Human Microbiome Journal 19 (2021) 100078

Contents lists available at ScienceDirect

Human Microbiome Journal

journal homepage: www.sciencedirect.com/journal/human-microbiome-journal

Original Article

Reduced metagenomic sequencing (RMS) approach to determine the gut-associated phageome in mother-child

Prasanth Manohar [a,b], Nachimuthu Ramesh [c,*], Sebastian Leptihn [a,f], Anuradha Ravi [d], Knut Rudi [e,*]

[a] Zhejiang University-University of Edinburgh (ZJU-UoE) Institute, Zhejiang University, International Campus, Haining, Zhejiang 314400, PR China
[b] Second Affiliated Hospital Zhejiang University (SAHZU), School of Medicine, Hangzhou, Zhejiang, PR China
[c] Antibiotic Resistance and Phage Therapy Laboratory, School of Biosciences and Technology, Vellore Institute of Technology (VIT), Vellore, Tamil Nadu, India
[d] Quadram Institute Bioscience, Norwich Research Park, Norwich, UK
[e] Faculty of Chemistry, Biotechnology and Food Science (KBM), Norwegian University of Life Sciences (NMBU), Norway
[f] University of Edinburgh Medical School, Biomedical Sciences, College of Medicine & Veterinary Medicine, The University of Edinburgh, 1 George Square, Edinburgh EH8 9JZ, United Kingdom

ARTICLE INFO

Keywords:
Bacteriophages
Phageome
Reduced metagenomic sequencing
Caudovirales
Gut microbiome

ABSTRACT

The role of the human gut phageome (HGP) for a healthy gut microbiome is not well-established. This study aims to identify phages based on Reduced Metagenome Sequencing (RMS) fragments from an Indian mother and child cohort. For this study, fecal samples were collected from 17 mother-infant pairs at Nishanth Hospital, Tamil Nadu, India. RMS data analysis and shotgun sequencing approaches were used to assemble and identify the genome fragments. Out of the 156,926 RMS fragments, 434 were classified as bacteriophages by Kraken 2. Mapping of virus sequences in NCBI and *de novo* assembly with subsequent taxonomic assignment revealed 41 different phage species. The prevalence (>50%) of three bacteriophages was observed in mother and child; overall four phages were more prevalent in the mothers while one phage was more prevalent in the children. Even at the species level, mothers were found to have more diverse phage species than children. No significant association was observed for mother–child sharing of phages. This study highlights the prevalence of *Caudovirales* phages in healthy HGP and also the use of the RMS approach to study the phageome composition.

1. Introduction

The human gut microbiome is known to consist of *Bacteria*, *Archaea* and *Eukarya* but also contains viruses, the virome [1]. Studies on the gut microbiome (GM) have received more attention in the recent past; however, the knowledge about gut virome and its relevance in shaping human health are lagging behind [2]. The gut virome includes both eukaryotic viruses and bacterial viruses (bacteriophages or simply phages). The adult human gut is known to contain at least 10^{15} phages (phageome) and potentially helps in determining the bacterial colonization that forms the healthy GM [3]. Earlier studies on the human gut 'phageome' showed that the majority of the phage population belongs to dsDNA families, mainly *Caudovirales* (family: *Ackermannviridae*, *Autographiviridae*, *Chaseviridae*, *Demerecviridae*, *Dxexlerviridae*, *Herelleviridae*, *Myoviridae*, *Podoviridae* and *Siphoviridae*), and ssDNA family, *Microviridae*, and recent study showed the abundance of *CrAss-like* phages (Cross-Assembled phage) in the human gut microbiome [1,2,4,5].

