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*Original Article*

# Differential virome composition and richness between children's diarrheagenic stools kept at ultra-low temperatures for long-term

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## Abstract

Introduction: Diarrhoeal illness is the second cause of morbidity/mortality among children from less-developed regions worldwide. Nonetheless, there is scarce information regarding their gut microbiome.

Aim: Microbiome characterization, with an emphasis on the virome, of children's stools with diarrhoea, by a commercial microbiome array.

Methodology: Nucleic acids extraction, optimised for viral identification, of stool samples from 20 Mexican children with diarrhoea (10 children < 2 and 10 ≥ 2-years-old), collected 16 years ago and kept at -70 °C, were analysed for the presence of viruses, bacteria, archaea, protozoa, and fungi species sequences.

Results: Only viral and bacterial species sequences were identified among children's stools. Most stool samples harboured species belonging to the bacteriophages (95%), anellovirus (60%), diarrhoeagenic viruses (40%), and non-human pathogens viruses (45% avian virus and 40% plant viruses) groups. Among the children's stools, virome inter-individual species composition was observed, even in presence of illness. The < 2-years-old children group has significantly higher viral richness ($p = 0.01$), conferred mainly by bacteriophages and diarrheagenic-viruses ($p = 0.01$) species, in comparison with the ≥ 2-years-old group.

Conclusions: The virome of stools of children with diarrhoea revealed inter-individual viral species composition. Similarly, to the few virome studies in healthy young children, the bacteriophages group was the most abundant. A significantly higher viral richness, conferred by bacteriophages and diarrheagenic-viral species, was observed among < 2-years-old children in comparison with older children. Stools preserved at -70 °C for long term can successfully be used for microbiome studies.

**Key words:** Children with diarrhoea; gut virome; bacteriophages; anelloviruses; plant viruses.

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## Introduction

Diarrhoeal diseases are one of the main causes of morbidity and mortality among children under five years of age, particularly in less developed areas of the world, including Mexico [1,2]. Diarrheal illness has been associated with malnutrition and stunting (impaired growth, cognitive development, and school performance) in children from these regions [3]. Because 72 % of diarrhoea deaths occur in the first two years of life, targeting this age group will yield the greatest future impact on the identification of diarrheal aetiology agents and mortality [4]. Therefore, studies of aetiology and incidence of diarrheal diseases in children are usually divided into children < 2-years-old and ≥ 2-years-old. A recent study, using 16S rRNA sequencing (bacteriome), to analyse faecal samples of children at four time-points during their first 2 years of life, and from their mothers, revealed, that mode of delivery accounted for some of the inter-individual variations in early childhood, but with a pronounced attenuation over time, as well as there is major shift in gut microbiota (diverse microbial community) composition during the first 2 years of life [5].

The human gastrointestinal tract (GT) is the anatomical site that hosts the most abundant, complex, and diverse microbiota, which is composed of a vast

number of different viruses and bacteria and, to a lesser extent archaea, fungi, algae, and small protists [6-8], while the microbiome is a term that describes the genome of all the microorganisms, symbiotic and pathogenic, living in the GT. In this anatomical site, viruses outnumber bacterial organisms by as much as 10:1 and include eukaryotic viruses, which infect eukaryotic cells, endogenous retroviruses, bacterial viruses (bacteriophages), and viruses that infect archaea (archaea viruses) [9].

It was recently reported that at birth, the GT of healthy neonates usually lacks viruses, but after delivery is rapidly colonized by viruses and bacteria [10]. Studies of intestinal viral genomes (virome) from both healthy and sick infants are scarce; even so, these studies have revealed a great variety of intestinal virus genomes species (richness) [10]. Furthermore, it seems that intestinal viruses interact with the host immune system and might play an important role in healthy infant development [8]. According to metagenomic viral analysis, most constituents of the children's virome are inferred to belong to bacteriophages. However, other studies stated that it is uncertain whether they belong to bacteriophages or other virus species, due to the lack of bacteriophage sequences in databases [11]. Among the few studies of virome characterization of stools of healthy children, besides bacteriophages, it has been frequently identified pathogenic and non-pathogenic vertebrate viruses, as well as viruses that infect plants [11-15].

