bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

DNA extraction and virome processing methods strongly influence recovered human gut viral community characteristics

Short title –Processing Method Shapes Gut Virome Data

Luke S. Hillary[1], Trina A. Knotts[2,3,4], Sean H. Adams[2,3,4], Mohamed R. Ali[2,3,4], Matthew R. Olm[5], Joanne B. Emerson[1*]

1 - Department of Plant Pathology, University of California, Davis, USA

2 - Department of Surgery, University of California, Davis School of Medicine, Sacramento, USA

3 – Center for Alimentary and Metabolic Science, University of California Davis School of Medicine, Sacramento, California, USA

4 –Nutrition for Transformative Healthcare Program, University of California Davis School of Medicine, Sacramento, USA

5 – Department of Integrative Physiology, University of Colorado, Boulder, USA

Contact details:

*Corresponding author

jbemerson@ucdavis.edu

Department of Plant Pathology, Hutchison Hall, 425 Storer Mall, University of California, Davis CA, 95616, USA

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

Abstract

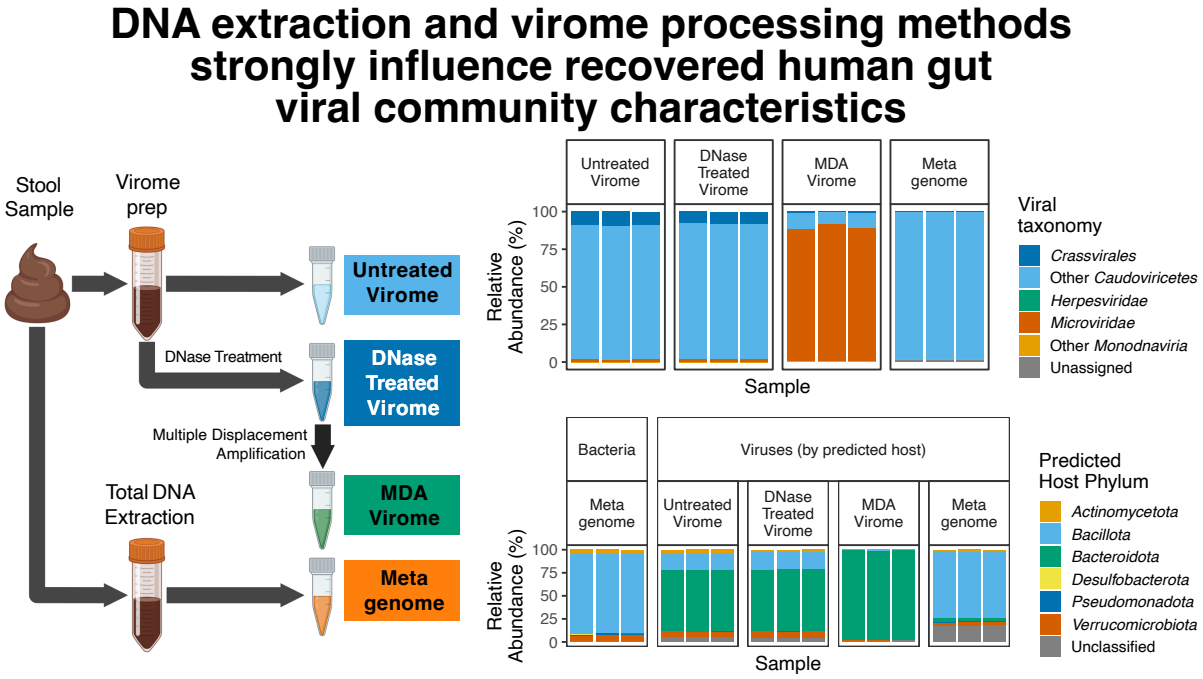
Accurately characterising the human gut virome is critical to understanding virus-microbiome-host interactions. However, widely used methods introduce biases that complicate data interpretation and limit cross-study comparability. For instance, multiple-displacement amplification (MDA) preferentially amplifies single-stranded DNA viruses, while total metagenomes are dominated by non-viral sequences, reducing viral signal. These traditional methods have not been systematically compared to viral size-fraction metagenomes (viromes) prepared without MDA. To address this, we applied four common methods for characterising human gut viral community composition (total metagenomes, viromes with/ without DNase treatment (to remove free DNA), and MDA viromes) to a human stool sample, with technical triplicates for each approach. MDA biased viral community composition to a shocking degree: *Microviridae* formed ~90% of MDA viromes compared to just 2% of non-MDA viromes. Removing ssDNA viruses from data analyses substantially reduced, but did not eliminate, MDA bias. Metagenomes were enriched for putative temperate phages and predicted *Bacillota-phages*, whereas predicted *Bacteroidetes*-phages dominated all viromes, suggesting that metagenomes and viromes select for different populations within the total viral community. DNase treatment had little-to-no effect on virome richness or community composition. This proof-of-principle experiment demonstrates that preparatory methods for viral community analysis can lead to substantially different conclusions from the same faecal sample, and we provide a comprehensive omic data analysis framework for comparing laboratory methodologies for viral ecology. With sufficient DNA yields now easily achievable from human gut viromes without the use of MDA, our results suggest that this biased amplification method should be avoided in human gut virome studies.

Keywords

Human gut virome, metagenomics, viral ecology, multiple displacement amplification, DNase treatment, virus-host interactions, sequencing methods

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

Graphical Abstract



List of Abbreviations

AMG – Auxiliary Metabolic Gene

ANI – Average Nucleotide Identity

dsDNA – double-stranded DNA

MDA - Multiple Displacement Amplification

ssDNA – single-stranded DNA

VLP - Virus-Like-Particle

vOTU – viral Operational Taxonomic Unit

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

Introduction

The viruses present in the human gastrointestinal tract, otherwise known as the gut virome, have the potential to regulate the composition, activity, and functional capacity of the gut microbiome through cell lysis, altered cellular metabolism, and horizontal gene transfer (1,2). Viral dysbiosis, a disruptive imbalance of the human gut virome (e.g. lower diversity), is associated with various disease states and may act as a diagnostic indicator in inflammatory bowel disease and pancreatic cancer (3–6). Phage therapy, via individual phages, phage cocktails, and whole-virome transplants, has the potential to address issues related to antimicrobial resistance and targeted interventions in gastrointestinal diseases (7,8). Therefore, accurate characterisation of the gut virome composition and dynamics is of critical importance.

Human gut virome studies often use either faecal shotgun metagenomics or size-selective viromics to characterise viral community composition, functional capacity, and ecological dynamics (9–11). Comparative studies in soils and aquatic environments show that while total metagenomes capture integrated prophages, giant viruses excluded by size-selection, and actively-replicating viruses often absent from the viral size fraction, they can recover less fine-scale diversity than size-selective viromes (12–14). Thus, metagenomics and viromics can differ in recovered viral community compositions (15), but systematic comparisons across different techniques remain limited.

Previous work on improving viral community characterization has focused on optimizing sample handling (16), viral concentration methods (17,18), nucleic acid extraction (19,20), PCR amplification cycles (21), and sequencing library preparation (22). As many of these studies focused on mock communities or viral spike-ins, viral recovery and diversity metrics were the primary evaluation criteria. This leaves clear knowledge gaps in how different methods affect recoverable virome diversity within complex human gut communities.