Few virome studies showed the uniqueness of the phageome within the healthy human gut, and the diversity within the phage population has received little attention [3,6,7]. As the number of studies on the phageome are minimal, very little is known about the transmission of phages during child-birth (mother to infant) [8,9], and the phageome evolution from birth to adulthood is poorly understood [10]. During the first few months of child growth (0–4 weeks), the gut phageome was found to be rich and diverse, mostly dominated by *Caudovirales* phages [7]. But later during growth (24 months or older), there was an increased relative abundance in *Microviridae* phages which may be due to the rapid increase of the gut microbiome mainly due to the bacterial community. It was also found that there is a high level of bacteriophage diversity during infancy, (compared to eukaryotic viral population) which decreases with age [11]. The infant phageome is believed to develop in parallel with the microbiome (here mostly related to

* Corresponding authors.
E-mail addresses: drpnramesh@gmail.com (N. Ramesh), knut.rudi@nmbu.no (K. Rudi).

https://doi.org/10.1016/j.humic.2021.100078

Available online 16 January 2021

bacteria), and other sources such as diet and environment play a critical role in shaping the adult gut phageome [11]. Therefore, studies on the phageome in different age groups of the human host will provide more knowledge about the role of the phages in human health and disease.

Recently, reduced representation of microbial genomes [12,13] has been adapted for reduced metagenome sequencing (RMS) of microbial assemblages [14,15]. Although the RMS approach is comparable to shotgun sequencing [15] and 16S rRNA gene sequencing [16], RMS has not yet been used to determine the virome composition. Until now, shotgun sequencing has been the only option for analyzing the virome. Unfortunately, however, shotgun sequencing approaches have notoriously been associated with assembly errors that need a considerable amount of user input for correction and thus present obstacles to analytical processes [17]. It should also be mentioned that there are technical challenges that are associated with gut virome analysis with recent studies also focusing on long-read shotgun metagenome sequencing [18,19]. Therefore, we chose RMS as a simple and reliable approach for taxonomic assignment [20].

The current study aimed to identify viruses based on RMS fragments from an Indian mother and child cohort [21]. Our approach was to identify all unique RMS fragments and classify them using Kraken 2.

2. Materials and methods

The reduced metagenome sequencing was previously published [21]. Briefly, the protocol involves cutting DNA with a rare and a frequent cutting restriction enzyme, with the sequencing of only the fragments between the frequent and the rare cutter [21]. The sequence information used is available in NCBI with GenBank accession numbers KS790030 to KS946955.

2.1. Cohort description

The cohort consists of 17 mother-infant pairs. The infants were born full-term at Nishanth Hospital, India. Twelve of the 17 infants were born through C- section. Fecal samples were collected from late pregnant women (gestational age 32–36 weeks), and at day 4 after birth for all the infant, and at 15, 60 and 120 days after birth from respectively 4, 3 and 3 infants (S. Table 3). A written informed consent form before the fecal sample collection was given by the parents.

2.2. RMS data analysis

RMS fragments were obtained by fragmenting genomic DNA using an enzyme combination of EcoRI and MseI, followed by an adapter ligation [21]. The RMS data were processed using a CLC Genomic Workbench (Qiagen, Hilden, Germany) to identify all RMS-fragments in the dataset. Read-counts for each sample over the set of library RMS-fragments were obtained by mapping the merged reads back to the library of all identified RMS fragments. These data were arranged into an RMS-table, similar to an OTU-table, but where OTUs are replaced by RMS-fragments. Finally, the read counts for all the samples were normalized by dividing on the total number of reads detected for each sample. These analyses were done as a part of the previously published work [21].

For identification, we classified each of the RMS fragments using Kraken-2 in the Patric environment. All the fragments identified as bacteriophages were classified. Finally, we binned and summed the counts of the fragments assigned to the same bacteriophage taxon using Matlab.

2.3. Shotgun sequencing

Ten samples were selected for shotgun sequencing. Sequencing was done using the Nextera XT sequencing, while processing and assembly were done using the CLC genomic workbench module for combined taxonomy- and abundance-based binning. Taxonomic assignments of assembled contigs were done using Blast search towards the NCBI virus database.

2.4. Statistical analyses

Statistical analyses were done using both Matlab and Minitab. These include a Kruskal Wallis test for comparing medians of distributions and binominal test for comparing prevalence data.