Until now very few studies have characterized the gut microbiome of children with diarrhoea [16,17]. Besides, most gut virome studies have focused on diarrheagenic viruses, rather than non-pathogenic eukaryotic viruses or bacteriophages [18-20]. It is not clear, which changes may occur in the infant gut virome upon diarrhoea and if these changes are similar between children. Therefore, the study aims were: 1) to characterise the microbiome, with an emphasis on the virome, of children's stools under five years of age with diarrhoea, by a commercial microbiome array (Axiom Microbiome Array) and 2) to evaluate the association between viral species and richness with: children gender, age, type of birth, breastfeeding duration, age of initiation of complementary feeding, and clinical and anthropometrical data.

## Methodology

*Patients and stool samples*

The 20 children's stool samples analysed in this study, are part of a large cross-sectional study of diarrheal aetiology conducted in Mexico City. Stools, clinical and anthropometric data were collected from children with acute gastroenteritis attending three hospitals of the Mexican Institute of Social Security (IMSS) between March 1998 and December 2000 [21]. Informed consent was obtained from parents or tutors, and the protocol was reviewed and approved by the Internal Review Board at the Paediatric Hospital (protocol: FP0038/673). All stool samples were aliquoted and kept at -70 °C until use in 2016. Pathogens tested by traditional microbiologic methods included: *Salmonella enterica*, *Shigella* spp., *Vibrio cholerae*, *Campylobacter* spp., *Aeromonas* spp., *Isospora* spp., *Entamoeba histolytica*, and *Giardia lambia* [22]. Rotavirus, astrovirus, and adenovirus were tested by ELISA using monoclonal antibodies (IDEIA, DAKO Diagnostics, Ely, UK) [21]. Norovirus, Sapovirus, and the diarrheagenic *Escherichia coli* pathotypes (DEPs) were identified by molecular methods [21,23].

*Nucleic acid extraction and cDNA synthesis*

From stool samples from 20 children with diarrhoea, all negative for 17 diarrheagenic pathogens, nucleic acids (DNA and RNA) were extracted, as previously described [24]. Briefly, faecal samples were thawed and 100 mg of each were added to tubes with 150-212 µm glass beads (G1145 Sigma-Aldrich, St. Louis, US), chloroform (10µL), and phosphate-buffered saline (PBS) up to 1 mL. Samples were homogenised in a bead beater (Biospec-Products, Bartlesville, US) and clarified by centrifugation at 2,000 g for 10 minutes. Supernatants were recovered and filtered in Costar Spin-X (pore size 0.45 µm; CLS8162 Sigma-Aldrich, St. Louis, US) at 5,000 g for 15 minutes. Filtered samples were treated with Turbo DNase (AM2238 Ambion, Thermo Fisher Scientific, Waltham, US) and RNase (R4875 Sigma-Aldrich, St. Louis, US) at 37 °C for 30 min, and then chilled on ice. Nucleic acids were extracted from 400µL of the treated sample using PureLink Viral-DNA/RNA kit (12280050 Invitrogen, Thermo Fisher Scientific, Waltham, US), according to the manufacturer's instructions, quantified, and stored at -70 ºC until used. cDNA was generated from extracted nucleic acids using SuperScript® VILO cDNA Synthesis Kit (11754050 Invitrogen Thermo Fisher Scientific, Waltham, US), following Axiom® Microbiome Solution Guide (Affymetrix, Thermo Fisher Scientific, Waltham, US). The entire 20µL product of cDNA synthesis was sent to Thermo Fisher Scientific, Inc. (Santa Clara, US) to be processed by the Axiom Microbiome Array (AMA).

