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2 **Characterization of the gut DNA and RNA viromes in a cohort of Chinese residents and**
3 **visiting Pakistanis**

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5 Short title: Differences of gut virome between Chinese and visiting Pakistanis

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24

25 **Abstract**

26 **Background:** Trillions of viruses inhabit the gastrointestinal tract. Some of them have been
 27 well-studied on their roles in infection and human health, but the majority remain unsurveyed. It
 28 has been established that the composition of the gut virome is highly variable based on the
 29 changes of diet, physical state, and environmental factors. However, the effect of host genetic
 30 factors, e.g. ethnic origin, on the gut virome is rarely investigated.

31 **Methods and Results:** Here, we characterized and compared the gut virome in a cohort of local
 32 Chinese residents and visiting Pakistani individuals, each group containing 24 healthy adults and 6
 33 children. Using metagenomic shotgun sequencing and assembly of fecal samples, a huge number
 34 of viral operational taxonomic units (vOTUs) were identified for profiling the DNA and RNA
 35 viromes. National background contributed a primary variation to individuals' gut virome.
 36 Compared with the Chinese adults, the Pakistan adults showed higher macrodiversity and different
 37 compositional and functional structures in their DNA virome and lower diversity and altered
 38 composition in their RNA virome. The virome variations of Pakistan children were inherited from
 39 the that of the adults but also tended to share similar characteristics with the Chinese cohort. We
 40 also analyzed and compared the bacterial microbiome between two cohorts and further revealed
 41 numerous connections between virus and bacterial host. Statistically, the gut DNA and RNA
 42 viromes were covariant to some extent ($p < 0.001$), and they both influenced the holistic bacterial
 43 composition and vice versa.

44 **Conclusions:** This study provides an overview of gut viral community in Chinese and visiting
 45 Pakistanis and proposes a considerable role of ethnic origin in shaping the virome.

46 **Keywords:** virus-like particle, gut virome, viral community, RNA virus, metagenomic sequencing,
47 bacterial microbiome, nationality

48

49

50 **Background**

51 The human gut is a large reservoir of microorganisms, containing 10^{11} - 10^{12} bacterial cells [1, 2],
52 10^9 - 10^{12} viral particles [3, 4], and small quantities of archaea and eukaryotes in per gram of feces
53 [5]. Benefiting from the development of high throughput sequencing techniques (e.g. amplicon or
54 whole-metagenomic sequencing), the gut bacterial community have been well studied over the
55 past years [6-8]. Gut bacteria was shown to exert profound effects on regulating host metabolism
56 [9, 10], and thereby had been linked to host health and diseases [11, 12]. However, as another part
57 of the gut microbial ecosystem, the holistic viral community of enteric microbiome (or “gut
58 virome”) was less well characterized [13]. Virus has a very flexible small genome ranging from a
59 few to several hundred kilobases [14], which corresponds to approximately 1% of the bacterial
60 genome (in average, 2-4 Mbp) [15, 16]. The gut virome was predominantly composed of two taxa
61 of bacteriophages, double-stranded DNA *Caudovirales* and single-stranded DNA *Microviridae*,
62 which constituted over 80% relative abundance of viral populations in human intestine [17]. The
63 *crAssphage* and *crAss-like* phages, a type of *Caudovirales* members that characteristically infect
64 *Bacteroides* spp., represented the highest abundance in healthy human gut [18, 19]. In addition to
65 bacteriophages, eukaryotic viruses, archaeal viruses, and RNA viruses were also important
66 components of gut virome [20, 21].

67

68 Due to the limitation of viral abundance in human gut, routine whole-metagenomic sequencing of
69 fecal microbiome can produce only a small proportion of viral sequences for further analysis.
70 Recently, virus-like particle (VLP) enrichment and subsequently metagenomic sequencing
71 provided a prospective application for fully delineating the gut virome [22, 23]. Based on the VLP
72 technique, studies had showed that the normal gut virome was partly inherited from mother [24,
73 25], potentially transferred between twins [4], and continuously expanded during the first years of
74 life [21]. In addition, longitudinal analysis revealed that the gut virome of healthy adults was
75 highly diverse, temporally stable, and individually specific [14]. Disease-induced alterations of the
76 gut virome had also be reported in multiple gastrointestinal and systemic disorders, including
77 colorectal cancer [26, 27], inflammatory bowel disease [17, 28], type I diabetes [29], and coronary
78 heart disease [30]. These studies suggest a significant role of gut virome in human health, however,
79 some essential issues of human gut virome, such as population heterogeneity and impacts of
80 geography, lifestyle or environment, is still in shortage.
81
82 By studying the gut microbiome of migrated or short-term visiting peoples, previous studies had
83 shown that their microbiota was markedly remodeled upon environmental change, but yet
84 accompanied with maintenance of numerous individual or ethnic microbial characteristics [31-34].
85 Herein, we depicted the compositional differences of gut virome between Chinese residents (n =
86 24) and visiting Pakistani (n = 24) individuals living in the same city and also examined the
87 repeatability of these differences in their child offsprings (respective n = 6). We quantified the
88 DNA and RNA viromes from fecal VLPs, and parallelly measured the bacterial microbiome for
89 virus-bacteria association analysis. This pilot study provided evidences for the effect of ethnic
90 backgrounds on human gut virome.

91

92 **Results**

93 **Population characteristics and study design**

94 This study included 30 Chinese residents and 30 visiting Pakistani individuals who were recruited
 95 at Dalian Medical University in March 2019. Both cohorts consisted of 24 healthy adults and 6 of
 96 their child offsprings (**Table 1**). All adults were students or young teachers of the Dalian Medical
 97 University, and the Pakistani adults and children had arrived in China for 0-18 months (average of
 98 11 months) and 0-15 months (average of 9 months), respectively. Notably, the Chinese and
 99 Pakistani adults showed significant differences on their body mass index (BMI), dietary habit, and
 100 drinking and smoking rates (**Table 1; Table S1**), which seemed to be due to ethnic and lifestyle
 101 differences.

