidence indicating that *Prevotella spp.*

402　show capacity to digest complex carbohydrates and display enzymatic potential to break down

403　cellulose and xylan from foods.[39] A specific strain, *Prevotella copri,* is one of the strongest

404　driver species associated with branched chain amino acid biosynthesis in the gut and insulin

405　resistance,[40] and vitamin A and β-carotene from bananas and mangos can stimulate the growth

406　of both *P. copri* and *P. stercorea*.[41] Furthermore, the faecal metagenomes of the rural subjects

407　were also enriched in genes associated with thiamine metabolism. It is feasible that thiamine

408　deficiency, which is likely to be prevalent in the Korku, may be leading to a host driven

409　compensatory increase in thiamine producing microbiota in the gut.

410　Ecological studies of macro-organisms consistently demonstrate the importance of predators

411　within environments. Nonetheless, the majority of human microbiome studies only consider its

412　bacterial fraction and do not concomitantly study this ecosystem's predators, viruses. In this

413　study, we identified and analysed 8,746 viral sequences grouped into 1,344 putative genera

414　termed Viral Clusters (VCs). Similar to previous studies of the human faecal virome, the vast

415　majority of viruses detected are tailed phages of the order *Caudovirales* that infect bacteria

416　(Figure 5A).

417　Phage predation has been proposed to modulate bacterial populations within ecosystems

418　through various predator-prey interactions.[42-43] The faecal virome diversity of Central India

419　rural inhabitants was greater than their urban counterparts (Figure 5B). A similar observation

420　is described by Rampelli *et al* (2017), whereby two hunter-gatherer communities also had a

421　higher faecal viral diversity compared to two Western society cohorts.[44]

422   The changes in the relative abundance of VCs demonstrates specific viruses are strongly

423   associated with urban and rural communities, and also with diarrhoeal status (Figure 5D). The

424   identification of VCs' host bacteria through CRISPR spacers is in agreement with the bacterial

425   analysis of Indian faecal microbiomes. The relative abundance of viruses targeting *Bacteroides*

426   and *Parabacteroides* is greater amongst urban residing individuals, while viruses targeting

427   *Eubacterium* and *Prevotella* are more abundant amongst rural inhabitants (Figure 5E).

428   The abundance of unique proteins associated with VC representative sequences demonstrates

429   the majority of functions are shared between urban and rural viruses (Figure 6A), with

430   geography best explaining the observed differences (Figure 6B). The most abundant functional

431   annotations associated with Indian faecal viromes correspond to 'function unknown' and

432   'replication, recombination and repair' (Figure 6C). However, recent studies have highlighted

433   the auxiliary metabolic potential of phages. Oceanic virome studies have demonstrated phages

434   enhance the fitness of infected bacteria through augmenting their photosynthetic capability and

435   energy production.[42,45] Therefore, we investigated the energy harvesting potential encoded by

436   human gut viruses. Specific pathways for amino acid and carbohydrate transport and

437   metabolism are more abundant in rural VCs (Figure 6D & E). The increased abundance in rural

438   associated VCs may be attributed to a narrower repertoire of encoded functions.

439   There were several limitations to this study. Co-morbidity data were unknown and we were

440   unable to capture BMIs for all participants (see Supplementary metadata). Detailed dietary

441   information was not available using a standard FFQ approach. Further, the control population

442   comprised mainly hospitalized patients without diarrhoea and thus do not represent healthy

443   controls. It was also not possible to achieve identical sampling strategies across both rural and

444   urban populations, particularly in view of lack of hospital facilities in Melghat. Due to lack of

445   diagnostic facilities, we were unable to determine the etiological cause of acute diarrhoea or in

446   the case of *C. difficile* positive samples, undertake further strain characterisation studies.

447 Finally, due to limitations related to specimen collection and preparation, we were unable to

448 assess other components of the microbiome, including RNA viruses and intestinal parasites.

449 **Conclusions**

450 Here we report the most comprehensive study to date that has simultaneously examined the

451 enteric bacteriome, DNA virome and antibiotic resistome in divergent populations in Central

452 India, a region of the world that has been grossly understudied. Together, these data suggest

453 that not all rural traditional societies display a healthy gut microbiota as exemplified by a lack

454 of significant difference in bacterial diversity between our rural and urban cohorts and the

455 presence of a core set of AMR genes. Our findings will help assess progress towards meeting

456 the goals of global and national action plans to tackle AMR and the burden of infectious

457 diarrhoea in India, including CDI. These results may also be useful in laying the foundations

458 for implementing culturally acceptable One Health-inspired interventions to improve

459 healthcare outcomes in this region of the world.