*Axiom Microbiome Array (AMA) characteristics and processing*

AMA (902903, Thermo Fisher Scientific, Waltham, MA) harbours a total of 1.38 million probes, including 135,555 target sequences (genomes, contigs, segments, or plasmids) from 12,513 microbial species (archaea, bacteria, fungi, protozoa, and viruses). Controls were included on all array plates, one positive Axiom Reference Genomic DNA 103 (Ref103) and one no template negative control (NTC), and were used for generating assay quality control (QC), and array QC metrics following standard manufacturer protocols. AMA also includes human-specific probes used to generate the QC metric Dish QC (DQC) for the Ref103 control. DQC is calculated based on the intensities of the probe sequences in non-polymorphic human genome locations and values of less than 0.82 indicate a possible issue with the processing of the plate. Microarray data were analysed using the Axiom™ Microbial Detection Analysis Software (MiDAS) (Thermo Fisher Scientific, Waltham, US), based on the Composite Likelihood Maximization algorithm. The array uses a threshold of signal intensities greater than the 99th percentile of the negative controls and at least 20% of probes detected to determine a positive detection. Axiom MiDAS output is a list of microbial organisms likely to be present in a sample as previously described [25].

*Statistical analysis*

Children with diarrhoea were divided into two age groups: 10 children were < 2 years old (ranging from 1-23 months) and 10 children aged ≥ 2 years (ranging from 24-54 months). Then, virome richness, taxa composition, patients' anthropometrical and clinical data, gender, mode of delivery, breastfeeding duration, and age of complementary feeding, were compared between the age groups (Table 1). Contingency tables were constructed and analysed by two-tailed Fisher's exact test, 95% confidence intervals (CI), and odds ratio (OR) were calculated by the Woolf method, when one or more values were zero, by the Baptista-Pike method. A $p$ value of $< 0.05$ was considered statistically significant. Analyses were performed using GraphPad Prism version 8 (San Diego, US).

**Table 1.** Children´s clinical profile and their viral composition.

| Child | Gender | Type of birth | Months | | | Number of viral taxa (richness) | Plant virus (PV), CAV or AGV | Diarrheagenic viral species | Group |
|---|---|---|---|---|---|---|---|---|---|
| | | | Age | Breast feeding duration | Initiation of complementary feeding | | | | |
| 1 | F | V | 2 | 0 | 0 | 8 | 0 | *Enterovirus C, Norwalk virus, Rotavirus A, Sapporo virus* | < 2*§ |
| 2 | M | C | 8 | 1 | 5 | 13 | 3 PV, CAV | *Astrovirus* MLB1, *Norwalk virus*, *Rotavirus A* | |
| 3 | F | V | 10 | 10 | 6 | 15 | 5 PV, CAV | *Rotavirus A*, *Rotavirus C* | |
| 4 | F | V | 12 | 1 | 3 | 12 | 3 PV, CAV / AGV | Astrovirus MLB1, *Norwalk virus* | |
| 5 | F | NA | 14 | 1 | 3 | 5 | 2 PV | -- | |
| 6 | F | C | 16 | 5 | 6 | 5 | 0 | *Mamastrovirus* 1 | |
| 7 | F | NA | 18 | 2 | 3 | 7 | 3 PV | -- | |
| 8 | F | C | 18 | 12 | 4 | 6 | CAV | -- | |
| 9 | F | C | 19 | 9 | 3 | 3 | CAV | -- | |
| 10 | M | C | 22 | 0.25 | 6 | 9 | 1 PV, CAV / AGV | *MW polyomavirus, MX polyomavirus* | |
| 11 | M | NA | 24 | 24 | 3 | 4 | CAV | *MW polyomavirus, Rotavirus A* | ≥ 2 |
| 12 | M | V | 27 | 0 | 12 | 2 | 0 | *Mamastrovirus 1, Rotavirus A* | |
| 13 | M | V | 31 | 2 | 3 | 2 | 0 | -- | |
| 14 | F | V | 36 | 6 | 3 | 2 | 0 | -- | |
| 15 | F | C | 39 | 9 | 3 | 5 | 0 | *Rotavirus A* | |
| 16 | F | V | 40 | 6 | 4 | 7 | 0 | -- | |
| 17 | M | V | 45 | 24 | 3 | 11 | CAV / AGV | -- | |
| 18 | M | C | 48 | 6 | 0.5 | 5 | 4 PV, CAV / AGV | -- | |
| 19 | F | V | 48 | 3 | 3 | 13 | 3 PV | -- | |
| 20 | F | C | 54 | 6 | 12 | 2 | 0 | -- | |