The amount of faecal material available for DNA extraction can be limited, leading to an assumed need for amplification to achieve sufficient yields for sequencing. This is often accomplished via high

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

numbers of PCR cycles (>6-8) during sequencing library preparation or through an initial treatment of multiple displacement amplification (MDA) before library construction (21,23). However, MDA is known to bias viromes towards amplifying small circular DNA, leading to the presumed but rarely quantified overrepresentation of single-stranded DNA viruses, such as *Microviridae*, in MDA-viromes from diverse environments (24,25). Given this known bias, systematic tests comparing viral communities recovered from MDA viromes and non-MDA viromes are needed.

Although the effects of DNase treatment have not been systematically evaluated for human gut viromes, studies in soil show that freezing can lead to viromic DNA yields below detection limits after DNase treatment (26,27), presumably because freezing compromises virions and exposes viral genomic DNA to enzymatic degradation. In agricultural soils, DNase treatment on fresh samples resulted in a 53% reduction in viral diversity, although broader community structure and ecological patterns remained similar with and without DNase treatment (28). As most faecal samples from human studies are stored frozen for logistical reasons, and storage above freezing has been shown to alter microbial community composition (29), the effects of DNase treatment on previously-stored faecal viromes warrant further consideration. Given that omitting DNase treatment improves viral recovery from frozen soil samples, the potential for skipping DNase treatment on faecal samples stored frozen should be evaluated, especially since low DNA yields might make biased MDA more tempting.

Here we leveraged our soil viromics protocol (largely similar to existing protocols in the human gut literature - Conceição-Neto et al., 2015; Kleiner et al., 2015; Wang et al., 2023) to: (i) compare two types of virome preparations (with and without DNase treatment – Sorensen et al., 2021), (ii) add a multiple-displacement amplification (MDA) treatment to aliquots from the DNase-treated viromes, an approach commonly applied in the human gut virome literature (31), and (iii) compare all of the virome preparations to "total metagenomes," commonly used for viral community recovery from a variety of ecosystems, including soil and the human gut (32,33). With analyses considering percent viral reads, viral contigs assembled *de novo* vs. recoverable through read mapping to a reference set, viral genome quality,

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

viral richness, viral community beta-diversity, viral taxonomy, predicted host taxonomy, predicted viral replication strategies, library k-mer complexity, functional annotations, viral genome coverage depth to enable strain-level analyses, and comparisons to a comprehensive gut viral genomic database, we also provide an example for rigorous comparisons of the downstream omic data resulting from methodological differences. Since the faecal material here was stored frozen (experiencing a single freeze-thaw), we hypothesised that DNase treatment would yield insufficient DNA for sequencing without further amplification and that viromes without DNase treatment would recover the highest viral diversity. We hypothesised that these viromes would recover vastly more viral species than total metagenomes and that MDA viromes would preferentially recover *Microviridae* (small, single-stranded circular DNA viruses), rendering MDA viromes non-quantitative and not representative of the non-MDA viral community. To test these hypotheses and provide guidance for future studies, we systematically compared untreated, DNase-treated, and MDA viromes against metagenomes from the same single faecal source material. This design allowed us to directly assess how upstream processing choices shape downstream characterisation of the gut virome, providing an observational benchmark to inform future experimental design.

Materials and methods

Sample collection and study design

Faecal sub-samples were generated from a single stool sample collected from a healthy female participant recruited from the UC Davis bariatric surgery clinic during the pre-operative period, and while consuming a typical diet. The participant provided informed consent, sample collection protocols were approved by the UC Davis Institutional Review Board, and the studies conform to the Declaration of Helsinki (34). The stool material was stored intact at -80 °C until processing. A 100 g stool sample was thawed overnight at 4°C and manually homogenised, followed by sub-sampling for DNA extraction.

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

In total, four approaches to measure viral community composition were compared in triplicate (see Supplementary Fig. S1): untreated viromes, DNase-treated viromes, MDA viromes (DNase-treated viromic DNA that was subsequently treated with multiple-displacement amplification, MDA), and stool total metagenomes.

Virome sample processing

For viromes, virus-like particles (VLPs) were purified from six 10 g sub-samples of thawed stool as previously described for soil (35). Briefly, VLPs were purified from stool by three sequential rounds of suspension in 9 mL of protein-supplemented PBS buffer (PPBS - 2% bovine serum albumin, 10% phosphate-buffered saline, 150 mM $MgSO_4$) in 50 mL conical tubes, each followed by 10 minutes of orbital shaking at 300 rpm, 4°C, and 10 minutes centrifugation at 4,000 × g, 4 °C. Supernatants from the first and second rounds were stored in separate 50 mL conical tubes at 4 °C during subsequent rounds of resuspension, shaking, and centrifugation. Supernatants from each aliquot were combined, centrifuged at 10,000 × g at 4°C for 8 minutes, and the supernatant was retained, followed by a second round of centrifugation. Supernatants were filtered sequentially through 5 µm, 0.45 µm, and 0.2 µm PES (polyethersulfone) syringe filters and pooled in 50 mL conical tubes prior to being transferred to 26.3 mL polycarbonate round-bottomed ultracentrifuge tubes (Beckman-Coulter Life Sciences), followed by ultracentrifugation at 35,000 rpm (112,000 × g) at 4 °C for 145 minutes using an Optima LE-80K ultracentrifuge and 50.2 Ti rotor (Beckman-Coulter Life Sciences). Supernatants were discarded, and pellets resuspended in 600 µL nuclease-free water. DNase treatment was performed on three 100 µL aliquots of VLP concentrate by incubating them with 10 µL RQ1 DNase and 10 µL RQ1 DNase buffer (Promega) for 30 minutes at 37 °C. DNase was inactivated by the addition of 10 µL of RQ1 DNase stop solution (Promega).

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

Virome and metagenome DNA extraction, multiple-displacement amplification, and library construction

DNA was extracted from 100 µL aliquots of VLP concentrates (untreated viromes), the DNase-treated virome preparations, or 0.25 mg of stool (for metagenomes), using the PowerSoil Pro DNA extraction kit (Qiagen) per manufacturer's instructions. Samples were incubated with lysis buffer for 10 minutes at 65 °C followed by vortexing for 10 minutes at maximum speed. Further steps were carried out according to the manufacturer's instructions. DNA was quantified using a Qubit 1x High Sensitivity DNA quantification assay and Qubit 4 fluorimeter (Thermo Fisher Scientific, Inc.).

To generate the MDA viromes, three 1 µL aliquots of DNA from each of the three DNase-treated virome preparations were used as templates for MDA, for nine reactions in total. MDA was performed using the GenomiPhi V2 DNA amplification kit (Cytiva), according to the manufacturer's instructions. The nine reactions were then pooled into three treatment replicates (three MDA reactions per DNase-treated virome), yielding three MDA virome preparations.