3. Results

3.1. Taxonomic assignments of bacteriophages

Out of the 156,926 RMS fragments identified (from 17 mother–child pairs) 434 were classified as bacteriophages by Kraken-2. The RMS fragments belonged to 48 bacteriophage species, with a dominance of three phages belonging to the family *Myoviridae* (Fig. 1; S. Table 1). The identified (n = 48) *Caudovirales* phages (dsDNA) belonged to 41.66% *Siphoviridae*, 39.58% *Myoviridae* and 18.75% *Podoviridae* (including *CrAss-like* phages).

For the shotgun data, 87,372 out of 15,316,276 reads were mapped towards virus sequences in NCBI. *De novo* assembly, with subsequent taxonomic assignment revealed 41 different phage species, identified in six of the libraries (S. Table 2).

3.2. Mother-child distribution of bacteriophages

Three bacteriophages showed a prevalence of >50% for both mothers and children. Four of the phages were significantly more prevalent in mothers than in children, while only one phage was more prevalent in the children compared to mothers (Fig. 2A). Three phages showed a median fragment number of >0.01% for mothers, while only one *Clostridium* phage for children (Fig. 2B). Overall, the number of observed bacteriophage species was higher in mothers than in children; while the beta-diversity across mothers was lower (Fig. 3). With respect to mother–child sharing of bacteriophages, we did not identify any significant association (FDR corrected chi-square test p-value > 0.5).

4. Discussion

The interest in phageome and phage biology has put attention on the role of bacteriophages in human health and disease. The human gut phageome (HGP) plays a vital role in maintaining a healthy gut microbiome [3,6,7]. The studies on HGP are mainly focused on the distribution of phages in the healthy human gut [5,8,22,23]. However, very little progress has been made to understand the difference in the phageome of a mother and child; or the mother–child overlap/transmission of the phageome. This study was undertaken to analyze the phageome distribution in mother (pregnant women) and child (newborn) using the RMS approach. In this study, 434 bacteriophage fragments were identified which belong to dsDNA phages of the order *Caudovirales*. Recent studies also reported that only 7–13% of the viral contigs could be assigned to available viral families in the DNA virus database, mostly belonging to the *Caudovirales* [1]. There are other studies that showed the distribution of both dsDNA and ssDNA phages in the human fecal samples [1,23–26] highlighting the dominance of dsDNA, that is, *Caudovirales* phages in the infants and the gain of ssDNA, that is, *Microviridae* phages during child growth (after 24 months from birth). The presence of *Caudovirales* phages in this study strongly correlates with other studies on HGP, and this is one of the few studies to report mother–child phageome using; a) fecal samples that were collected from late pregnant women (gestational age 32–36 weeks), b) the source of samples for infants, from 4 days and up to 120 days (app. 4 months). The host range for *Caudovirales* phages is usually very broad [1,4] and here, we found myo-, sipho- and podo- viruses infecting *Enterobacteria*, *Cronobacter*,