NA: Not available. F: Female; M: Male; C: Caesarean-section; V: Vaginal delivery; CAV: *Chicken anemia virus*; AGV: *Avian gyrovirus* 2; PV: plant virus. *p value < 0.05 viral richness between < 2-years-old (n = 10) and ≥ 2-years-old (n = 10) group. OR = 21, 95% CI = 1.7-248, Fisher exact test. §p value < 0.05 bacteriophages and diarrheagenic-viruses between < 2-years-old (n = 10) and ≥ 2-years-old (n = 10) group. OR = 21, 95% CI = 2.1-255, Fisher exact test.

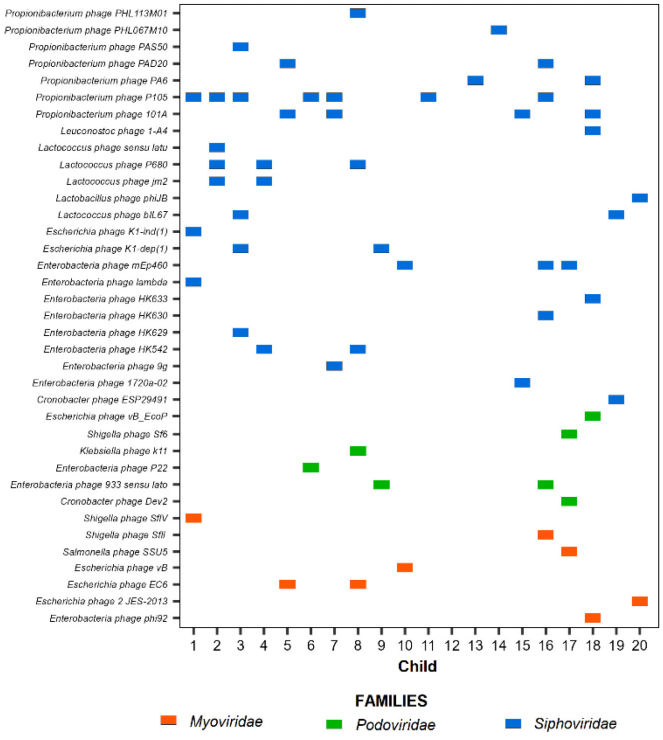
## Results

*Children's virome composition*

The microarray analysis revealed the presence of bacteria and virus nucleic acids and the absence of archaea, fungi, and protozoa. Since nucleic acid extraction was done with an emphasis on virus identification, we focus on virome composition. Of the 100 different virus families, included in the microarray, 21 (21%) families were identified in the children's stool samples.

As it is illustrated in Figure 1, each child had a unique gut virome composition. Bacteriophages were the most prevalent group detected, of note child-20 only harboured species belonging to this group, while child-12 was negative for bacteriophage species. In total, 37 different bacteriophage species were found in 19 stool samples (95%), most of them belonging to the *Siphoviridae* and *Myoviridae* families that were identified in 19 and 8 stool samples, respectively (Figure 1). Furthermore, phages and their potential bacterial hosts were found in 16 stool samples (80%): in six samples phages infecting *Propionibacterium* spp., in five samples phages infecting *Enterobacteriaceae* species, plus together phages that infect *Propionibacterium* spp. and *Enterobacteriaceae* and their hosts, in five samples (Figure 1).