102

103 Fecal samples of all participants were collected and treated using a unified approach (see
 104 Methods). To depict the gut viral characteristics of healthy individuals, we extracted DNA and
 105 RNA from fecal VLP fractions and performed high throughput shotgun sequencing using the
 106 Illumina platform. To extend the content of total microbial community, the bacterial microbiome
 107 of feces was also profiled using whole-metagenomic sequencing. The analytical workflow of the
 108 DNA virome, RNA virome, and bacterial microbiome was shown in **Figure 1**. Focusing on the
 109 comparison of gut viromes between Chinese and Pakistani individuals, overall, this study included
 110 six sections to elaborate the results:

111 1-2. DNA virome and its functional characteristics.

112 3-4. RNA virome and the concordance between DNA and RNA viromes.

113 5-6. Bacterial microbiome and the virus-bacteria associations.

114

115 **Comparison of DNA viral community**

116 We obtained 782 million high-quality non-human reads (12.1 ± 0.5 million per sample) through
 117 shotgun sequencing of the DNA viral community of 60 fecal samples. The reads were *de novo*
 118 assembled into 182,471 contigs with the minimum length threshold of 1kbp, of which 45.0%
 119 (82,119) were recognized as highly credible viral fragments based on their sequence features and
 120 homology to known viral genomes (**Figure 1**). The remaining contigs were from bacterial or
 121 eukaryotic contaminations (26.8%) and dependency-associated sequences (6.0%), and 22.2%
 122 contigs were still unclassifiable. Despite that, average 82.3% of sequencing reads in all samples
 123 were captured by the viral contigs, revealing well representativeness of the high-abundance viral
 124 contents in human gut DNA virome. The viral contigs were further clustered into 54,947 “viral
 125 operational taxonomic units (vOTUs)” (a phylogenetic definition of discrete viral lineage that
 126 corresponds to “species” in prokaryotes, also named “viral population” [35]) by removing the
 127 redundant contigs of 95% nucleotide similarity. These vOTUs represented an average size of
 128 $3,054 \pm 2,868$ bp (**Figure S1**), which was comparable with similar studies [14] but remarkable
 129 lower than that of the available viral genomes (average 38.5 kbp for ~6,500 complete virus
 130 isolates from the RefSeq database), suggesting that the vOTUs were mostly fragmented genomes.
 131 Only 33.6% of vOTUs could be annotated into specific family, highlighting a considerable novelty
 132 of gut virome.

133

134 Rarefaction analysis showed that, despite the rarefaction curve was unsaturated under current
 135 number of samples in each group, the vOTU richness was significantly higher in Pakistani adults
 136 than in Chinese adults ($p=0.008$, **Figure 2a**). The within-sample diversity pattern of gut DNA

137 viromes was assessed by macrodiversity (Shannon index) and microdiversity (nucleotide diversity
138 or π [35]) at the vOTU level. The Chinese adults showed a lower Shannon index than the Pakistani
139 adults, similarly for the children (**Figure 2b**), but no significant difference in microdiversity was
140 detected between Chinese and Pakistanis (**Figure 2c**).

141
142 Next, we undertook a non-metric multidimensional scaling (NMDS) analysis to further understand
143 the differences in fecal DNA viral communities between Chinese and Pakistanis. Clear separations
144 were revealed in the viromes of both adults and children between Chinese and Pakistanis (*adonis*
145 $p < 0.001$ for both adults and children; **Figure 2d**). Notably, we also found that 1) the viral
146 communities of Chinese adults and children were similar, but those of Pakistani adults and
147 children were differed, and 2) the viral communities of Pakistani children were closer to Chinese
148 subjects when compared with those of Pakistani adults. These findings were validated by the
149 permutational multivariate analysis of variance (PERMANOVA) (**Figure 2e**).

150
151 We finally compared the DNA virome composition of Chinese and Pakistani at the family level,
152 ignoring the family-level unclassified vOTUs (which represented only 33.1% of total sequences).
153 The most dominant viral families in all samples were *Podoviridae*-crAssphage (average relative
154 abundance, $27.0 \pm 30.7\%$), *Siphoviridae* ($24.8 \pm 25.5\%$) and *Adenoviridae* ($23.7 \pm 28.1\%$) (**Figure**
155 **2f**). Compared with the Chinese adults, the viral communities of the Pakistani adults showed a
156 significant increase of *Adenoviridae*, *Anelloviridae*, *Marseilleviridae*, and *Lavidaviridae*, and a
157 remarkable depletion of *Circoviridae* and *Rudiviridae* (Mann-Whitney U test, $q < 0.05$; **Figure 2g**).
158 *Adenoviridae*, *Myoviridae*, *Phycodnaviridae*, *Mimiviridae*, *Herelleviridae*, and *Inoviridae* were

159 significant higher in viral communities of Pakistani children (**Figure 2h**), as compared with the
160 Chinese children, while no viral family was lower.

161

162 **Functional analysis of DNA virome**

163 To better elucidate the functional capacity of the DNA viromes, we predicted a total of 221,418
164 protein-coding genes from the vOTUs (average of 4 genes per vOTU) and annotated functions of
165 24.2% of these genes based on the KEGG (Kyoto Encyclopedia of Genes and Genomes) [36]
166 database. Analysis on KEGG pathway level B showed that functions involved in genetic
167 information procession and signal and cellular processes are dominant in all samples (**Figure 3a**),
168 suggesting that these are core functions of the gut DNA virome. Compared with the Chinese adults,
169 viral functions in the Pakistani adults were significantly decreased involving “protein families:
170 metabolism”, amino acid metabolism, antimicrobial drug resistance, cell motility, and substance
171 dependence, and increased in immune disease (Mann-Whitney U test, $q < 0.05$; **Figure 3b**). For
172 example, a putative hemolysin enzyme (K03699) that encoded by several *Myoviridae* and
173 *Siphoviridae* viruses showed over 10-fold enrichment in the virome of Chinese adults compared to
174 that of Pakistani adults. When compared with the Chinese children, a number of important
175 functions, including carbohydrate metabolism, signal transduction, and cell growth and death,
176 were significantly higher in the viral communities of Pakistani children, while the “protein
177 families: genetic information processing” were lower (**Figure 3c**).