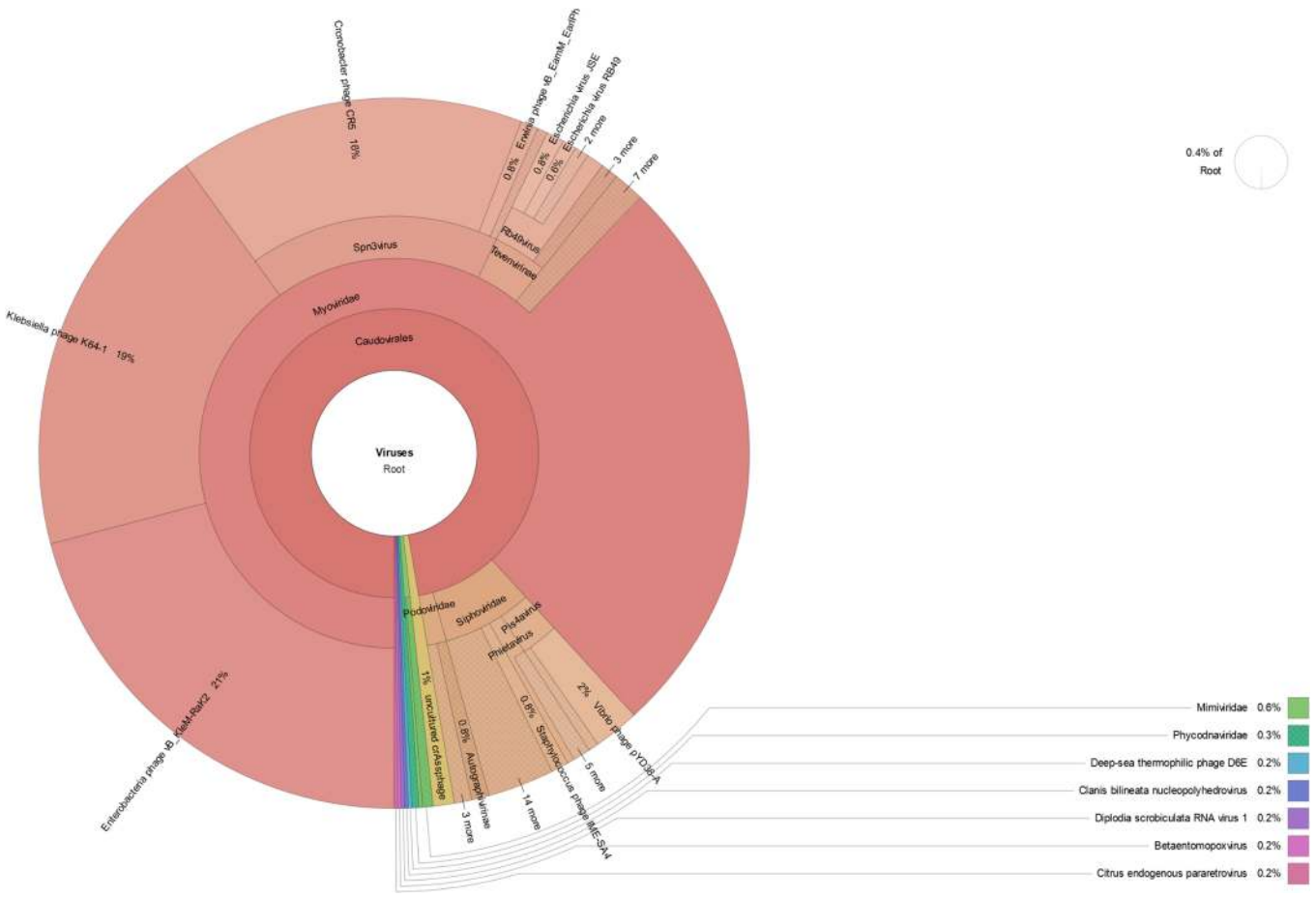

Fig. 1. Krona representation of RMS fragments classified as bacteriophages. Kraken 2 derived taxonomy represents the input for the taxonomic visualization.

Klebsiella, *Pseudomonas*, *Enterococcus*, *Escherichia*, *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Vibrio*, *Clostridium*, *Shigella*, *Salmonella*, and *Citrobacter* etc. Interestingly, the highly abundant dsDNA phage *CrAss-like* phages were also identified in this study, which are grouped under the *Podoviridae* (shared > 50% genome function). The relative abundance of *Microviridae* (ssDNA) in the phageome studies remains controversial [3,27] as their role in healthy and diseased humans is still controversial, although our study here deals with dsDNA phages only.