Libraries for all four methods (3× untreated viromes, 3× DNase-treated viromes, 3× MDA viromes, 3× metagenomes) were prepared using the KAPA DNA HyperPrep library kit (Roche) by the DNA Technologies & Expression Analysis Core, UC Davis, and 150 bp paired-end sequencing was performed to a target depth of 20 Gbp per library using the Illumina NovaSeq 6000 platform.

Sequencing data quality control

Detailed data processing settings are provided in Supplementary Table S2. Raw reads were trimmed and filtered using BBDuk v39.1, error-corrected using Tadpole, and deduplicated using Clumpify (all part of BBTools v39.1 Bushnell 2018). Raw and processed read quality was assessed using FastQC v0.12.1 (Andrews 2010) and MultiQC v1.14 (Ewels et al. 2016).

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

Library complexity, rRNA gene read, and human read analyses

K-mer (k=31) frequencies were calculated using khmer v2.1.1 (36). Ribosomal RNA gene reads were identified using SortMeRNA v4.3.6 (37). Taxonomic profiling of raw reads was performed using SingleM v0.18.3 (38). To identify human host reads, error-corrected reads were mapped to the human genome (GCF_009914755.1, Nurk et al., 2022) using Minimap2 v2.26 (40) and Samtools v1.17 (41). Count data were aggregated using CoverM v0.6.1 (42).

Assembly and viral contig identification

Reads from each library were separately assembled (12 assemblies) using MEGAHIT v1.2.9 (43), and summary statistics were produced using Quast v5.2.0 (44). Viral contigs were identified from each assembly using geNomad v1.7.0 (45) and additionally filtered by length ≥10 Kbp (46) or with a requirement for both a length between 1 and 10 Kbp and a geNomad phylum = *Monodnaviria*. The latter requirement facilitates inclusion of single-stranded DNA viruses that typically possess genomes <10 Kbp (47).

vOTU clustering, read mapping, and community compositional analyses

All viral contigs were clustered together into vOTUs, using a combination of MegaBLAST v2.14.0 and custom Python scripts (see Data and Code Availability), requiring a minimum average nucleotide identity (ANI) of 95% across 85% of the length of the shortest contig (48–50). Quality-filtered and error-corrected reads from each library were mapped to dereplicated vOTUs using minimap2 (40) and relative abundances calculated using transcripts per million (TPM) values generated by CoverM v0.6.1 (42).

The presence of vOTUs in a sample was determined using two complementary criteria: detection by read mapping and recovery of viral contigs through a combination of mapping and assembly. For vOTUs categorised as “mapped” a vOTU was considered shared between two libraries if ≥75% of its length was covered at ≥ 1x read depth and 90% ANI in both libraries. vOTUs were considered “assembled” in a

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

library if, in addition to reads mapping to the vOTU sequence according to the aforementioned thresholds, a viral contig from the same vOTU cluster was assembled from that same library.

To identify known vOTUs from the Unified Human Gut Virome catalogue (UHGV, https://github.com/snayfach/UHGV, accessed on June 12th 2025) (31,49,51–60) within libraries from this study, all UHGV vOTUs >10Kbp in length or >50% complete were downloaded from https://portal.nersc.gov/UHGV/ on June 12th 2025. These UHGV vOTUs were clustered with all 2,480 predicted viral contigs from this study using the same parameters as above, and reads from our libraries were mapped to this dereplicated set. A UHGV vOTU was considered detected in our dataset if it clustered with at least one viral sequence from this study or if read mapping covered the vOTU in at least one sample at the same detection thresholds described above.

Read depth analysis

Raw reads were randomly sub-sampled to depths of 1 Gbp increments from 1-17 Gbp using seqtk v1.4-r122 (https://github.com/lh3/seqtk), and quality control, assembly, viral contig identification, vOTU clustering, and read mapping were performed on each subsampled dataset as described above.

Viral translated protein annotation

As the gene content of vOTU cluster sequences can differ from the vOTU representative sequence, all viral translated proteins from predicted viral contigs by geNomad were annotated in bulk using Pharokka v1.7.1 with database v1.4.0 (61).

vOTU lifestyle and host predictions

Viral lifestyle, i.e., whether a virus is virulent or temperate, was predicted using BACPHLIP v0.9.6 (62). vOTUs were classified as putatively virulent or temperate if the confidence score for either assignment was ≥0.95; otherwise, they were labelled unclassified. vOTU host prediction was performed using iPHoP v1.3.2, with a combined prediction confidence score to genus level of ≥90/100 (63). When

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

two hosts were predicted, the host with higher confidence was selected. If confidence scores were equal, host prediction was converted to the lowest common taxonomic level.

Data analysis and visualization

Statistical analyses and data visualization were performed using R v4.4.0, RStudio v2024.09.1-394, ggpubr (64), and tidyverse (65). Statistically significant differences with p-values <0.05 were identified using analysis of variance (ANOVA) and post-hoc Tukey tests and converted into compact letter displays using multcompView (66). Venn diagrams were visualised using ggVennDiagram (67). Principal Coordinates Analyses (PCoA) of pairwise Bray-Curtis dissimilarity matrices were performed using Vegan (68), and significant differences were identified by permutational multivariate analysis of variance (PERMANOVA).

Results and discussion

Recovered viral communities differed across all processing methods, except between DNase-treated and untreated viromes

To evaluate the impacts of total metagenomics, virus-like particle (VLP) fractionation (viromics), DNase treatment, and multiple displacement amplification (MDA) on recoverable faecal viral community composition (Supplementary Fig. S1a), 12 libraries were generated from one stool sample, sequenced to an average depth of 21.1 ± 2.3 Gbp, and analysed. A total of 2,480 viral contig sequences were identified using geNomad (45) and clustered into 605 viral operational taxonomic units (vOTUs; Supplementary Fig. S2). Full results of statistical tests (ANOVA and Tukey post hoc comparisons) for all analyses are provided in Supplementary Tables S5–S6.

Because all preparations were performed on aliquots of the same faecal sample, observed differences reflect the influence of processing method rather than inter-individual variation, which is a known major

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

source of variability in gut virome studies (2). While this single-sample design limits generalisability, it provides a clear view of methodological biases in isolation, supporting observational conclusions about their effects on detectable viral community composition and downstream analyses typically employed in intervention-based human gut virome studies.

The similarity between DNase-treated and untreated viromes following frozen stool sample storage was unexpected, as DNase treatment is often discouraged for environmental samples stored frozen since it can result in a 10- to 100-fold loss in viromic DNA yield (69). The negligible impact observed here likely reflects stool-specific factors, such as (i) higher viral loads and organic content, supporting higher intact viral particle recovery (15) and/or new virus production during thawing or (ii) a naturally high ratio of encapsidated to free DNA due to DNase I secretion in the small intestine (70). Unlike soils, where freeze-thaw cycles may damage a greater proportion of viral capsids (potentially due to higher viral diversity and overall lower biomass), stool VLP concentrates appear to retain sufficient encapsidated viral DNA to permit effective sequencing of frozen samples, even after DNase treatment. Together, these findings are particularly relevant to studies using frozen, biobanked, or transported samples without guaranteed cold-chain preservation above 0 °C. Of course, further tests using additional samples and comparisons between fresh and frozen stool samples would be required to assess the generalizability of these results. As DNase-treated and untreated viromes were virtually indistinguishable in each of our analyses in this study, we generally describe their results together and refer to them collectively as non-MDA viromes, but we retain them separately in calculations and figures.