**Figure 1.** Phage species detected by Axiom Microbiome Array from children's stools. Samples from children are according to their age in months. The family to which each virus species belongs is indicated on the downside.

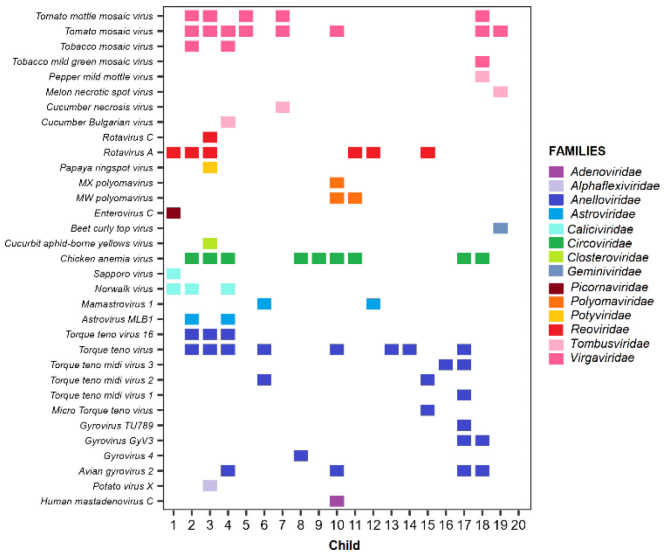



As shown in Figure 2, ten species of anelloviruses were present in 12 (60%) children's stools: *Avian gyrovirus 2*, *Gyrovirus TU789*, *Gyrovirus 3*, *Gyrovirus 4*, *Micro torque teno virus*, *Torque teno virus, Torque teno midi virus 1, Torque teno midi virus 2, Torque teno midi virus 3,* and *Torque teno virus 16*. Child-17 harboured the highest number (five) of anellovirus species (Figure 2).

Nine children's stools (45%) harboured pathogenic avian viruses such as *Chicken anemia virus* (CAV) and *Avian gyrovirus 2* (AGV2) of the *Circoviridae* family and anelloviruses group, respectively (figure 2). The nine children were ≥ 8 months old and all had initiated complementary feeding (Table 1).

Virus species sequences that cause diarrheal illness were identified in eight (40%) children, most of them younger than 22 months (Table 1). The most prevalent diarrheagenic viruses were *Rotavirus A* and *Rotavirus C* of the *Reoviridae* family, followed by two recently described diarrheagenic *Astroviridae* species (*Astrovirus MBL1, Mamastrovirus 1*), then *Caliciviridae* members (*Norovirus* and *Sapovirus*) and in one child *Enterovirus C* of the *Picornaviridae* family. Two children, child-1 and -2, younger than 24 months harboured three or more diarrheagenic virus sequences (Table 1). Furthermore, in two children, sequences of two species belonging to the *Polyomaviridae* family (*MW-* and *MX-polyomavirus*) were identified, species that have been previously isolated from the stools of children with diarrhoea.

**Figure 2.** Eukaryotic viral species detected by Axiom Microbiome Array from children's stools. Samples from children are according to their age in months. The family to which each virus species belongs is indicated on the right side.



As illustrated in Figure 2, plant viruses were found in the faeces of eight (40%) children, all ≥ 8 months old, and that had initiated complementary feeding (Table 1). In total 11 species of plant viruses were detected belonging to six families: *Virgaviridae* (*Tomato mosaic virus*, *Tomato mottle mosaic virus*, *Tobacco mild green mosaic virus,* and *Pepper mild mottle virus*); *Tombusviridae* (*Cucumber necrosis virus*, *Cucumber Bulgarian virus*, and *Melon necrotic spot virus*); *Potyviridae* (*Papaya ringspot virus*); *Alphaflexiviridae* (*Potato virus X*); *Closteroviridae* (*Cucurbit aphid-borne yellows virus*); and *Geminiviridae* (*Beet curly top virus*). Noteworthy, the only eukaryotic viruses identified in three children (child-5, -8, -18) were those infecting plants (Figure 2).