In this study, the prevalence of three bacteriophages belonging to the *Myoviridae* (*Enterobacteria*, *Cronobacter* and *Klebsiella*) was observed between mothers and children (4 days old). The possible reason may be the vertical transmission of phageome during childbirth [8,10]. The dominance of only four bacteriophages within the samples collected from mothers shows the uniqueness of phageome between the individuals [23,27] which strongly correlates with the increased number of bacteriophage species in mothers while beta-diversity was lower. In this study, only *Clostridium* phage (*Siphoviridae*) was found to be more prevalent in children across the samples. During growth as the gut microbiome diversifies, the phages kill the dominant commensal bacteria [10], possibly explaining the abundance of other *Caudovirales* phages. While the observed bacteriophage species was fewer in children (compared to mothers), the dominance of *Siphoviridae* phages in the newborn was reported earlier [2]. Earlier studies showed the correlation between mother–child transmissions of phageome either during birth or through milk (breastfeeding) [1,28]; However, in our study no significant mother–child overlap of phages was observed, while the milk microbiome was not analysed. A possible explanation for the discrepancies of the studies may be due to the metagenomic approach taken for this study while the earlier study described a PCR-based approach to determine mother–child transmission of phages [28]. The birth mode can also impact the microbiome [22] but the impact of C-section or vaginal delivery in phageome is not well-established and in this study, mode of birth was not taken into consideration during data analysis. One of the limitations of this study is the analysis of dsDNA phages (only) and the use of Kraken 2. To the best of our knowledge, this is the first study to report the metagenomic analysis of phageome in cohort samples collected in India and the first study to report the use of RMS approach to investigate the distribution of phages in mother–child pairs.

5. Conclusion

This study highlights the prevalence of *Caudovirales* phages in the human gut from the fecal samples collected from mother-child pairs. Importantly, this study highlights the use of the simple and reliable RMS approach for the detection of dsDNA phages from the stool samples. While this work did not investigate ssDNA phages, it should be noted that dsDNA phages play the most important role in maintaining a healthy gut phageome. Surprisingly, we could not observe a shared phageome community in mothers and newborns; more studies on the transmission of phageome between mother–child will provide insights on the mechanisms of the early phageome acquisition and subsequent development.

CRediT authorship contribution statement

Prasanth Manohar: Conceptualization, Validation, Writing - original draft. **Nachimuthu Ramesh:** Conceptualization, Investigation, Supervision, Validation, Writing - review & editing. **Sebastian Leptihn:** Writing - review & editing. **Anuradha Ravi:** Formal analysis, Investigation, Resources, Validation. **Knut Rudi:** Conceptualization, Funding