460

461 **Materials and Methods**

462 *Experimental design and aim of study*

463 The main aim of this observational cohort study was to use shotgun metagenomics to

464 characterise the gut bacteriome, DNA virome and antibiotic resistome of two highly divergent

465 populations in Central India; rural agriculturalists in Melghat and an urban population in

466 Nagpur. We also sought to investigate comparative differences in microbiome profiles in

467 subjects with and without diarrhoea, including the impact of CDI.

468

469 *Human participants*

Inclusion and Exclusion Criteria

471   During participant selection, inclusion criteria were (i) adults aged from 18 to 70 years who

472   could provide written or thumb-print acknowledged informed consent, (ii) HIV, hepatitis B or

473   C negative, and (iii) not pregnant or breast-feeding.

474   For the diarrhoeal group, a presumptive diagnosis of infective diarrhoea was defined as 3 or

475   more loose stools in a 24-hour period accompanied by other gastrointestinal symptoms such as

476   nausea, vomiting, abdominal cramps, tenesmus, bloody stools, or fever (oral temperature

477     $\geq38^{\circ}$C). All subjects in the *C. difficile*-infected group had diarrhoea and a positive stool *C.*

478   *difficile* (enzyme immunoassay) for toxin.

479   The exclusion criteria for this group were (i) any individual with a known non-infectious cause

480   of diarrhoea such as inflammatory bowel disease, (ii) those unable to provide a stool sample,

481   (iii) or if the sample is formed stool. For the non-diarrhoeal control group, the exclusion criteria

482   were (i) presence of acute diarrhoea at the time of or within 2 weeks of recruitment or (ii) those

483   unable to provide a stool sample. It was acknowledged that such individuals could be recruited

484   from the in- or outpatient population and could have been exposed to antibiotics in the recent

485   past (within 3 months of recruitment), although ideally not at the time of recruitment.

486   Immunosuppression was defined as those with cancer, were receiving chemotherapy or on

487   prednisolone (>5mg/d), immunomodulators (azathioprine, methotrexate, calcineurin inhibitor)

488   or biologics.

489   Potential participants from Nagpur were identified with the assistance of project fellows at the

490   Central India Institute of Medical Sciences (CIIMS) who approached all consecutive cases of

491   diarrhoea presenting to CIIMS as either an in-or outpatient. Similarly, all non-diarrhoeal cases

492   were recruited to this study via the assistance of inpatient or outpatient clinical teams who

493   closely liaised with the project fellows at CIIMS. All rural participants who provided stool

494   samples in this study were directly recruited by community village health care workers trained

495   by MAHAN Trust, which is a non-governmental organisation providing medical expertise to

496   the disparate tribal population of the Melghat region in their own homes.

497

498

499   *Human Geography - Nagpur*

500   Nagpur is the third largest city of the Indian state of Maharashtra and the 13th largest city by

501   population (2.5M) in India. It is located at the exact centre of the Indian peninsula (zero

502   milestone) and enjoys a tropical savannah climate where temperatures can reach in excess of

503   48 $^{\circ}$C in the summer months. Hinduism is the main religion followed closely by Buddhism and

504   Islam, with smaller contributions from Christianity, Jainism and Sikhism.

505   Nagpur is an emerging metropolis attracting significant commercial inward investment and is

506   a major education hub in Central India. It is also home to the Central Indian Institute of Medical

507   Sciences (CIIMS). Nagpur was declared open defecation free in January 2018 and is one of the

508   cleanest and most livable cities in India, as a leader in healthcare, green spaces and public

509   transportation. The majority of households have good drinking water and sanitation facilities,

510   and use clean fuel for cooking.

511

512   *Human Geography - Melghat*

513   Melghat Tiger Reserve, with its diverse flora and fauna, is located in Amaravati district of

514   Maharashtra and is home to approximately 250,000 members of the Korku tribe spread across

515   two talukas, Dharni and Chikaldhara and 300 villages, and extends across 4,000 square km. By

516   road, it is approximately 250 km from Nagpur.