178

179 We identified a total of 11,242 CAZymes (Carbohydrate-active enzymes [37]) from the viral genes,
180 including 5,437 glycoside hydrolases, 3,270 glycosyl transferases, 1,993 carbohydrate binding,
181 396 carbohydrate esterases, 120 polysaccharide lyases, and 26 auxiliary activities (**Figure 3d**).

182 The majority (65.9%) of CAZymes were encoded by unclassified vOTUs, followed by
183 *Siphoviridae* (12.1%) and *Myoviridae* (8.2%), suggesting their important roles in carbohydrate
184 metabolism in gut viral ecosystem. Moreover, we also identified 37 acquired antibiotic resistance
185 genes (ARGs) from the DNA vOTUs (**Table S2**). Most of these ARGs were related to tetracycline
186 resistance (n = 12), macrolide resistance (n = 7), beta-lactamase (n = 7), and aminoglycoside
187 resistance (n = 6). Taken together, these findings revealed that the DNA virus can widely express
188 the carbohydrate metabolism-associated genes and are potentially involved into carrying and
189 transmission of antibiotic resistance genes.

190

191 **Comparison of RNA viral community**

192 For RNA virome, we performed shotgun metatranscriptomic sequencing of 60 fecal samples
193 described above and obtained 671 million reads (11 ± 3.4 million per sample) after removing the
194 low-quality reads and bacterial ribosomal RNA contamination. A total of 99,454 contigs with
195 minimum length threshold of 500 bp were assembled, 3,442 (3.5%) of which were identified as
196 highly credible RNA viral fragments via blasting against the available RNA viral genomes and
197 searching of the RNA-dependent RNA polymerase (RdRp) sequences (**Figure 1**). 25.4% of these
198 RNA viruses contained at least one RdRp gene, while 28 viral RdRp genes had no homology with
199 any known virus in NCBI database. We obtained 569 RNA vOTUs based on clustering at 95%
200 nucleic acid level similarity. The average size of these vOTUs was $1,162 \pm 916$ bp, which was
201 fragmented compared with the available RNA viral genomes (average 7.4 kbp from ~4,000
202 isolates). Furthermore, considering that only average 24.8% reads of all samples were covered
203 from the RNA vOTUs, we also used the available RNA viral genomes from the RefSeq database
204 as a reference for analyzing of the gut RNA virome. 118 available RNA viruses were observed in

our samples, which covered additional 1.3% reads (in average) for further analysis.

Rarefaction analysis showed that the detection of RNA virus was increased with the number of samples, and the accumulative curve was nearly saturated at nearly 10 samples (**Figure 4a**). This is due to our RNA virus pipeline mainly focused on the known species and the sequence containing a RdRp gene, but high proportions of virus remain untagged and many of them are independent on RdRp gene [38]. Compared with Pakistanis, the macrodiversity (Shannon index) was significantly higher in Chinese adults, but there was no statistical difference in that of children (**Figure 4b**).

NMDS analysis on the overall RNA vOTUs composition captured significant separation of adults between Chinese and Pakistanis (*adonis* $p < 0.001$; **Figure 4c**), but of children the separation was visible but not significant (*adonis* $p = 0.2$). Likewise, the viral communities of Chinese adults and children were closer, yet of Pakistani adults and children.

Finally, to investigate the gut RNA viral signatures between Chinese and Pakistanis, we compared two cohorts on viral composition. At the family level, the dominant family *Virgaviridae* consisted of average 83.7% relative abundance in all samples (**Figure 4d**), which was slightly but significantly enriched in Chinese adults compared with that in Pakistani adults (**Figure 4e**). Three other families, *Betaflexiviridae*, *Picornaviridae*, and *Astroviridae*, was reduced in Chinese adults than in Pakistani adults (Mann-Whitney U test, $q < 0.05$ for all), while *Picornaviridae* was also reduced in Chinese children than in Pakistani children. At the species level, the plant-associated virus, including *Pepper mild mottle virus* (average relative abundance, $37.5 \pm 23.1\%$), *Tomato*

227 *mosaic virus* ($27.1 \pm 27.4\%$), and *Tobacco mild green mosaic virus* ($14.1 \pm 12.4\%$), composed of
 228 the dominant species in all samples (**Figure 4f**). Compared with the Chinese adults, the viral
 229 communities of the Pakistani adults showed a significant increase of *Shallot latent virus*,
 230 *Picornavirales Tottori-HG2*, *Aichivirus A*, and *Astrovirus VA3*, and a remarkable depletion of
 231 *Paprika mild mottle virus*, *Peach virus T*, *Enterovirus C*, and *Cosavirus A* (**Figure 4g**). When
 232 compared with the Chinese children, 9 species were significantly higher in viral communities of
 233 Pakistani children (**Figure 4h**), with no species that was lower.

234

235 **Concordance between DNA and RNA viromes**

236 Having characterized the differences of DNA and RNA viromes between local Chinese residents
 237 and visiting Pakistanis, we wanted to examine the existence of concordance between DNA and
 238 RNA viromes. Although the DNA and RNA viromes were irrelevant in Shannon diversity index
 239 (Pearson $r=0.04$, $p=0.7$; **Figure 5a**), the overall compositions of two types of viral community
 240 were strongly correlated (Procrustes correlation $M^2=0.37$, $p<0.001$; **Figure 5b**). And this
 241 correlation was reproducible across nationality and age. Moreover, we identified 24 co-abundance
 242 correlations between 6 DNA and 9 RNA viral families (Spearman correlation test $q<0.05$; **Figure**
 243 **5c**), including some positive correlations between *Adenoviridae* and several RNA viruses and a
 244 negative correlation between *Herpesviridae* and *Tombusviridae*. The significance of these
 245 relationships required further studies.