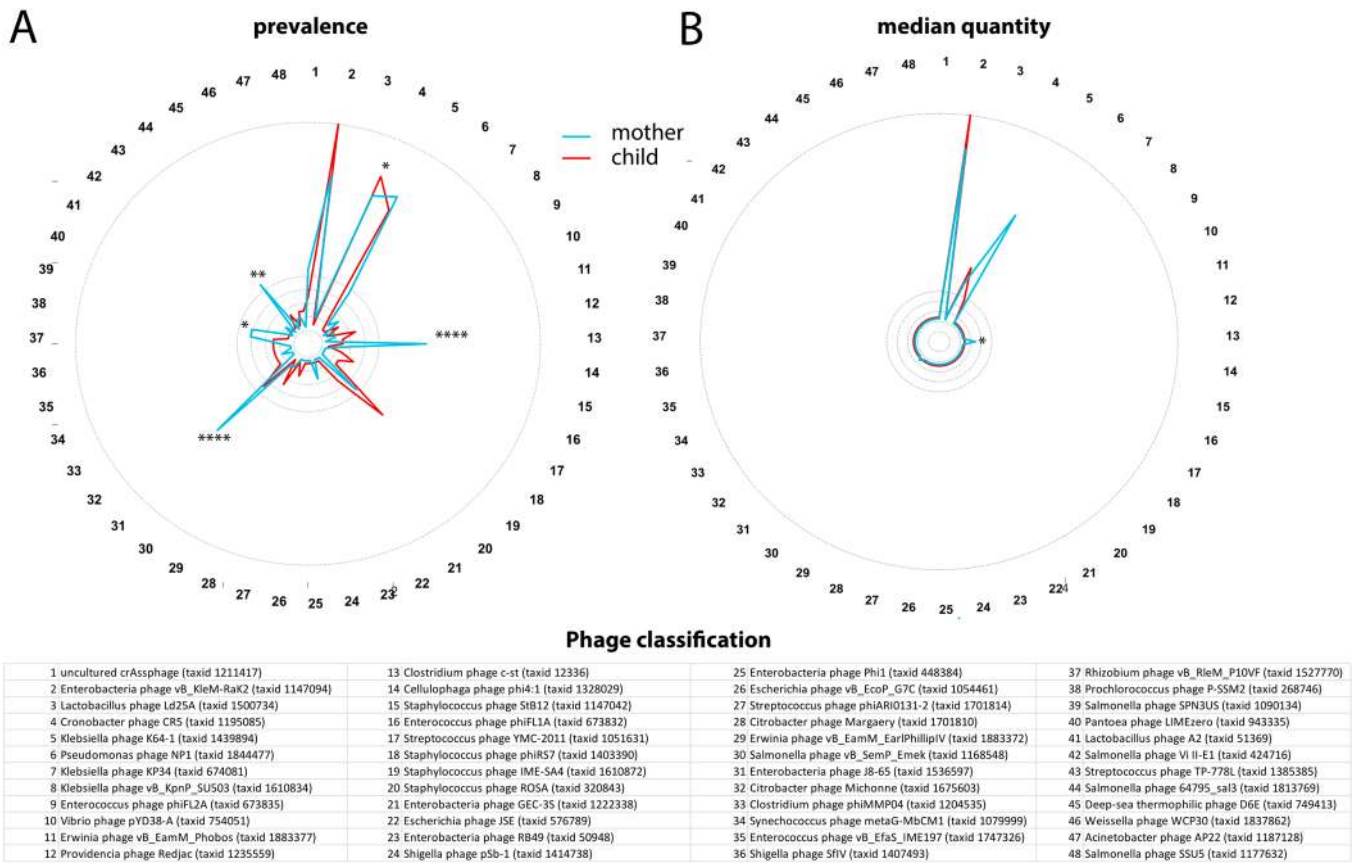

Fig. 2. Prevalence (A) and quantity (B) for the bacteriophages identified. The asterisks represent FDR corrected p-values with $p < 0.05$ represented by *, <0.01**, <0.001*** and <0.0001****.

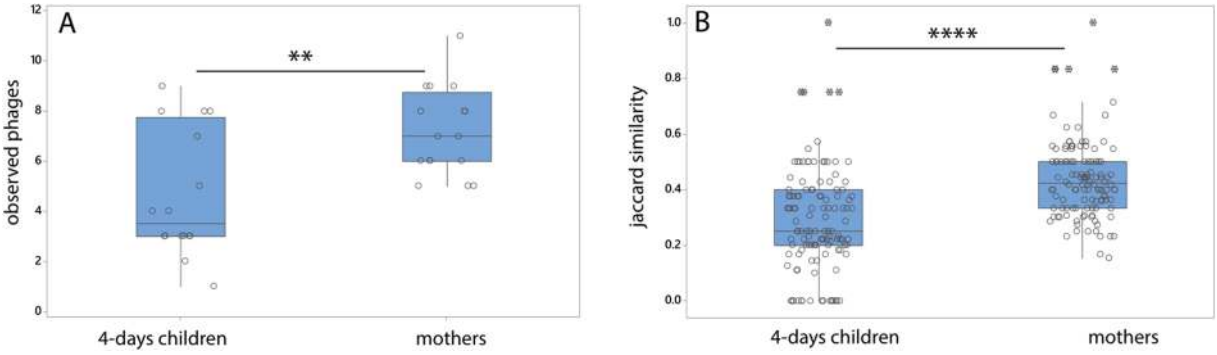

Fig. 3. Observed phages (A) and Jaccard similarity (B). The asterisks represent p-values for the Kruskal Wallis test; p-value < 0.01**, and < 0.0001**.

acquisition, Investigation, Methodology, Resources, Software, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank the Norwegian government for the scholarship provided to Anuradha Ravi. We would also like to thank the doctors and nurses at Nishanth Hospital, India for doing the sampling and collecting the information.