All rural Melghat subjects within the Melghat Tiger Reserve of Maharashtra identify as members of the Korku Scheduled Tribe and practice Hinduism mixed with ancestral worship. The Korku are an Adivasi ethnic group, speak Korku dialect, and are primarily an agriculturalist community of low socioeconomic status, high rates of illiteracy and malnutrition and possess poor access to medical and educational facilities. They live in small huts typically made of mud, grass and bamboo frames which lack an electricity or running water supply or proper sanitation systems and possess unique and distinct cultural knowledge, beliefs, and customs.

Metadata collection (Metagenome study)

Site-specific project coordinators were assigned to review health records form each participant. Basic demographic details including age, gender, geographic location, hospitalisation exposure, antibiotic usage during and before (within 3 months) of study recruitment, and *C. difficile* (GDH positive, toxin-positive) detection rates were recorded for urban and rural diarrhoeal and control participants.

In addition, BMI, immunosuppression status, and environmental details: type and location of home dwelling, number in family, drinking water supply, hygiene practices and number and type of domestic animals were also recorded for all participants. A description of the dietary information for the sampled cohorts is presented in the Supplementary methods.

Faecal Sample Collection and Storage

All specimens were anonymised and assigned a study code number linked to participant demographic details. Human faecal samples were collected from urban participants with and

23

540   without diarrhoea that were either in- or outpatients from the Central Indian Institute of Medical

541   Sciences (CIIMS), Nagpur or from other hospitals within a 20 km radius of CIIMS. Similarly,

542   faecal samples were also collected from participants with and without diarrhoea in Melghat

543   with the assistance of research fellows based at the Mahatma Gandhi Tribal Hospital, which

544   hosts a CIIMS satellite laboratory and other neighbouring hospitals within Melghat. Suitable

545   recruits were identified by the research fellows who interacted daily with village healthcare

546   workers to facilitate participant recruitment and sample collection. Up to two samples (3-5

547   grams each) were collected in UV sterilised dry plastic containers at the time of recruitment

548   from each participant and placed in a cool box. As per the standard operating procedures, all

549   stool specimens were stored at 4°C immediately after collection to avoid enzymatic degradation

550   prior to detection of toxigenic *C. difficile* and genomic DNA extraction which were performed

551   within 24 hours of sample collection.

552

553   *Detection of Clostridioides difficile GDH antigen and free toxin in diarrheal stool samples*

554   All diarrhoeal samples in the metagenome study (58/105) were tested for *Clostridioides*

555   *difficile* infection (detection of glutamate dehydrogenase antigen and toxins A/B) using the C.

556   DIFF QUIK CHEK COMPLETE-enzyme immunoassay (QCC; TechLab, Blacksburg, VA,

557   USA) in accordance with the manufacturers' instructions, including the use of appropriate

558   controls as specified in the package insert. Briefly, ~25 ml of stool sample was added to a tube

559   containing the diluent and conjugate and the mixture was transferred to the device sample well.

560   After incubation for 15 min at room temperature, the wash buffer followed by the substrate

561   were added to the reaction window. The results were read after 10 min. The GDH antigen

562   and/or toxins were reported as positive if a clear visible band was seen on the antigen and toxin

563   side of the device display window, respectively, confirming the presence of toxigenic *C.*

564   *difficile* as per manufacturer guidelines.

565

566

567   *Faecal DNA extraction*

568   DNA was extracted from 1 to 1.5g of feces and homogenised in lysis buffer (Tris HCl, EDTA,

569   NaCl and SDS). The content was centrifuged at 7,000 $x\,g$ for 10 min. The supernatant was then

570   transferred to a 1.5mL tube containing a mixture of Isopropanol and Sodium acetate (5M) and

571   incubated at -20°C for 30 min. Following removal of the supernatant the pellet was dried for

572   about an hour. The pellet was suspended in 1X Tris EDTA buffer (pH 8) and incubated at 65°C

573   for 15 min. An approximate equal volume (0.5- 0.7 ml) of Phenol: Chloroform- Isoamyl

574   alcohol (24:1) was added, mixed thoroughly and centrifuged for 10 min at 12,000 $x\,g$. The

575   aqueous viscous supernatant was carefully transferred to a new 1.5mL tube. An equal volume

576   of Chloroform-Isoamyl alcohol (1:1) was added, followed by centrifugation for 10 min at

577   12,000 $x\,g$. The supernatant was mixed with 0.6x volume of Isopropanol to aid precipitation.