246

247 **Comparison of bacterial microbiome**

248 For bacterial microbiome, we obtained a total of 1,236 million reads (20.6 ± 7.7 million per
 249 sample) from the samples and quantified the relative abundances of a total of 833 taxa, including

12 phyla, 22 classified, 41 orders, 81 families, 179 genera, and 498 species, using MetaPhlAn2

[39]. Comparison on Shannon index showed that the bacterial microbiome of Chinese adults

exhibited a significantly higher diversity than that of the Pakistanis (**Figure 6a**), similarly but not

significantly trend was observed in that of children. NMDS analysis on the overall bacterial

composition also revealed significant separation between Chinese and Pakistan adults (*adonis*

$p < 0.001$; **Figure 6b**), as well as between Chinese and Pakistan children (*adonis* $p < 0.001$).

Consistent with the observations in DNA and RNA viromes, the bacterial microbiome of Pakistan

children was also close to that of Chinese subjects in tendency.

Taxonomically, the bacterial microbiome of Chinese adults showed significant enrichment of

Lachnospiraceae, *Ruminococcaceae*, *Eubacteriaceae*, *Enterobacteriaceae*, *Tannerellaceae*,

Rikenellaceae, *Acidaminococcaceae*, *Clostridiaceae*, and *Sutterellaceae* and depletion of

Prevotellaceae, *Bifidobacteriaceae*, *Coriobacteriaceae*, *Lactobacillaceae*, *Oscillospiraceae*,

Selenomonadaceae, and *Atopobiaceae*, compared with that of Pakistani adults (linear discriminant

analysis [LDA] score > 3 ; **Figure 6c**). Similarly, *Clostridiaceae*, *Eubacteriaceae*, and

Ruminococcaceae were enriched in Chinese children compared to Pakistani children, and

Coriobacteriaceae was depleted. At the species level, the Chinese adults exhibited 28 enriched

bacterial species and 19 decreased species when compared with the Pakistani adults, while the

Chinese children showed 11 enriched species and 12 decreased species compared with the

Pakistani children (**Table S3**). The exhibition of enormous differential taxa led to a dramatic

distinction of enterotype constitution between Chinese and Pakistanis. The Chinese subjects was

characterized by a high proportion of *Bacteroides*/*Firmicutes*-type (75% and 100% in adults and

children, respectively), whereas almost of all Pakistani subjects were *Prevotella*-type (100% in

adults and 66.7% in children) (**Figure 6d**).

Virus-bacteria associations

To study the virus-bacteria correlation, first, we predicted the bacterial hosts of virus by searching the potential viral CRISPR spacers from bacterial metagenomic assemblies (see Methods). This approach allowed host assignments for 3,948 DNA and 4 RNA vOTUs, representing 7.2% and 0.7% of all DNA and RNA viruses, respectively. We revealed a large connection network of family-level known virus ($n = 392$) and its bacterial host (**Figure 7a**), facilitated by frequent acquisition of phage/prophage in bacterial genomes and spread of phages across bacterial hosts. Members of *Faecalibacterium*, *Prevotella*, *Ruminococcus*, *Bifidobacterium*, *Dialister*, and *Streptococcus* were the most common host for human gut virome. Meanwhile, the *crAss-like* phages had infected the highest number of bacteria.

Then, we performed the PERMANOVA-based effect size analysis between gut virome and microbiome. DNA vOTUs ($q < 0.10$), including members of *Siphoviridae*, *Phycodnaviridae*, and *Podoviridae*-*crAss*phage, and 25 RNA vOTUs ($q < 0.10$) showed significant affection on the bacterial microbiome communities (**Figure 7b-c**). More importantly, combination of these DNA and RNA vOTUs explained 20.2% and 18.2% of the microbiome variance, respectively (**Figure 7d**), suggesting that the effect size of the gut virome on bacterial microbiome is considerable. Similar effect sizes were found in subjects from two nations. Parallely, 117 bacterial species were identified that significantly impact the holistic composition of DNA and RNA viromes, accounting for 13.2% virome variance (**Figure 7d**). These species included *Bifidobacterium angulatum*, *Streptococcus salivarius*, *Bacteroides coprophilus*, and *Prevotella copri* (**Figure 7e**).

295

296 **Discussion**

297 Both ethnic origin and residential environment have negligible effects on individual's gut
 298 microbiome [32, 40-42]. To extend this finding on gut virome, our study focused on the viral
 299 community of a cohort of Chinese and visiting Pakistanis. Despite sharing the residential
 300 environment, the viral diversity and composition of Chinese and Pakistanis were dramatically
 301 differed, suggesting that the ethnicity-specific characteristics of virome enable to maintain over an
 302 extended period (average 11 and 9 months for Pakistani adults and children, respectively). This
 303 result was in accordance with an earlier study showing that the individual characteristics of gut
 304 virome can be relatively stable for at least one year [14].

305

306 Using *de novo* assembly and discovery approaches, we identified a huge number of viruses from
 307 the subjects' fecal samples, including approximately 55,000 non-redundant complete and partial
 308 DNA viral genomes and 569 non-redundant RNA viruses, particularly the number of DNA vOTUs
 309 increased over 8-fold compared with the isolated viral sequences in RefSeq database. The majority
 310 of viruses were unclassified even at the family level, in agreement with previous observations of
 311 extensive novelty of viral world in multiple environments as well as in human gut [43-45].