*Virome richness*

The alpha diversity of each sample was determined according to the number of taxa observed (richness). Comparisons between children's general characteristics (Table 1) and viral richness were statistically analysed. Revealing that children belonging to the < 2-years-old group had a significantly higher viral richness (> 6 viral species OR = 21, 95% CI = 1.7-248, *p* value = 0.01), and a higher prevalence of phages and diarrheagenic virus species (> 2 viral species, OR = 21, 95% CI = 2.1-255, *p* value = 0.019) as well, in comparison with children of the ≥ 2-years-old group (Table 1 and Figure 1).

## Discussion

An alternative method to characterize the microbiome from biological samples, particularly for virome studies is the Axiom Microbiome Array (AMA); since the metagenomic analysis of viruses from biological samples is extremely expensive, plus it is very difficult to identify virus species by the metagenomic analysis due to lack of bacteriophages and eukaryotic virus species sequences in the databases [25]. One of the advantages of the AMA array is that detects up to 12,513 microbial species, including archaea, bacteria, fungi, protozoa, and viruses, but its disadvantage is that abundance of each species is not determined. AMA has successfully been used for the identification of microbial species in swine stool samples [25] and more recently in saliva from human adults [26].

One of the main limitations of the present study was that the stool samples were kept frozen at -70° C for approximately 16 years, nevertheless, we successfully characterized the gut virome of 20 children with diarrhoea, which allowed the identification of several viral species sequences including a great diversity of bacteriophages, anellovirus, and diarrheagenic virus species. In line, the bacteriome from stool samples of New Zealander adults kept at -20° C for almost 14 years, was also efficaciously obtained by the 16S RNA method [27]. Furthermore, from dried human 1,000–2,000 years old palaeofaecal samples, was possible to discover and characterise previously undescribed gut microorganisms from ancient microbiomes and even genome reconstruction assembly [28,29]. Together these observations suggest that DNA from dried or frozen faecal specimens preserved for long periods of time successfully can provide microbial profiles.

In the present study, a high inter-individual gut virome diversity among the 20 children with diarrhoea requiring hospitalization was observed, similar to the few reports on gut virome among healthy children [15,30]. Inter-individual viral diversity was observed despite the illness, as previously reported for the bacteriome composition of patients [31, 32]. The main factor driving inter-individual bacteriome diversity among younger children (< 2 years-old) is the mode of delivery, hence the virome structure and composition of young children may also be influenced by this factor, whereas in older children several intrinsic/extrinsic factors may have a significant effect on the virome structure, including sex, lifestyle, and diet, as has been recently described for the virome of healthy Japanese adults [33].

Bacteriophage species were the most prevalent viruses found in the 20 children with diarrhoea, with 37 species identified, in accordance, it has been reported that bacteriophages are the most prevalent group in the stools of healthy children [34]. Furthermore, we also found that the stools of 16 children, harboured phages, and their bacterial host, as has been identified in the faeces of healthy children; revealing that bacteriophages coexist with their bacterial hosts in both healthy and sick children [31,34]. However, as expected phage diversity and viral richness were not similar between healthy and sick children, thus altered phage diversity and viral richness may contribute to dysbiotic enteric virome [35,36]. Therefore, studies are needed to understand the role of phage diversity in infants with diarrhoea.

The second most prevalent viral group in the children's stools was anellovirus, with ten different species detected such as *Torque teno virus* (TTV), *Avian gyrovirus 2, Gyrovirus 3*, and *Micro torque teno virus* that have also been found in the faeces of three Mexican healthy infants by metagenomic analysis [15]. TTV was the most prevalent species among our

children, also previously found among the stools of children with gastroenteritis [37-39] and immunosuppressed adults [40]. Six other anellovirus species were detected in the stool samples: *Gyrovirus TU789*, *Gyrovirus 4*, *Torque teno midi virus 1*, *Torque teno midi virus 2*, *Torque teno midi virus 3*, and *Torque teno virus-16.* Anelloviruses are an extremely diverse group, that has not yet been associated with human diseases, but can infect most humans [37,38]; suggesting that maybe anellovirus species are a major component of the human virome.