578   The precipitated nucleic acids were washed with 75% ethanol, dried and re-suspended in 50μL

579   of TE buffer.

580

581   *Whole-Genome Shotgun (WGS) Sequencing*

582   Sequencing was carried out by Source Biosciences (Nottingham, U.K.). High quality genomic

583   DNA was quantified using Qubit Broad Range (Invitrogen, U.K.) and prepared for Illumina

584   paired end sequencing following the TruSeq DNA Nano manufacturers protocol (Rev D, June

585   2015) (Illumina Inc, San Diego, U.S.A.). The DNA was sequenced using a standard HiSeq

586   4000 150bp PE flowcell. Raw data has been submitted to the European Nucleotide Archive

587   under the accession number https://www.ncbi.nlm.nih.gov/bioproject/PRJNA564397

588   *Generation of taxonomic, resistome and functional profiles from metagenomic shotgun data*

589   Raw Fastq files (average 13,410,735 reads per sample) were assessed for quality using

590   skewer,[46] trimming adaptor reads and regions of quality below a phred of 30. The filtered reads

591   (average 10,635,653 reads per sample) were then assessed for taxonomic assignments using

592   Metaphlan2 [47] and for the presence of antimicrobial resistance genes using ARIBA[48] with the

593   MegaRes database.[49]

594   Functional analysis was performed using MOCAT2 (v2.1.3).[50] Briefly, trimmed and filtered

595   reads were assembled into contigs with SOAPaligner (v2.21). These contigs are initially

596   corrected for indels and chimeric reads using BWA (v0.7.5a-r16) and screened against the

597   human hg19 reference to filter out reads which originated from the host using USEARCH

598   (v5/v6). Genes were predicted using Prodigal (v2.60). Single copy marker genes are extracted

599   using fetchMG (v1.0) and clustered using CD-HIT (v4.6). The gene catalogues were annotated

600   using DIAMOND (v0.7.9.58) against multiple functional databases including eggNOG[51] and

601   KEGG.[52] The abundance of genes annotated to specific KEGG orthologs (KO) was determined

602   using the insert mm dist among unique norm setting in MOCAT2, normalising by read length

603   and sequencing depth and allowing for multiple mappers.

604

605   *Analysis of taxonomic contributions to functional shifts*

606   Functional shifts between groups and predicted taxonomic contributions were calculated using

607   the FishTaco package,[53] taking the species-level taxonomic table produced by Metaphlan2 and

608   the normalised KO abundance table from MOCAT2 as inputs. Only 49 taxa which exceeded a

609   minimum proportional abundance of greater than 0.1 in any single sample were included in the

610 final model. Enriched pathways were identified using the Wilcoxon rank sum test at FDR

611 corrected p<0.05. Taxonomic contributions were predicted by *de novo* inference in FishTaco,

612 inferring genomic content through a permutation-based approach and performing a total of 50

613 permutations per differentially abundant pathway.

614 For comparison of gene copy number for enriched metabolic pathways, KO gene copy numbers

615 for 8 gut-associated annotated reference genomes were obtained from the Integrated Microbial

616 Genomes and Microbiomes (IMG) database[54] as follows; *Prevotella stercorea* DSM 18206

617 (IMG: 2513237318), *Prevotella copri* CB7 DSM 18205 (IMG: 2562617166), *Eubacterium*

618 *rectale* DSM 17629 (IMG: 650377936), *Ruminococcus bromii* L2-63 (IMG: 650377966),

619 *Escherichia coli* UM147 (IMG: 2728369554), *Klebsiella pneumoniae* YH43 (IMG:

620 2687453226), *Bacteroides vulgatus* mpk (IMG: 2687453192), *Parabacteroides distasonis*

621 2b7A (IMG: 2660238380). KO gene copy numbers associated with each enriched metabolic

622 pathway were aggregated to yield overall pathway gene counts.