Method-dependent impacts on vOTU recovery, proportions of viral and rRNA gene reads, and viral community profiling

To evaluate the effects of virome and metagenome preparation methods on vOTU recovery, we assessed vOTU detection using two metrics: “assembled” vOTUs or “mapped” vOTUs. Notably, vOTUs only detected via read mapping for a given processing method would not have been detected if other processing methods had not been performed to generate the reference set of 605 vOTUs, whereas

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

263 "assembled" vOTUs would have been detected from that method alone (see Methods and Supplementary
264 Fig. S3). Both metrics produced similar patterns across processing methods. DNase-treated and untreated
265 viromes recovered highly overlapping vOTUs, with 97% shared by mapping, confirming highly similar
266 compositions. In contrast, 50% of vOTUs assembled uniquely in MDA viromes, and 62% of vOTUs
267 uniquely assembled in metagenomes were not detected by other methods, even after read mapping. Only
268 25% of vOTUs were detected in at least one replicate from all four methods via read mapping (Fig. 1a-b).
269 Metagenomes and non-MDA viromes assembled similar numbers of vOTUs (292-304), approximately
270 half of the total, while MDA viromes only assembled 166 vOTUs (27%). By both metrics, MDA viromes
271 shared more vOTUs with non-MDA viromes than with metagenomes, while metagenomes yielded the
272 most unique vOTUs. These findings indicate that metagenomes and viromes recover distinct portions of
273 the viral community, and differences persist when using a shared reference database for read mapping.

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

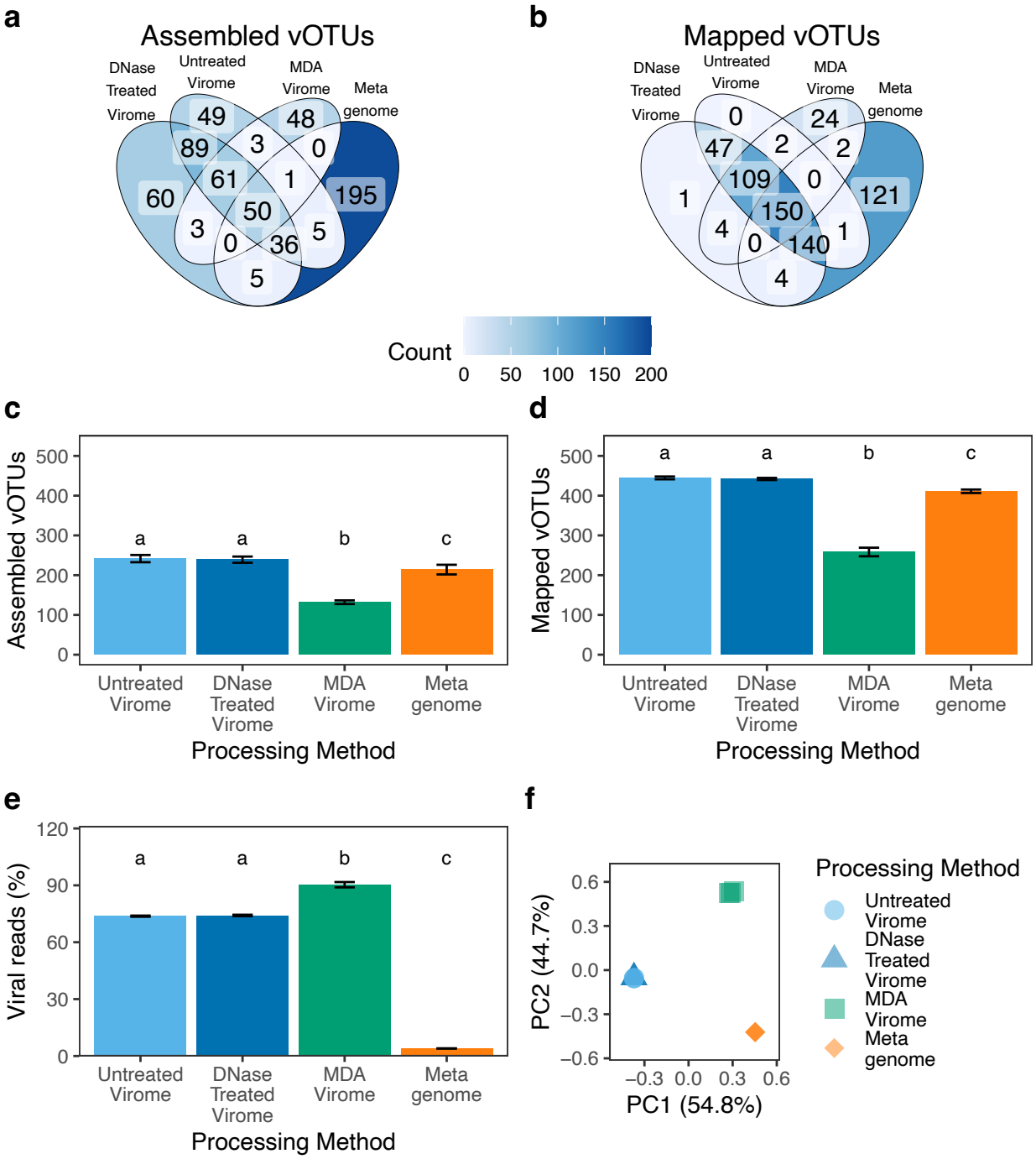


Figure 1. Comparison of vOTU recovery, read composition, and viral community structure across methods. (a-b) Venn diagrams showing the number of vOTUs shared between methods based on (a) assembly and (b) read mapping to a dereplicated set of 605 vOTUs. (c-d) Mean number of vOTUs detected per library in each method by (c) assembly and (d) read mapping. (e) Percentage of reads per

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

library mapped to vOTUs by method. For panels (c-e): bars = mean, error bars = standard deviation, letters represent groupings according to Tukey HSD where differences require $p_{adjusted}<0.05$. (f) Principal Coordinates Analysis (PCoA) of Bray-Curtis dissimilarities between samples with percent variance explained by PC1 and PC2. A total of 12 samples are shown, but due to the extreme similarity between some, differences among technical replicates are not visible (e.g., all three metagenome samples directly overlap). Significant differences among methods were tested by PERMANOVA, $R^2 = 0.996$, $p < 0.001$.

Other alpha-diversity metrics support vOTU detection metrics, namely that non-MDA viromes, MDA viromes, and total metagenomes captured distinct parts of the viral community, with DNase-treated and untreated viromes statistically indistinguishable. On a per-library basis, both types of non-MDA viromes yielded significantly higher viral richness (number of vOTUs detected per library) than did MDA viromes or metagenomes by both "assembled" (Fig. 1c; ANOVA, $F_{3,8}$=85.3, p < 00001; Tukey HSD, all $p_{adjusted}$ < 0.001) and "mapped" (Fig. 1d; ANOVA $F_{3,8}$ = 31.5, p < 0.00001, ; Tukey HSD, all $p_{adjusted}$ < 0.01 for non-MDA vs. MDA viromes) vOTU detection metrics. The MDA viromes had a mean of 132 assembled vOTUs, or only 55% of the vOTUs from the DNase-treated viromes from which the MDA virome DNA was sourced.