312

313 The DNA viral macrodiversity of Chinese adults was lower than that of Pakistani adults, whereas
 314 an opposite phenomenon was observed in the diversity of bacterial community. This result was in
 315 conflict with the observation in US adults which exhibited strong correlation between gut virome
 316 and microbiome diversities [4]. As most of the DNA viruses were bacteriophages (in this study,

the bacterial hosts of at least 7.2% DNA viruses were verified) [4], the degree to which bacterial microbiome drives the virome diversity is considerable. The explanation for high DNA viral diversity in Pakistani adults was unknown, but reason for the enrichment of some eukaryotic viruses in their gut was speculated (see the following discussion). In contrast to DNA virome, the RNA viral diversity was higher in Chinese adults than in Pakistani adults. This observation could be due to the difference of dietary habits between two groups, as in fact the gut RNA viruses were generally plant-associated viruses in our cohort.

Significant compositional differences were observed in DNA and RNA viromes, so was bacterial microbiome between Chinese residents and visiting Pakistanis. In DNA virome, the Pakistani adults showed remarkable enrichment of two eukaryotic viruses, *Adenoviridae* and *Anelloviridae*. Members of *Adenoviridae* were the most prevalent human-associated viruses that can cause respiratory infection, gastroenteritis, and multi-organ diseases [46-48]; while some members of *Anelloviridae* were also associated with human viral infections [49]. *Adenoviridae* was also highly abundant in the gut of Pakistani children but was rare in that of Chinese children, suggesting potential transmission of such viruses from Pakistani parents to their offsprings. In RNA virome, some members of the plant-associated virus *Virgaviridae* were enriched in Pakistanis but some others were reduced. This finding was thought to be connected to the difference of dietary habits between two cohorts. For example, the abundance of *Shallot latent virus* was higher in Pakistani adults than in Chinese adults, as the shallot (e.g. onion, leek) is commonly used in halal foods in the school canteen but rarely appeared in Chinese foods (based on the authors' experience). In addition, some members of the Pakistani adult-enriched *Picornaviridae*, including *Picornavirales* *Tottori-HG2*, *Enterovirus C*, and *Cosavirus A*, and *Astroviridae* were well-known human

340 enteroviruses that can cause diarrhea and enteric infections [50-52]. In bacterial microbiome, the
 341 enterotype distribution of Chinese and Pakistanis was deviated, characterized by a high proportion
 342 of *Bacteroides/Firmicutes*-type (associated with diets enriched animal carbohydrates [53, 54]) and
 343 low proportion of *Prevotella*-type (associated with plant fiber-enriched diets [55]) in Chinese
 344 subjects. Combination of these findings suggested that the dietary habits may be a key driver for
 345 shaping the gut RNA virome and bacterial microbiome. Of course, more proof-of-principle studies
 346 are needed in future.

347
 348 One striking observation was that the DNA virome of Pakistan children is closer to that of Chinese
 349 subjects, when compared with the degree of deviation between Chinese and Pakistan adults. This
 350 phenomenon was also observed in RNA virome and bacterial microbiomes in tendency. These
 351 findings suggested that the virome and microbiome of children was more changeable than that of
 352 adults, despite the fact that the Pakistan adult participants seemed to live a bit longer in China. In
 353 accordance with the previous studies, the infant or child gut microbiome was less stable under the
 354 changes of environmental, dietary pattern, and antibiotic usage [56-58]. In addition, dynamic
 355 development of the infant gut virome towards a more stable adult-like gut virome was also
 356 confirmed by recent studies [21, 59, 60].

357
 358 We characterized the functional capacity of gut virome by identifying over 53,000 KEGG
 359 annotated protein-coding genes, of which the core functions seemed consistent with previous
 360 findings in the gut phage catalog [61]. Different from the observation in DNA viral composition,
 361 the Chinese adults revealed a more diver functional profile than that of the Pakistani adults, as
 362 revealed by more metabolism-associated genes in Chinese adults. In addition to general functions,

we also identified over 11,000 CAZymes and 37 antibiotic resistance genes from all DNA viruses.

To the best of our knowledge, the appearance of extensive CAZymes in gut virome was first found in this study. Potential viral contributions to complex carbon degradation were validated in ocean and soil ecosystems [62, 63]. Thus, our findings further highlight the importance of viral carbohydrate metabolism capacity in human gut. Moreover, the virus-encoded ARGs was also directly relevant to human health, consistent with previous studies [64].

Not only bacteriophages but also free-living viruses in human gut can influence bacterial microbiome structure and therefore indirectly affect health status [65, 66]. We confirmed remarkable connections between viruses and bacterial hosts in our study cohort, including the previous-known parasitic relations (e.g. *crAss-like* phages and Bacteroidetes members [18, 67]) and many novel connections. Noticeably, the Pakistani-dominated genus *Prevotella* connected the largest number of viruses and was responsible for a large part of variance in the virome composition, in agreement with the previous studies showing that the high relative level of *Prevotella* lead to a higher prevalence of temperate bacteriophages and increased virome macrodiversity [14]. One the other hand, we also statistically revealed that the gut virome was also an important determinator of the bacterial microbiome.

As all participants shared the residential environment, we were only able to study the effect of nationality on their gut virome. Through collecting samples from the visiting Pakistani before they arrived China or from other local Pakistani residents, future research is believed to confirm the effect of environment on gut virome. Other limitations in this study included 1) the relatively

385 small sample size, 2) the lack of longitudinal sampling for the individuals, and 3) the inadequacy
386 of viral reference database. These limitations did not affect the robustness of results in the current
387 cohort, but follow-up studies in wider populations will still complement some deficiencies of the
388 current study and provide more new findings.

389 **Summary**

390 In conclusion, we systematically described the baseline gut virome in a well-characterized cohort
391 of Chinese and visiting Pakistanis and demonstrated that the national background contributed a
392 primary variation to gut virome. The mechanisms underlying the difference between two cohorts
393 remain unclear, but the ethnic factor must be proposed and considered in designing future studies
394 of the virome.