623

624 *Detecting viruses in whole community metagenomic shotgun data*

625 Sequencing reads were processed using Trimmomatic (version 0.36),[55] to remove Illumina

626 adaptors and prune sequences where the Phred score dropped below 30 across a 4bp sliding

627 window. All surviving reads less than 70bp were discarded. Fastq reads were assessed pre- and

628 post-processing using fastqc[56]. Both the paired and unpaired, forward and reverse reads, from

629 samples were assembled individually using metaSPAdes (version 3.11.1)[57]. Only contigs

630 greater than 1,000bp were examined further.

631 Two approaches were employed to find viruses within whole community metagenomic

632 assemblies. A standard reference-based similarity search was performed to detect sequence

633 relatedness to known viruses, while a reference-independent approach was undertaken by

634 searching for sequences which encode a high density of viral proteins. For the reference-based

635 search, nucleotide sequences were queried locally using BLAST (version 2.6.0+)[58] against the

636 viral RefSeq database (version 89; E-value 1E-10),[59] the complete Reference Viral Database

637 (C-RVDB version 14.0; E-value 1E-05),[60] and 249 crAss-like phages previously described as

638 the human gut's most abundant viruses (E-value 1E-05).[61]

639 For the reference-independent approach, proteins for all contigs were predicted using Prodigal

640 (version 2.6.3)[62] with the 'meta' option enabled for small contigs and Shine-Dalgarno training

641 bypassed. Proteins were subsequently queried against the prokaryote Viral Orthologous

642 Groups database (pVOGs)[63] using HMMER (version 3.1b2),[64] with a minimum score

643 requirement of 15. Putative reference-independent discovered viruses needed to fulfil three

644 basic requirements: (i) ≥1.5kb, (ii) encode 2 distinct proteins with similarity to 2 unique

645 pVOGs, and (iii) encode ≥2 pVOGs per 10kb-equivalent genome length. Additional stringent

646 dynamic filtering was applied to contigs based on their actual genome length. For contigs <5kb,

647 it was required that there were at least ≥5 distinct pVOG hits; contigs ≥5kb and <10kb, ≥6

648 pVOG hits; contigs ≥10kb and <20kb, ≥7 pVOG hits; contigs ≥20kb and <40kb, ≥8 pVOG

649 hits; contigs ≥40kb and <60kb, 9 pVOG hits; and contigs ≥60kb, 10 pVOG hits.

650 All putative viral contigs detected using the reference-dependent and -independent methods

651 were pooled and made non-redundant as follows: following a BLASTn all-v-all, the larger of

652 two contigs were retained when the blast identity and coverage between two sequences

653 exceeded 90%. Subsequently, any putative viruses encoding a ribosomal protein (BLASTp, E-

654 value 1E-10) was removed from further analysis. This was performed for stringency despite

655 recent research showing specific viruses can encode ribosomal proteins[65]. In addition, any

656 contig encoding a protein with similarity to all available Pfam sequences (version 32.0) of

657 plasmid replication proteins PF01051, PF01446, PF01719, PF04796, PF05732, and PF06970,

658 were removed (HMMER, score 15).

659 Viral contigs were grouped into Viral Clusters (VCs) using vContact2 (version 0.9.8)[27],

660 implemented through the CyVerse Discovery Environment. Protein clusters were identified

661 amongst VCs using default settings (Diamond, E-value 0.0001), and with the inclusion of

662 known viruses (Bacterial and Archaeal Viral RefSeq 85, with ICTV and NCBI taxonomy).

663 Following vContact2, only viral clusters that contain viral sequences from two or more of the

664 study's complete cohort (n=105) were analysed further. This was designed to remove singleton

665 and spurious viral sequences that may be transiently associated with diet, but are not abundant

666 or stable components of the faecal microbiome. The final Indian faecal virome was visualised

667 as a network through Cytoscape (version 3.7.1),[66] with viral sequences as nodes and shared

668 protein clusters as edges. The edge distance between connected viruses is calculated by

669 Cytoscape as their 'interaction'.

670

671 *Discerning differences in virome diversity and abundance*

672 Quality filtered reads, both paired and unpaired, were mapped onto the final Indian faecal

673 virome using bowtie2 in 'end-to-end' mode (version 2.3.4.1).[67] The read alignment outputs

674 were converted to sorted bam files through samtools (version 1.7).[68] The abundance and

675 breadth of coverage of reads mapping to each contig was determined using the bedtools

676 coverage function (version 2.26.0). [69] Subsequently, in order to determine if a viral sequence

677 was indeed present in a faecal virome, a breadth of coverage filtering was applied. This was

678 designed to remove viruses where potentially 100s of reads could map onto a single conserved

679 region. Therefore, for viral sequences ≤5kb, 75% of the genome needed to be covered by

680 aligned reads; sequences >5kb and ≤50kb, 50% of the genome needed to be covered; and

681 >50kb, 25% of the genome needed to be covered.