Ethical statement

A written informed consent form before the fecal sample collection was given by all the participants.

Funding

This research work was not funded by any external agencies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humic.2021.100078.

References

[1] Shkoporov AN, Hill C. Bacteriophages of the human gut: the "Known Unknown" of the microbiome. Cell Host Microbe 2019;25(2):195–209.

[2] Beller L, Matthijnssens J. What is (not) known about the dynamics of the human gut virome in health and disease. Curr Opin Virol 2019;37:52–7.

[3] Manrique P, Bolduc B, Walk ST, van der Oost J, de Vos WM, Young MJ. Healthy human gut phageome. Proc Natl Acad Sci USA 2016;113(37):10400–5.

[4] Minot S, Bryson A, Chehoud C, Wu GD, Lewis JD, Bushman FD. Rapid evolution of the human gut virome. Proc Natl Acad Sci 2013;110(30):12450–5.

[5] Guerin E, Shkoporov A, Stockdale SR, Clooney AG, Ryan FJ, Sutton TDS, Draper LA, Gonzalez-Tortuero E, Ross RP, Hill C. Biology and taxonomy of crAss-like bacteriophages, the most abundant virus in the human gut. Cell Host Microbe 2018;24(5):653–64.

[6] Milani C, Duranti S, Bottacini F, Casey E, Turroni F, Mahony J, Belzer C, Delgado Palacio S, Arboleya Montes S, Mancabelli L, Lugli GA, Rodriguez JM, Bode L, de Vos W, Gueimonde M, Margolles A, van Sinderen D, Ventura M. The first microbial colonizers of the human gut: composition, activities, and health implications of the infant gut microbiota. Microbiol Mol Biol Rev 2017;81(4). e00036–17.

[7] Lim ES, Wang D, Holtz LR. The bacterial microbiome and virome milestones of infant development. Trends Microbiol 2016;24(10):801–10.

[8] Ferretti P, Pasolli E, Tett A, Asnicar F, Gorfer V, Fedi S, Armanini F, Truong DT, Manara S, Zolfo M, Beghini F, Bertorelli R, De Sanctis V, Bariletti I, Canto R, Clementi R, Cologna M, Crifò T, Cusumano G, Gottardi S, Innamorati C, Masè C, Postai D, Savoi D, Duranti S, Lugli GA, Mancabelli L, Turroni F, Ferrario C, Milani C, Mangifesta M, Anzalone R, Viappiani A, Yassour M, Vlamakis H, Xavier R, Collado CM, Koren O, Tateo S, Soffiati M, Pedrotti A, Ventura M, Huttenhower C, Bork P, Segata N. Mother-to-infant microbial transmission from different body sites shapes the developing infant gut microbiome. Cell Host Microbe 2018;24(1):133–45.

[9] Mueller NT, Bakacs E, Combellick J, Grigoryan Z, Dominguez-Bello MG. The infant microbiome development: mom matters. Trends Mol Med 2015;21(2):109–17.

[10] Mukhopadhya I, Segal JP, Carding SR, Hart AL, Hold GL. The gut virome: the 'missing link' between gut bacteria and host immunity? Therap Adv Gastroenterol 2019;12. 175628481983662.

[11] Lim ES, Zhou Y, Zhao G, Bauer IK, Droit L, Ndao IM, Warner BB, Tarr PI, Wang D, Holtz LR. Early life dynamics of the human gut virome and bacterial microbiome in infants. Nat Med 2015;21(10):1228.

[12] Janssen P, Coopman R, Huys G, et al. Evaluation of the DNA fingerprinting method AFLP as an new tool in bacterial taxonomy. Microbiol 1996;142:1881–93.

[13] Vos P, Hogers R, Bleeker M, Reijans M, Lee TVd, Hornes M, Friters A, Pot J, Paleman J, Kuiper M, Zabeau M. AFLP: a new technique for DNA fingerprinting. Nucl Acids Res 1995;23(21):4407–14.