396 **Methods**

397 **Subject and sample collection**

398 This study received approval from the ethics committee of Dalian Medical University, and written
399 informed consent was obtained from each participant. The methods were carried out in accordance
400 with the approved guidelines. Thirty healthy Pakistani from Dalian Medical University and thirty
401 BMI-, dietary habit-, alcohol intake- and frequency of smoking-matched Chinese healthy controls
402 were recruited for this study. Each cohort was consisted of 24 healthy adults and 6 of their healthy
403 child offsprings. Fresh fecal samples were collected from each subject and were immediately
404 stored at a -80°C freezer.

406 **Experimental procedures for DNA and RNA viromes**

407 *Virus-like particles enrichment.* The procedure of VLPs enrichment was performed on ice. Add
 408 0.1g fecal sample into 1 ml HBSS buffer (137 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl₂, 0.3 mM
 409 Na₂HPO₄·2H₂O, 0.5 mM MgCl₂·7H₂O, 0.4 mM KH₂PO₄, 0.6 mM MgSO₄·7H₂O, 4.2 mM
 410 NaHCO₃, 5.6 mM D-glucose), centrifuge at 10000 g twice to obtain supernatant. After filtering to
 411 sterilize, the sterilized filtrate was mixed with the same volume of HBSS buffer and centrifugated
 412 at 750,000 g for an hour, the supernatant was stored at -80°C. The pellet was collected for DNA
 413 extraction.

414
 415 *Viral DNA and RNA extraction.* The DNA and RNA of virus were extracted by using TIANamp
 416 Virus DNA / RNA Kit (TIANGEN) according to the manufacturer's protocols. Prepare the
 417 mixture contained extracted viral DNA, 1µl 20 mM random primers D2-8N (5'-
 418 AAGCTAAGACGGCGGTTCGGNNNNNNNN-3'), 1 µl 10xRT mix, 1 µl 10 mM dNTP and 11.5
 419 µl DEPC H₂O. To synthesize the first strand of viral DNA, desaturated mixture at 95 °C for 5 min,
 420 add Klenow fragment solution (0.15 µl 10x Klenow Buffer, 0.5 µl Klenow fragment, 0.85 µl
 421 DEPC H₂O) at 37 °C. The procedure should be performed twice to obtain two-strand viral DNA.
 422 The extracted RNA was reverse transcribed by using Vazyme HiScript II 1st Strand cDNA
 423 Synthesis Kit (+gDNA wiper) with the same random amplification primer. The two-strand of
 424 cDNA could be synthesized by the same approach.

425
 426 *cDNA preparation.* Add the mixture contained rSAP and exonuclease-1 into viral two-strand DNA
 427 and cDNA at 37 °C, respectively, to remove the remained dNTP and primer D2-8N. After 1 hour,
 428 add 10 µl 5X Q5 Reaction Buffer, 3 µl 50 mM MgCl₂, 1.5 µl 10 mM dNTP, 3 µl 20 mM primer

429 D2 (5'-AAGCTAAGACGGCGGTTCGG-3'), 1.25 µl Q5 High-Fidelity DNA Polymerase and
 430 23.25 µl DEPC H₂O to amplify the viral DNA and cDNA by polymerase chain reaction (PCR).
 431 DNA and cDNA were stored at -20°C freezer. The DNA and RNA concentration and purity
 432 were quantified with NanoDrop2000. DNA and cDNA quality were examined with a 1% agarose
 433 gel electrophoresis system.
 434
 435 *Shotgun sequencing of viromes.* All the DNA and cDNA viral samples were subjected to shotgun
 436 metagenomic sequencing by using the Illumina HiSeq 3000 platform. Libraries were prepared
 437 with a fragment length of approximately 350 bp. Paired-end reads were generated using 150 bp in
 438 the forward and reverse directions.

439

440 **Bioinformatic analysis of DNA and RNA viromes**

441 *DNA virome assembly, identification, clustering and taxonomy.* The quality control of DNA
 442 virome sequences was performed using fastp [68], and the human reads were removed based on
 443 Bowtie2 [69] alignment. Each sample was individually assembled using metaSPAdes [70].
 444 Proteins of the contigs were predicted using Prodigal [71]. After that, the assembled contigs
 445 (>1,000 bp) were identified as viruses when it satisfied one of the following criteria: 1) at least 3
 446 proteins of a contig (or at least 50% proteins if the contig had less than 6 proteins) were assigned
 447 into the viral protein database integrating from NCBI reference viral genomes and the virus
 448 orthologous groups database (<http://vogdb.org>), with a maximum pairwise alignment e-value
 449 1e-10 based on DIAMOND [72]; 2) score >0.7 and p-value <0.05 in the VirFinder [73], a k-mer
 450 based tool for identifying viral sequences from assembled metagenomic data; 3) at least 2 proteins

451 were uncharacterized from the integrated databases of KEGG [36], NCBI-nr, and UniProt [74].

452 Viral contigs were pairwise blasted and the highly consistent viruses with 95% nucleotide identity

453 and 80% coverage of the sequence were further clustered into vOTUs using inhouse scripts. The

454 longest viral contig was defined as representative sequence for each vOTU. Proteins of the vOTUs

455 were aligned with the available viral proteins using blastp (minimum score 50), and the family

456 level taxonomy of a vOTU was generated if more than a third of its proteins were assigned into

457 the same viral family.

458

459 *Macrodiversity and microdiversity of DNA virome.* The macrodiversity (Shannon diversity index)

460 of virome was calculated using *vegan* package in R platform, with a uniformed number of reads (1

461 million) for each sample. The microdiversity (nucleotide diversity, π) for representative sequence

462 in each vOTU was calculated based on the methodology developed by Schloissnig *et al.* [75], and

463 microdiversity of a sample was generated by averaging from the viruses that presented

464 (depth >10x) in that sample.