Virus species sequences that cause diarrhoea were identified in eight (40%) children. The most prevalent sequences belonged to the *Reoviridae* and *Caliciviridae* families, which are the most common agents associated with diarrhoea among children. Epidemiological studies of *Rotavirus* illness worldwide, have revealed a similar prevalence in children from industrialized and less developed regions of the world, including Mexico, and it seems that *Norovirus* (*Caliciviridae*) illness has a similar prevalence in children from these regions, as well [41-45]. Other sequences that were found among our children included *Mamastrovirus* 1, which has been associated with approximately 5% of diarrheal episodes in Spanish children < 5 years old [46], while *Astrovirus* MLB1 was identified in 6% of Kenyan children with diarrhoea [46,48]. One child stool sample was positive for *Enterovirus C*, which has been reported to be the most prevalent virus present in faeces samples of Thai and Indian children with acute gastroenteritis [49] and hospitalized diarrhoeal cases, respectively [50]. Two stool samples were positive for sequences of viruses of the *Polyomaviridae* family, *MW polyomavirus* and *MX polyomavirus*, but their role as causative agents of diarrhoea is not yet clear [51,52]. Of interest, *MW polyomavirus* was first described in 2012 from the stool of a Mexican young child collected between 2006-2009 [52].

Forty-five percent of children's stools harboured avian pathogenic viruses such as CAV or AGV2, these viruses do not cause disease in humans. The presence of CAV has been reported in children's stools, for example, in 35% of Chilean children with diarrhoea and in 25% without diarrhoea [53]. Furthermore, CAV is highly prevalent in chicken meat from the US and the presence of AGV2 has also been reported in these meat samples [54]. As shown in table 1, plant viruses that are not pathogenic for humans were found in 40% of the children's faeces. In a longitudinal faecal virome study from birth until 12 months of three Mexican children, plant viruses were first identified at five months of age [15]. In our study, avian and plant viruses were only identified in children ≥ 8 months old, and that had initiated complementary feeding. The presence of *Pepper mild mottle virus* in water, a virus identified in this study, is an indicator of faecal contaminated water [55]. Among Mexican infants, vegetables and fruits are introduced as complementary food feeding as early as 4 months of age, while chicken meat is introduced between 6-9 months [56]. Together our findings and previous reports suggest that plant and avian viruses would have been acquired after consumption of contaminated water, vegetables, and meat given to the children

Children of the < 2-years-old group had significantly higher viral richness and a higher prevalence of bacteriophages. It seems that very early children are colonised with viruses, since it has been reported that the first bacteria that colonise the gut of neonates commonly harbour integrated prophages that, after being excised from the bacterial chromosome, lead to lytic growth, providing the first pool of viral particles in the neonate's gut and by the fourth month of life, the viral community of most infants has changed dramatically [12]. Furthermore, it has been reported that the richness and diversity of bacteriophages diminish with the child's age (0-2 years old) concomitant with the increased detection of eukaryotic human viruses [34,57].

## Conclusions

The present study confirmed that stools from 20 children with diarrhoea, preserved at -70 °C for long-term, can successfully be used for virome studies. It seems that anellovirus that are non-pathogenic to humans is a major component of the children's virome. Virome inter-individual species composition was observed among the children's stools, even in presence of illness. The < 2-years-old children group has significantly higher viral richness, conferred by bacteriophages and diarrheagenic-virus species, compared to the ≥ 2-years-old group. Furthermore, non-human pathogenic avian and plant viruses may have been acquired after the child was complementary feed. We considered that our work adds to recent studies reporting that the gut of infants is frequently colonized with diverse virus species.

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