682   In addition to 105 faecal metagenomes, two negative control samples (water) were sequenced.

683   While these samples contributed no contigs to the final Indian faecal virome, the breadth of

684   coverage of sequencing reads from these samples was used to remove potential contaminant

685   sequences. Any viral sequence, from any sample, which 'passed' the breadth of coverage

686   filtering using reads derived from either water sample were removed from further analysis.

687   Any viral sequence from a faecal microbiome sample which failed the breadth of coverage

688   filtering was recorded as zero reads, while if the filtering step was passed, the observed number

689   of reads aligned were used to populate the read count matrix. Due to differences in sequencing

690   depth between samples, the read count matrix was normalised per sample using the DESeq2

691   ratio of means method.[70] The reads aligned to individual viral sequences were aggregated by

692   their vContact2 determined VCs. DESeq2 was subsequently used to calculate the VC changes

693   between cohorts. The normalised VC read count matrix was used to determine the diversity

694   and statistical differences observed between Indian faecal microbiome cohorts (see 'Statistical

695   Analyses' below).

696

697   *Determining phage-host pairs and viral encoded functions*

698   CRISPR spacers from bacterial contig assemblies were predicted using PILER-CR (version

699   1.06). [71] Putative CRISPR spacer predictions <20bp and >100bp were discarded. The CRISPR

700   spacers were queried locally using BLASTn against all individual viral sequences which

701   formed the Indian faecal virome VCs. Due to the use of short nucleotide sequences, only

702   CRISPR spacers with an E-value ≤0.001 and ≤1 mismatch were considered as significant. In

703   order to determine the taxonomy of the original assembled bacterial contigs, or the pre-

704   assembled contigs from the Pasolli *et al*. (2019) study,[72] contig kmer MinHash sketches were

705   queried against JGI taxonomy server using the BBMap sendsketch function (version 38.44).[73]

706 The bacterial enterotypes of Indian microbiomes were calculated using the Jensen-Shannon

707 divergence (JSD) to cluster the samples, followed by partitioning around medoids (PAM) to

708 cluster the abundance profiles. [28]

709 The functions associated with Indian faecal viruses were determined using eggNOG-mapper

710 v1 (online submission portal) using the eggNOG 4.5.1 database.[51] For each VC, the largest

711 viral sequence was chosen as a representative of that VC. In order to avoid the confounding

712 effect of viral abundance fluctuations within the faecal microbiome, the relative abundance of

713 VCs observed at the specific sampling time-point were not taken into consideration. Only the

714 overall presence-absence and abundance of viral-encoded functions were considered. The

715 similarity between virome-encoded functions, with respect to presence-absence, were assessed

716 through PCoA using the Jaccard index. The abundance of specific metabolic genes were

717 compared between cohorts, with statistical difference determined by the Mann-Whitney U test

718 with Bonferroni correction using the 'ggpubr' compare means function in R.

719

720 *Statistical Analyses and Graphic Generation*

721 All statistical analyses were conducted in R (64-bit, version 3.6.0; Foundation for Statistical

722 Computing, Vienna). The package 'vegan' was used for measures of taxonomic diversity

723 including alpha diversity (Inverse Simpson Index) and beta diversity (Principal Coordinates

724 Analysis with Bray Curtis Dissimilarity and Jaccard Similarity). Differences in alpha diversity

725 between study groups was assessed by ANOVA with Tukey's honest significance test. The

726 contribution of categorical variables to beta diversity was tested for using the Adonis function

727 (PERMANOVA) in vegan. Comparisons of proportional carriage of key taxa and resistance

728 genes between groups were assessed using the Chi-squared test. Generalised linear models

729 assuming a negative binomial distribution were used to identify differentially abundant taxa