465

466 *Functional profiles of DNA virome.* The viral proteins were aligned to KEGG [36] database (blastp

467 similarity >30%) for functional annotation. For functional profiling, the KEGG aligned proteins

468 were dereplicated with CD-HIT [76] (>95% identity and >90% sequence coverage) to construct

469 the custom viral functional gene catalog, followed by mapping the reads to the catalog using the

470 ‘very-sensitive-local’ setting in Bowtie2 [69]. The relative abundance of each functional gene in

471 sample was normalized by the total numbers of viral reads (the reads mapped to the viral sequence)

472 in the sample, and was transformed into centered log ratio (CLR) coordinates using *microbiome*

473 package in R platform. The carbohydrate-active enzymes and acquired antibiotic resistance genes
474 for the viruses were predicted from the CAZy [37] and CARD [77] databases, respectively, using
475 the same manner as functional assignment.
476
477 *RNA viromes assembly, identification, clustering and taxonomy.* The metatranscriptomic data of
478 RNA virome reads was trimmed using fastp [68]. The contamination of ribosomal RNA reads was
479 identified and removed by mapping to the small subunit sequences (bacterial 16S and eukaryotic
480 18S) on the latest SILVA database [78]. The rnaSPAdes was utilized in metatranscriptomic
481 assembly for each sample [79]. To identify RNA viruses, the assembled contigs (>500 bp) was
482 aligned to the reference RNA virus proteins downloaded from GenBank database using
483 DIAMOND (blastx e-value <1e-5). We also identified the RNA viral contigs by searching the
484 RNA-dependent RNA polymerase genes (RdRp genes, referred from Evan *et al.* [80]) using a
485 Hidden Markov Model approach [81]. Then, the RNA viral sequences were clustered based on 95%
486 identity and 90% coverage of the sequence.

487

488 Bacterial microbiome sequencing and analysis

489 All raw metagenomic data was trimmed and the human contamination sequences was removed
490 using the same methods in virome. MetaPhlan2 [39] was employed to generate the taxonomic
491 profile for each sample using default parameters. Enterotype analysis was performed at the
492 bacterial genus level composition based on the methodology developed by Costea *et al.* [55]. The
493 high quality microbiome data was assembled using metaSPAdes [70], and the resulting contigs
494 was searched against the NCBI-nt database to identity the bacteria sequence (>70% similarity

and >70% coverage at the phylum level). To search the potential bacterial host of virus, the CRISPR spacers in bacteria sequence was predicted using PILER-CR [82], and then the spacers were blasted to the viral sequences (“blastn-short” mode and bitscore >50) to identify the phage-bacterial host pairs. The matching bacterial host and viral sequence was summarized at the genus level. To avoid ambiguity, genus producing highest number of spacers hits was considered as primary host.

501

502 **Statistical analysis**

Statistical analyses were implemented at the R 3.6 platform (<https://www.r-project.org/>). Permutational multivariate analysis of variance (PERMANOVA) was performed with the *adonis* function of the *vegan* package, and the *adonis* *P*-value was generated based on 1,000 permutations. The method of effect size analysis was referred as Wang *et al.* [10]. The no-metric multidimensional scaling (NMDS) analysis was used as the ordination methods (*metaMDS* function in *vegan* package) for compositional data. The Procrustes coordinates analysis and significance were generated using the *procuste* and *procuste.randtest* functions in *vegan* package. The principal component analysis (PCA) was performed and visualized using the *ade4* package. The Wilcoxon rank-sum test was used to measure statistical differences in diversity and taxonomic levels between two cohorts. *P*-values were corrected for multiple testing using the Benjamini-Hochberg procedure.

514

515 **Data availability**

The raw sequencing dataset acquired in this study has been deposited to the NCBI SRA database under the accession code PRJNA641593. The sample metadata, vOTU and taxonomic

518 composition data, and the statistical scripts are available from the corresponding author on
519 reasonable request.

520

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526

527 **Author contributions**

528 T. M., S. L., Y. M., and Q. Y. conceived and directed the study. Q. Y., Y. W., X. C., G. W., T. A. and
529 X. L. developed and conducted the experiments. Q. Y., G. W. and T. A. performed sample
530 collection and investigation. H. J., K. G., Y. Z., and P. Z. carried out data processing and analyses.
531 S. L., Q. Y., and T. M. drafted the manuscript. Y. M., G. W., Y. L.; J. W.; G. C.; A. Z. and P. L.
532 participated in design and coordination, and helped draft the manuscript. P. Z., Y. S., M. X. and P.
533 L. revised the manuscript. All authors read and approved the final manuscript.

534

535 **Competing interests**

536 The authors declare no competing interests.

537

538

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764 **Table 1.** Characteristics of the subjects.

	Adults			Children		
	Chinese	Pakistani	P-value	Chinese	Pakistani	P-value
Number of subjects	24	24		6	6	
Sex, F/M	1/23	1/23	1.000	3/3	3/3	1.000
Age, years	26.0±4.3	29.1±3.7	0.011	2.8±1.8	2.8±1.7	1.000
Weight, kg	69.6±11.0	76.7±15.6	0.076	14±5.4	13.3±4.2	0.794
BMI, kg/m ²	22.8±2.8	25.6±4.5	0.011	16.0±2.0	17.4±3.1	0.396
Drinking, %	50%	8.3%	0.003	0%	0%	1.000
Smoking, %	16.7%	33.3%	0.030	0%	0%	1.000
Antibiotics (≤2mons), %	8.3%	8.3%	1.000	0%	0%	1.000
Prebiotics (≤2mons), %	58.3%	41.7%	0.387	66.7%	50%	1.000
Living in China, mons		11±4			9±6	

765 The data for age, weight, and BMI were presented as mean ± sd. *P*-values for age, weight, and
766 BMI were calculated by Student's t-test, and for sex, drinking, smoking, antibiotics, and prebiotics
767 were calculated by Fisher's exact test.