Despite this, MDA viromes had the highest proportion of viral reads (Fig. 1e; 91%), followed by non-MDA viromes (74% for both DNase-treated and untreated). As expected, metagenomes contained far fewer viral reads (4%; ANOVA, $F_{3,8}$ = 1,720.8, p < 0.00001; Tukey p < 0.0001 for all viromes vs. metagenomes). These trends were mirrored in rRNA gene read proportions (a proxy for cellular organism-derived DNA), which were highest in the metagenomes (Supplementary Fig. S4b; ANOVA, $F_{3,8}$ = 546.5, p < 0.00001). Human reads were slightly more abundant in the non-MDA viromes (0.22–0.25%) compared to the MDA viromes (0.14%) and metagenomes (0.12%; Supplementary Fig. S4c), but overall, these differences were not significant (ANOVA, $F_{3,8}$ = 1.42, p=0.31). These results show that non-MDA viromes best recover viral diversity while minimizing cellular DNA contamination.

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

Beta-diversity patterns based on Bray-Curtis dissimilarities clearly separated the dataset into the three methodological groups. Principal Coordinates Analysis (PCoA, Fig. 1f) revealed strong separation into three clusters corresponding to non-MDA viromes, MDA viromes, and metagenomes (PERMANOVA, $R^2 = 0.996$, $p < 0.001$). Replicates clustered with minimal differences within methods, with DNase-treated and untreated viromes grouping together (ANOVA on distances to centroids $F_{1,4} = 3.08$, $p = 0.091$). Within-method dissimilarities were consistently low (0.01-0.1), while between-group dissimilarities were substantially higher (0.88-0.96), except between DNase-treated and untreated viromes (0.04; Supplementary Fig. S5). These dissimilarity values are comparable to intra-subject or between-timepoint values from previous gut virome studies (71,72), suggesting that the processing method can overshadow biological signals in viral community composition.

Multiple-displacement amplification severely biased viral taxonomic diversity, but the biases were largely computationally correctable

Despite known MDA biases, limited quantitative comparisons may contribute to the continued use of MDA viromes. To address this, we offer an empirical comparison of MDA viromes and non-MDA viromes. As expected, high-level taxonomic classification by geNomad (45) revealed that MDA viromes were overwhelmingly dominated by Microviridae, a family of small, circular, single-stranded DNA viruses, forming 90% relative abundance from 23-24 vOTUs per sample, compared to just 2% in non-MDA viromes (Fig. 2a). The staggering dominance of Microviridae in the MDA-viromes (45 times that of non-MDA viromes) bears emphasis here and adds tangible, quantitative, empirical rigor to previous suggestions of MDA bias. Conversely, the widespread dsDNA phage order Crassvirales (73), known to infect Bacteroidota and typically characterised by circular genomes of ~100 kbp (alpha–gamma families) or ~145–192 kbp (epsilon and zeta families) (74,75), was largely absent from MDA viromes (1%) and even more so from metagenomes (0.3%). In contrast, *Crassvirales* comprised 8-9% of the viral communities in non-MDA viromes, suggesting that MDA viromes may undersample this group. Since k-

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

mer profiles reflect sequence diversity independently of taxonomic assignment (76–78), we also compared k-mer abundance distributions across methods to further assess how MDA affected sequence composition (Fig. 2b and Supplementary Fig. S4d). MDA viromes showed an underrepresentation of low-abundance k-mers compared to non-MDA viromes and metagenomes, consistent with lower sequence diversity in MDA viromes. Overall, MDA viromes were severely biased in recoverable viral taxonomic diversity compared to all other processing methods, leaving it difficult to justify using multiple-displacement amplification for viromics in the future if it can be avoided.

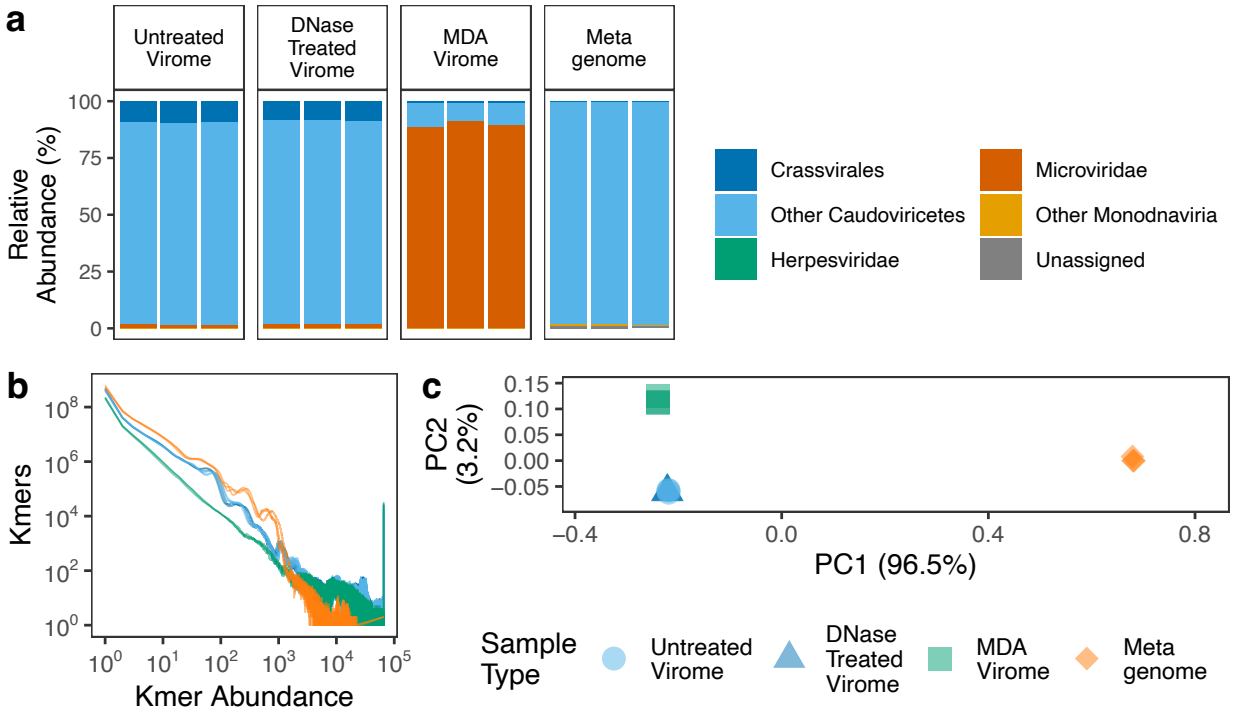


Figure 2. Impact of method on viral taxonomic composition and library k-mer profiles, and demonstrated potential to compensate for MDA compositional biases. (a) Relative abundances of viral taxa across methods, based on geNomad taxonomic classifications (45). (b) K-mer abundance profiles - MDA viromes in green show an underrepresentation of low-abundance k-mers (left side of the plot) compared to high abundance k-mers (right side of the plot). (c) Principal Coordinates Analysis (PCoA) of Bray-

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

Curtis dissimilarities after removal of ssDNA viruses and recalculation of relative abundances. Methods remained significantly different but with 96.5% of the variance explained by PC1, corresponding to differences between viromes and metagenomes (PERMANOVA, 999 iterations, $F = 1545.2$, $R^2 = 0.998$, $p = 0.001$).