[14] Aveshina E, Angell IL, Rudi K. Tools and approaches to achieve strain resolution analyses of the microbiota. J Int Soc Microbiota 2016;3:42.

[15] Liu MY, Worden P, Monahan LG, et al. Evaluation of ddRADseq for reduced representation metagenome sequencing. PeerJ 2017;5:e3837.

[16] Shah N, Tang H, Doak TG, et al. Biocomputing 2011. World Scientific, 2013, 165–176.

[17] Sutton TDS, Clooney AG, Ryan FJ, Ross RP, Hill C. Choice of assembly software has a critical impact on virome characterisation. Microbiome 2019;7(1). 12–12.

[18] Garmaeva S, Sinha T, Kurilshikov A, Fu J, Wijmenga C, Zhernakova A. Studying the gut virome in the metagenomic era: challenges and perspectives. BMC Biol 2019;17(1):84.

[19] Yahara K, Suzuki M, Hirabayashi A, et al. Long-read shotgun metagenome sequencing using PromethION uncovers novel bacteriophages, their abundance, and interaction with host bacterial immunity in the oral microbiota. bioRxiv 2020.

[20] Snipen L, Angell I-L, Rognes T, et al. Reduced metagenome sequencing for strain-resolution taxonomic proles. Res Square 2020.

[21] Ravi A, Avershina E, Angell IL, Ludvigsen J, Manohar P, Padmanaban S, Nachimuthu R, Snipen L, Rudi K. Comparison of reduced metagenome and 16S rRNA gene sequencing for determination of genetic diversity and mother-child overlap of the gut associated microbiota. J Microbiol Methods 2018;149:44–52.

[22] McCann A, Ryan FJ, Stockdale SR et al. Viromes of one year old infants reveal the impact of birth mode on microbiome diversity. PeerJ 2018;6:e4694.

[23] Minot S, Sinha R, Chen J, Li H, Keilbaugh SA, Wu GD, Lewis JD, Bushman FD. The human gut virome: inter-individual variation and dynamic response to diet. Genome Res 2011;21(10):1616–25.

[24] Reyes A, Haynes M, Hanson N, Angly FE, Heath AC, Rohwer F, Gordon JI. Viruses in the faecal microbiota of monozygotic twins and their mothers. Nature 2010;466(7304):334.

[25] Hoyles L, McCartney AL, Neve H, Gibson GR, Sanderson JD, Heller KJ, van Sinderen D. Characterization of virus-like particles associated with the human faecal and caecal microbiota. Res Microbiol 2014;165(10):803–12.

[26] Castro-Mejía JL, Muhammed MK, Kot W, Neve H, Franz CMAP, Hansen LH, Vogensen FK, Nielsen DS. Optimizing protocols for extraction of bacteriophages prior to metagenomic analyses of phage communities in the human gut. Microbiome 2015;3(1):64.

[27] Norman J, Handley S, Baldridge M, Droit L, Liu C, Keller B, Kambal A, Monaco C, Zhao G, Fleshner P, Stappenbeck T, McGovern DB, Keshavarzian A, Mutlu E, Sauk J, Gevers D, Xavier R, Wang D, Parkes M, Virgin H. Disease-specific alterations in the enteric virome in inflammatory bowel disease. Cell 2015;160(3):447–60.

[28] Duranti S, Lugli GA, Mancabelli L, Armanini F, Turroni F, James K, Ferretti P, Gorfer V, Ferrario C, Milani C, Mangifesta M, Anzalone R, Zolfo M, Viappiani A, Pasolli E, Bariletti I, Canto R, Clementi R, Cologna M, Crifò T, Cusumano G, Fedi S, Gottardi S, Innamorati C, Masè C, Postai D, Savoi D, Soffiati M, Tateo S, Pedrotti A, Segata N, van Sinderen D, Ventura M. Maternal inheritance of bifidobacterial communities and bifidophages in infants through vertical transmission. Microbiome 2017;5(1):66.