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Figure 1. Overview of the workflow for analyzing of DNA virome, RNA virome, and bacterial microbiome.

Figure 2. Differences in gut DNA virome between Chinese and Pakistanis. **a**, Rarefaction curve analysis of number of vOTUs on each group of samples. The number of identified vOTUs in different groups is calculated based on a randomly selected specific number of samples with 30 replacements, and the median and quartiles numbers are plotted. **b-c**, Boxplot shows the macrodiversity (**b**) and microdiversity (**c**) that differ among four groups. The significance level in the Student's t test is denoted as: *, $q < 0.05$; **, $q < 0.01$. **d**, NMDS analysis based on the composition of virome, revealing the separations between different groups. The location of samples (represented by nodes) in the first two multidimensional scales are shown. Lines connect samples in the same group, and circles cover samples near the center of gravity for each group. **e**, PERMANOVA analysis reveals that the virome of Pakistani children are similar with the Chinese subjects ($adonis\ p > 0.05$). The effect sizes and p -values of the $adonis$ analysis are shown. **f**, Composition of gut virome at the family level. **g-h**, Boxplot shows the differential viral families of adults (**g**) and children (**h**) when compared between Chinese and Pakistanis. C, Chinese individuals; P, Pakistani individuals. For boxplot, boxes represent the interquartile range between the first and third quartiles and median (internal line); whiskers denote the lowest and highest values within 1.5 times the range of the first and third quartiles, respectively; and nodes represent outliers beyond the whiskers.

Figure 3. Comparison of DNA viral functions between Chinese and Pakistanis. a, Composition of viral functional categories at the KEGG pathway level B. **b-c,** Boxplot shows the KEGG pathways that differed in abundance between Chinese adults and Pakistani adults (**b**) and between Chinese children and Pakistani children (**c**). Boxes represent the interquartile range between the first and third quartiles and median (internal line); whiskers denote the lowest and highest values within 1.5 times the range of the first and third quartiles, respectively; and nodes represent outliers beyond the whiskers. **d,** The taxonomic distribution of CAZymes. GH, glycoside hydrolase; GT glycosyl transferase; CBM, carbohydrate binding; CE, carbohydrate esterase; PL, polysaccharide lyase; AA auxiliary activity.

Figure 4. Differences in gut RNA virome between Chinese and Pakistanis. **a**, Rarefaction curve analysis of number of vOTUs on each group of samples. The number of identified vOTUs in different groups is calculated based on a randomly selected specific number of samples with 30 replacements, and the median and quartiles numbers are plotted. **b**, Boxplot shows the Shannon diversity index among four groups. The significance level in the Student's t test is denoted as: *, $q < 0.05$; **, $q < 0.01$. **c**, NMDS analysis based on the composition of virome, revealing the separations between different groups. The location of samples (represented by nodes) in the first

two multidimensional scales are shown. Lines connect samples in the same group, and circles cover samples near the center of gravity for each group. **d**, Composition of gut virome at the family level. **e**, Boxplot shows the differential viral families between Chinese and Pakistanis. **f**, Composition of gut virome at the species level. **g-h**, Boxplot shows the differential viral families of adults (**g**) and children (**h**) when compared between Chinese and Pakistanis. For boxplot, boxes represent the interquartile range between the first and third quartiles and median (internal line); whiskers denote the lowest and highest values within 1.5 times the range of the first and third quartiles, respectively; and nodes represent outliers beyond the whiskers.

Figure 5. Correlations between DNA and RNA viromes. **a**, Relationship of microdiversity between DNA and RNA virome. **b**, Procrustes analysis of DNA virome versus RNA viromes. Samples for DNA and RNA viromes are shown as circles and blue triangles, respectively; and samples from the same individual are connected by lines. Colors represent samples belong to different groups. **c**, Heatmap shows the co-abundance correlations between DNA and RNA viral families. The significance level in the Spearman correlation test is denoted as: *, $q < 0.05$; **, $q < 0.01$; ***, $q < 0.001$.

Figure 6. Differences in gut bacterial microbiome between Chinese and Pakistanis. a, Boxplot shows the Shannon diversity index among four groups. The significance level in Student's t test is denoted as: *, $q < 0.05$; **, $q < 0.01$. **d,** NMDS analysis based on the composition of bacterial microbiome, revealing the separations between different groups. The location of samples (represented by nodes) in the first two multidimensional scales are shown. Lines connect samples in the same group, and circles cover samples near the center of gravity for each group. **c,** Boxplot shows the bacterial families that differed in abundance between two cohorts. Boxes represent the interquartile range between the first and third quartiles and median (internal line); whiskers denote the lowest and highest values within 1.5 times the range of the first and third quartiles, respectively; and nodes represent outliers beyond the whiskers. **d,** Enterotype analysis of bacterial microbiome samples. The upper panel show the principal component analysis (PCA) of all samples, revealing the separation between two enterotypes. The lower panel show the composition of enterotypes in four groups.

Figure 7. Associations between virome and bacterial microbiome. **a**, Host range of viruses predicted through CRISPR spacer matches. Circles and triangles represent the bacteria and viruses, respectively; and the colors represent their taxonomic assignment at the phylum (for bacteria) or family (for viruses) levels. **b-c**, The 20 DNA (**b**) and RNA families (**c**) for which the highest effect size that significant impact the bacterial microbiome communities. **d**, The combined effect size of viruses on bacterial microbiome as well as bacteria on virome. To calculate the combined effect size, a set of non-redundant covariates (DNA vOTUs, RNA vOTUs, or bacterial species) is selected from the omic datasets, and then the accumulated effect size is calculated by *adonis* analysis using these selected covariates. **e**, The 20 bacterial species with highest effect size for impacting the viral communities.

Supplementary figure 1. Distribution of DNA viral contigs by length and depth of coverage.