To assess whether excluding ssDNA viruses mitigated MDA biases, we recalculated vOTU relative abundances after removing all ssDNA vOTUs, including the circular ssDNA families *Circoviridae, Genomoviridae, Inoviridae, and Microviridae*. No linear ssDNA viruses (*Spiraviridae, Bidnaviridae, Parvoviridae*) were detected. After filtering, 96.5% of the variance was attributable to differences between metagenomes and all viromes, regardless of DNase or MDA treatment (Fig. 2c). This increased observed compositional similarity across all viromes and separation of viromes from metagenomes after removing ssDNA viral data was confirmed by PERMANOVA ($R^2 = 0.998$, $p < 0.001$), suggesting that MDA viromes may still retain useful biological signal after dominant ssDNA vOTUs are excluded.

These findings highlight the substantial taxonomic distortions introduced by MDA. Still, it is encouraging that some biases can be reduced computationally to potentially make existing datasets (or future datasets if MDA is unavoidable) more representative. While MDA bias towards small circular ssDNA viruses such as *Microviridae* is known (79), our findings provide direct quantitative evidence of how this bias impacts downstream ecological interpretation and viral community structure in the human faecal virome. Although Wang et al. (21) reported similar diversity and composition between MDA viromes and non-MDA viromes, the universal use of a 3,000 bp contig cutoff likely excluded most ssDNA viruses, masking MDA biases. Our beta-diversity comparisons (Fig. 2c) confirm that such biases can be computationally addressed but not fully corrected for. Of course, as with all analyses presented here, the emphasis on comparisons across methods from the same sample does not allow for generalizability across individuals, but we have demonstrated that MDA can result in extreme bias.

Our findings suggest that while MDA viromes may still resolve broad compositional patterns, they remain inappropriate for detailed ecological and functional analyses, as they cannot fully compensate for

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

the loss of diversity or sequencing depth, nor can the simple approach of removing ssDNA viral sequences. Thus, while MDA viromes may still be informative for detecting broad-scale differences in community structure, especially in samples with low viromic DNA yields and/or in re-analyses of datasets already amplified with MDA, our results suggest that using MDA in future viromic studies is difficult to justify. Where its use is unavoidable, computational corrections may help.

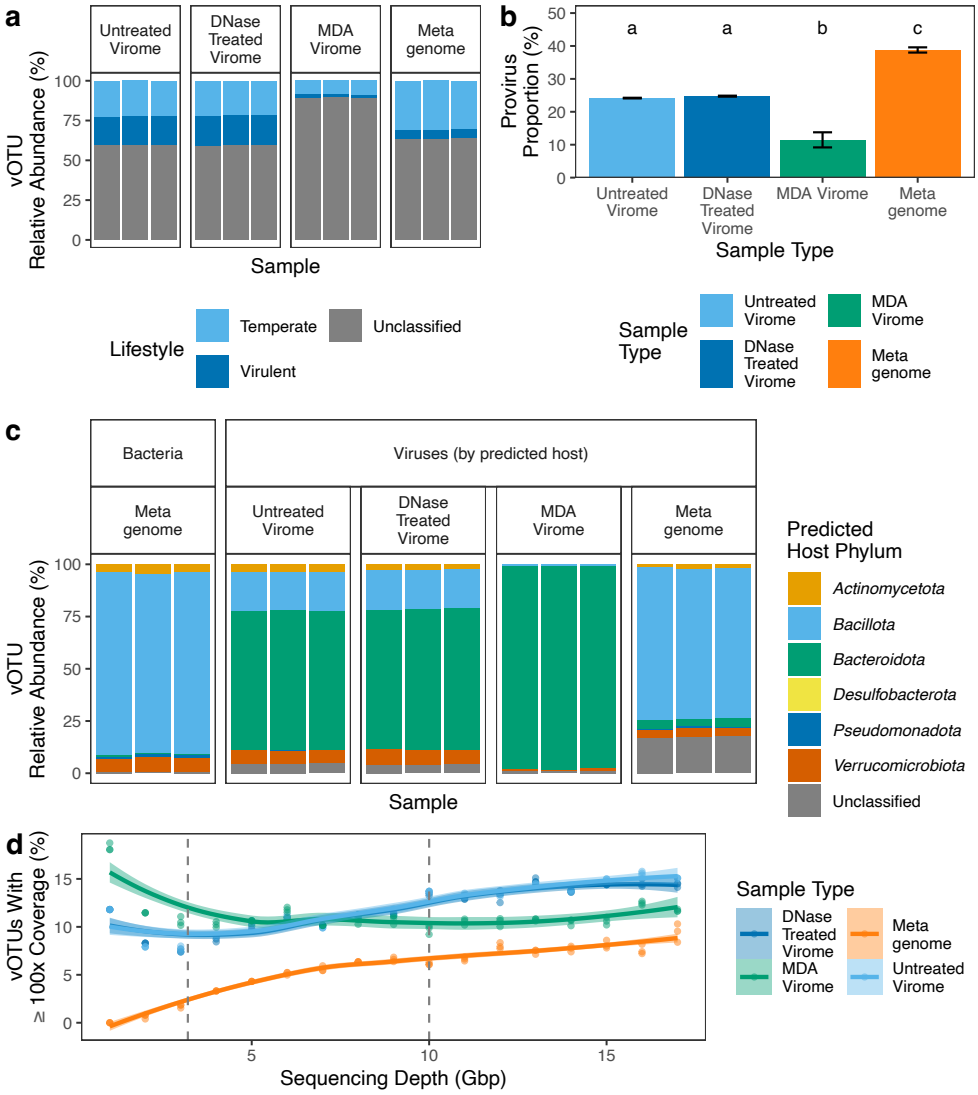
Metagenome-derived viral communities differ from viromes in predicted lifestyles, host associations, and genome annotations

It would be logical to presume that metagenomes would recover more temperate phages (viruses capable of both lytic replication and lysogenic integration into host genomes) than viromes that target free viral particles (80,81), and here we tested that assumption and explored the differences in viral communities recovered from metagenomes and viromes. We first used BACPHLIP (62) to predict the lifestyle (temperate, virulent, or unclassified) of each vOTU, which uses a random forest-based classifier trained on the presence or absence of lysogeny-associated protein domains. Consistent with previous reports of widespread lysogeny in the human gut (79), putative temperate phages were more abundant than those predicted to be virulent across all processing methods, including viromes (Fig. 3a), suggesting that many virions recovered from viromes retain the capacity for lysogenic infection. A substantially smaller proportion of the vOTUs from MDA viromes could be assigned a putative viral lifestyle, likely due to the dominance of *Microviridae*, which are expected to be challenging for viral lifestyle prediction algorithms. Specifically, while some *Microviridae* can integrate into host genomes via co-option of host cell XerC/XerD recombinases (82), they lack phage-borne integrases or transposases, which may prevent BACPHLIP and other prediction tools from predicting a viral lifestyle. To assess the prevalence of integration specifically, we next examined the percentage of temperate phages identified as integrated prophages by geNomad, which uses a conditional random field model to detect genomic regions enriched with viral markers and flanked by host chromosomal features (45). This analysis mirrored trends from the