730 between study groups as implemented in the R package 'mare'. Hierarchical clustering of

731 resistance gene abundances and heatmap generation was performed with the package

732 'heatmap3' using log-transformed Euclidean distance for distance matrix construction from

733 count data. For comparison of resistance gene and metabolic pathways counts between groups,

734 the Mann-Whitney U test was used. All p values obtained from testing with multiple

735 comparisons were corrected for false discovery rate (FDR, Benjamini-Hochberg). The fold

736 changes observed in the relative abundances of VCs across geographical and diarrhoeal status

737 cohorts were calculated using the 'gtools' package in R. Using the same package, the fold

738 changes were converted to log ratios (base 10). All graphical images were generated using

739 'ggplot2'.

740

741 **List of abbreviations**

742 CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

743 AMR: Antimicrobial Resistance

744 CDI: *Clostridioides difficile* infection

745 CDT: *C. difficile* toxin

746 CIIMS: Central India Institutes of Medical Sciences

747 BMI: Body Mass Index

748 PBP: Penicillin Binding Protein

749 ESBL: Extended Spectrum Beta-lactamases

750 VCs; Viral Clusters

751 ICTV: International Committee on Taxonomy of Viruses

752   MLS: Maximum Length Sequence

753   NDM-1: New Delhi Metallo-Beta-Lactamase 1 Enzyme

754   RNA: Ribonucleic Acid

755   DNA: Deoxyribonucleic Acid

756   rRNA: Ribosomal RNA

757   HCl: Hydrochloric Acid

758   NaCl: Sodium Chloride

759   EDTA: Ethylenediaminetetraacetic Acid

760   SDS: Sodium Dodecyl Sulphate Reagent

761   Tris: Tris[hydroxymethyl]aminomethane

762   WGS: Whole-Genome Sequencing

763   NCBI: National Center for Biotechnology Information

764

765

766 **Declarations**

767 **Ethics approval and consent to participate**

768 This study was approved by the Faculty of Medicine and Health Sciences Research Ethics

769 Committee at the University of Nottingham (REC No. 199-1901) and the Ethical Committee

770 of the Central India Institute of Medical Sciences, Nagpur.

771

772 **Availability of data and material**

773 Metagenomic sequencing datasets generated and analysed during the current study are

774 available in the European Nucleotide Archive under accession number:

775 [https://www.ncbi.nlm.nih.gov/bioproject/PRJNA564397]

776 All sequencing reads that map to the human reference genome have been removed from the

777 sequencing files.

778

779 **Funding details**

786

787

788   **Disclosure of interest**

796

797   **Author contributions**

798   T.M.M., T.J.S, S.S., and A.B. designed the study, analyzed the data and wrote the paper.

799   T.M.M., R.S.K., A.S., developed the clinical sample cohorts and R.B., R.N., S.M., J.G., and

800   P.J. managed sample and metadata collection, DNA extraction and quantification. A.B., T.J.S.,

801   S.S., analysed the WGS data. T.M.M., T.J.S., and S.S. performed the statistical analyses. S.A.,

802   R.D.E., M.W., L.A.D., and C.H., in addition to all other co-authors, reviewed the manuscript,

803   provided feedback, and approved the manuscript in its final form.

804

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813

814

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1055   **Figure legends**

1056   **Figure 1. Nagpur District.**

1057   Mapped locations of study participant home residences in Nagpur district**.**

1058

1059   **Figure 2. Variations in the gut microbiota by geographic location and diarrhoeal status.**

1060   **(A)** Principal coordinates analysis (PCoA) of microbiota profiles based on Bray-Curtis

1061   Dissimilarity of species-level taxonomic abundance. Subject profiles vary by both geographic

1062   location and diarrhoeal status. **(B)** Comparison of microbial diversity between diarrhoeal and

47

1063   non-diarrhoeal control subjects from both rural and urban geographic locations. * p.corr=0.05.

1064   **(C)** Summary of genus-level taxonomic profiles by subject. Subjects are grouped by

1065   geographic location and diarrhoeal status, with diarrhoeal subjects further subdivided into *C.*

1066   *difficile* toxin positive (CDT +ve) and negative (CDT –ve). Bacteroides dominant profiles are

1067   more frequent in urban subjects, while Prevotella dominant profiles are more frequent in rural

1068   subjects. **(D)** Differentially abundant taxa at species-level based on either geographic location

1069   (left, rural vs urban control subjects) or diarrhoeal status (right, non-diarrhoeal controls vs

1070   diarrhoeal). All taxa shown are significantly different between groups based on generalized

1071   linear models with FDR corrected $p<0.05$.