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

BACPHLIP results, i.e., a lower proportion of prophages were detected in MDA viromes and a much higher proportion in metagenomes (Fig. 3b; ANOVA, $F_{3,8} = 251.710$, $p < 0.00001$). The detection of putative integrated prophage sequences in viromes may reflect a combination of factors, including: (1) excised host DNA packaged into virions, (2) false-positive prophage boundary predictions, (3) prophages in ultra-small bacteria that may have passed through the 0.2 μm filter, and (4) the presence of residual host DNA/cellular contamination in VLP-concentrates (though we note that, for substantial contributions from free DNA, we would expect a difference between DNase-treated and untreated viromes, which was not observed). Overall, results suggest a greater proportion of integrated prophages recovered from the metagenomes compared to all viromes.

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.



399

400 *Figure 3. Method-dependent differences in viral lifestyle, host associations and sequencing coverage*
401 *depth. (a) Relative abundance of predicted viral lifestyles (temperate, virulent, or unclassified) across*
402 *methods, based on classification by BACPHLIP (62). (b) Percentage of temperate vOTUs predicted to be*
403 *prophages by geNomad (45). Bars show mean ± standard deviation, letters indicate significant*
404 *differences between groups (Tukey HSD, $p_{adjusted}<0.05$). (c) vOTU relative abundances by predicted host*
405 *phyla, according to processing method (left) and metagenome-derived prokaryotic community*
406 *composition (far right).*

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

We next explored whether predicted prokaryotic host taxa were similarly distributed in the viral communities recovered from each processing method and how these predicted host distributions compared to the prokaryotic community compositions recovered from the metagenomes. Hosts for each vOTU were predicted using iPHoP, and bacterial community profiles were characterised by SingleM (38,63) (Fig. 3c). Bacterial communities were dominated by *Bacillota* (formerly *Firmicutes* (83)), a pattern reflected in the predicted host associations of vOTUs identified in the metagenomes but not the viromes. This is consistent with the observed greater proportion of predicted prophages in the metagenomes compared to viromes, as viral and host abundance should be more highly correlated in the metagenomes, where they are more often coming from the same chromosome. Although viromes and metagenomes generally yielded comparable numbers of vOTUs predicted to infect each bacterial phylum, the relative abundance distributions of these vOTUs differed substantially across processing methods. For example, phages predicted to infect the phylum *Bacillota* comprised 72% of the viral community by relative abundance in metagenomes but only 19% in DNase-treated viromes, despite comparable proportions by vOTU counts (60% and 51%, respectively). In contrast, viruses predicted to infect the phylum *Bacteroidota* dominated all viromes (67% relative abundance of non-MDA viromes, 97% of MDA viromes) but were rare in metagenome viral communities (4%) and virtually absent in the prokaryotic communities (0.7%). Together, these results suggest that different processing methods could lead to wildly different interpretations of infection dynamics, since viral relative abundances differed dramatically between viromes and metagenomes when grouped according to predicted hosts.

We compared profiles of functionally annotated viral genes, including putative auxiliary metabolic genes (AMGs) (1), across processing methods. To more fully capture viral gene content, analyses were conducted at the assembled contig (as opposed to vOTU) level, after meeting viral prediction thresholds (see Methods). Using Pharokka (61), we found that 0.9-1.3% of genes across all viral contigs were annotated as moron elements (transferable elements within phage genomes (84)), accessory metabolic genes, or were involved in host takeover, with no significant differences across viromic methods (Tukey

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

HSD, $p > 0.05$). However, this category of gene was ~33% more frequently detected in metagenome-derived viral contigs compared to those from DNase-treated viromes (Supplementary Fig. S6a), suggesting greater prevalence in host-associated viral genomes (e.g., in integrated or actively replicating viruses), as previously suggested (1), and/or more false-positive viral predictions or errors predicting prophage boundaries in metagenomes. Accessory genes were rare overall, with significant differences only between viromes and metagenomes. This overrepresentation in metagenome-derived viral contigs may reflect false-positive detection of host-derived sequences rather than true viral gene content, and underscores the need for rigorous quality control when analysing putative AMGs (85,86).

Methodological Impact on Viral Prediction Confidence and Viral Recovery at Increasing Sequencing Depths

To assess whether vOTU quality might contribute to observed differences between viromes and metagenomes, we next examined viral prediction confidence scores across processing methods. geNomad confidence scores differed significantly (Fig. 4a, ANOVA, $F_{3,8} = 47.5$, $p = 1.92 \times 10^{-5}$), but the mean shift between processing methods was only a modest shift of 0.8–1.7%. Cumulative frequency diagrams (Fig. 4a) show that metagenomes contain a greater proportion of lower-confidence viral contigs, underscoring the need for additional caution when analysing metagenome-derived viral sequences, as a small shift in confidence score distribution can result in the inclusion of tens to hundreds of lower-quality viral sequences. Conversely, 2,261 contigs were excluded through length-filtering but had viral confidence scores >0.95. These shorter, high-confidence contigs may still be useful in specific contexts, but this choice must be clearly justified to avoid inclusion of false positives.

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

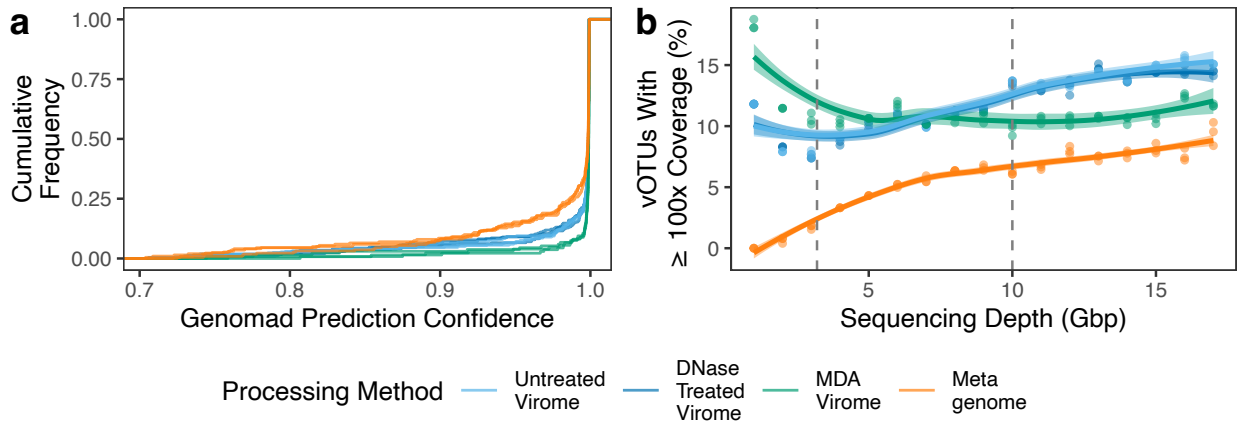


Figure 4. Impact of processing method on viral prediction and high-coverage viral contigs. (a) Cumulative frequency diagram of geNomad prediction confidence scores of viral contigs filtered for length ≥10 Kbp or between 1 and 10 Kbp and geNomad assigned phylum = Monodnaviria. (b) Number of vOTUs with ≥100 × coverage across subsampled sequencing depths (Gbp) for each method. Lines and shaded areas indicate fitted LOESS curves with 95% confidence intervals. Dashed vertical lines indicate commonly used viral dataset sequencing targets, 3.2 Gbp (56) and 10 Gbp (69,87–89)