1072

1073   **Figure 3. Analysis of antimicrobial resistance gene carriage by gut microbiota. (A)**

1074   Heatmap of antimicrobial resistance (AMR) gene abundance aggregated by antibiotic class.

1075   Individual columns show subjects grouped by geography (rural – yellow vs. urban – blue),

1076   diarrhoeal status (non-diarrhoeal - green vs. diarrhoeal – red) and antibiotic exposure (brown).

1077   Row order represents hierarchical clustering of resistance gene count data using a Euclidean

1078   distance matrix. MLS = Macrolides, Lincosamides and Streptogramins. **(B)** Heatmap of

1079   antimicrobial resistance gene cluster abundance for Beta-lactam antibiotics. Columns represent

1080   individual subjects, grouped as above. Individual gene cluster codes are shown in rows

1081   corresponding to MegaRes database entries. Beta-lactam resistance mechanisms for each gene

1082   cluster are indicated to the left of the heatmap; Ambler class A to D, Porin mutant or PBP

1083   (Penicillin Binding Protein). **(C)** Comparison of the Beta-lactam resistance gene counts which

1084   differed significantly between rural and urban subjects. All statistical comparisons between

1085   urban and rural subjects were made with the Mann-Whitney U test with FDR correction and

1086   results indicated in each panel. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

1087

Figure 4. Taxonomic contributions to differentially enriched metabolic pathways. The top 10 pathways enriched in either urban or rural subjects are shown with the predicted contribution of individual taxa to the overall pathway variance (red diamonds). For each pathway, the top and bottom bars indicate urban and rural associated taxa respectively, displaying the predicted contribution of each taxon to enrichment in either group; urban (positive) or rural (negative). For example, enrichment of Lipoic acid metabolism in urban subjects is associated with the positive contribution (a) of *Klebsiella pneumoniae* (Kp), *Parabacteroides distasonis* (Pd) and *Bacteroides vulgatus* (Bv), with only minor negative contributions from multiple other species (b). Rural associated taxa contributing to enrichment in urban subjects (c), most likely because they encode the function sparsely, include *Prevotella copri* (Pc) and *Eubacterium rectale* (Er). *Prevotella stercorea* (Ps) is predicted to enrich this pathway in rural subjects (d), acting against the total observed shift.

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Figure 5. Contrasting faecal viromes by geographic location and diarrhoeal status. (A) Network visualisation of viral clustering.Viral clusters (VCs) containing previously characterised viral sequences (viral RefSeq 85) are coloured by International Committee on Taxonomy of Viruses (ICTV) family-level taxonomic assignments. While *Microviridae* VCs are connected to *Caudovirales* through shared protein clusters, these taxa are unrelated. **(B)** Inverse Simpson diversity comparisons of subjects by diarrhoeal status and geographic location. **(C)** Principal coordinate analysis of VC profiles based on Bray-Curtis Dissimilarity. **(D)** The fold change (log10) of the top 25 most abundant rural and urban VCs, with superimposition of the same VC's association with either health or diarrhoeal status. **(E)** The fold change (log10) of all VCs relative abundance that are targeted by CRISPR spacers from

identifiable bacterial genera. Each point represents a VC, with size representing the aggregate number of CRISPR spacers targeting individual viruses within a cluster.

Figure 6. Examination of the auxiliary metabolic potential of human faecal viruses. (A) Shared proteins encoded by Viral Clusters (VCs) shared amongst 10 or more individuals within this study. **(B)** The VC-encoded metabolic functions were determined per individual virome, with the similarities between subjects visualised by principal coordinate analysis using the Jaccard index. **(C)** Relative abundance comparisons of the protein categorical-function predictions of VCs by residence. **(D & E)** The observed frequency of amino acid transport and metabolism functions, and carbohydrate transport and metabolism functional predictions encoded by individual virome VCs. Only statistically significant EggNOG functional predictions are displayed (Mann-Whitney U test with Bonferroni correction, p adj = 0.05).