One presumed advantage of viromes over metagenomes is improved recovery of strain-level microdiversity, which requires high coverage depth (90,91). To compare coverage depth for vOTUs across processing methods and sequencing effort, we subsampled the raw reads from each library in 1 Gbp increments between 1 and 17 Gbp and reprocessed each subset independently. Metagenomes consistently had the lowest proportion of vOTUs with ≥100 × average coverage (Fig. 4b), indicating, as expected, that viromic methods offer better access to sufficient coverage depth for strain-level analyses. At depths <7 Gbp, MDA viromes outperformed non-MDA viromes in recovering high-coverage contigs, reflecting lower viral diversity in MDA viromes and leading to higher coverage. However, this trend was reversed at higher sequencing efforts, with non-MDA viromes consistently capturing vOTUs with higher coverage. In non-MDA viromes, high-coverage vOTUs plateaued at ≤14% of all vOTUs after 12.5 Gbp, suggesting that per-sample strain-level analyses would likely only be possible for high-abundance

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

vOTUs. Our results further confirm that non-MDA viromes outperform both metagenomes and MDA viromes in generating high-coverage vOTUs suitable for detailed strain-level analyses.

The majority of recovered vOTUs were absent from existing human gut virome databases

The extent to which existing reference databases capture gut virome diversity is unknown, limiting our understanding of the human gut virosphere. To assess the novelty of viruses recovered by this study according to processing method, we clustered all 2,480 recovered viral contigs with the Unified Human Gut Virome catalogue (UHGV, https://github.com/snayfach/UHGV - accessed June 12th 2025), a non-redundant set of 168,536 vOTUs from 12 large-scale datasets (31,49,51–60). Species-level clustering yielded 603 vOTU clusters containing at least one viral contig from this study, compared to 605 vOTUs formed from our study alone. Only 35% (214 of 603) of clusters included UHGV sequences, meaning 65% of our recovered vOTUs were not present in the reference database (Supplementary Fig. S7). Read-mapping to the UHGV database identified a further 248 UHGV vOTUs that were not assembled *de novo* here (Supplementary Fig. S7c), with non-MDA viromes detecting 17% more than metagenomes and 130% more than MDA viromes (Supplementary Fig. S7d). These mapped-only vOTUs had similar coverage (Supplementary Fig. S7e) but were, on average, 50% shorter in length than clustered vOTUs (Supplementary Fig. S7f). These vOTUs may represent viral genomes with intrinsic features, such as high microdiversity or repetitive elements, that hinder complete assembly. Consistent with previous studies (2), our results indicate that a substantial fraction of human gut viral diversity remains uncharacterised and that non-MDA viromes best recover low-abundance viruses, both novel and reference-mapped but unassembled vOTUs.

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

Conclusions and outlook

We demonstrate how methodological choices in faecal DNA preparation can shape the composition of the recovered gut viral community and downstream interpretations. MDA introduced a striking bias toward *Microviridae*, consistent with prior studies of ssDNA overamplification (23–25,92–95). Although computationally filtering out short ssDNA contigs can reduce this distortion, MDA remains unsuitable for more detailed ecological comparisons. Viromes had higher viral richness, fewer putative temperate phages (viruses capable of switching between lytic replication and lysogeny), and fewer integrated prophages than metagenomes, emphasising the need to align methodological choices with study objectives.

We also showed that differences in viral community composition due to preparation methods can propagate through downstream analyses beyond community diversity metrics, with the potential for substantially influencing ecological and functional conclusions. We applied a suite of commonly used analytical approaches to examine how methodological choice affected interpretation, resulting in an analytical framework that could be useful for future methodological comparisons. For example, MDA viromes yielding fewer lifestyle predictions due to *Microviridae* dominance, and metagenomes showed substantially different predicted host compositions of the viral community at the phylum level compared to both non-MDA and MDA viromes. Had each method been used in separate studies, these differences could have led to conflicting interpretations, as they produce methodological artefacts that can be difficult to recognise in the literature without expert technical evaluation of study methodologies (19,96,97). Despite the study's limited scope, the consistency across replicates and the magnitude of the observed effects emphasise the importance of understanding the sources of methodological bias and potential mitigation methods. The more refined methods described herein will have significant value when applied to larger-scale human studies that explore interindividual variability of the gut virome and associations of the virome with health phenotypes.

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

Our findings complement and extend prior gut virome methods comparisons, which have often focused on individual steps, such as viral enrichment, DNA extraction, and sequencing library construction (15,19,21,30,98,99). No single method can capture all aspects of gut viral diversity, but by understanding the specific biases and strengths of each technique, researchers can make more informed methodological choices. As larger-scale, more integrative and multi-omic studies become more prevalent, careful method selection will become increasingly essential for generating robust insights into the role of the gut virome in human health and disease.

Acknowledgments

Elements of the graphical abstract and Supplementary Fig. S1 were produced in BioRender. The sequencing was carried by the DNA Technologies and Expression Analysis Core at the UC Davis Genome Center, supported by NIH Shared Instrumentation Grant 1S10OD010786-01. The authors also thank other members of the Emerson Lab for helpful discussions and general feedback.

Authors' contributions

LSH: conceptualisation, data curation, formal analysis, writing – original draft preparation, visualization

TAK: resources, project administration, writing – review and editing

SHA: resources, project administration, writing – review and editing

MRA: resources, project administration, writing – review and editing

MO: conceptualisation, formal analysis, visualization, writing – review and editing

JBE: conceptualisation, wet-lab methodology, investigation, writing – review and editing, supervision, funding acquisition, project administration

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

Funding

This research was supported by the NIH Common Fund (Human Virome Program), Award # U01DE034198. LSH was also partially supported by the U.S. Department of Energy (DOE), Office of Science, Office of Biological and Environmental Research (BER), Genomic Science Program, award number DE-SC0023127 (grant to JBE, grant PI Sydney Glassman). Research on the human microbiome by Drs. Adams, Knotts, and Ali is funded, in part, by NIH-NIDDK 1R01DK137173-01A1.

Data and Code Availability

All raw sequencing data have been deposited with the SRA under BioProject PRJNA1331857 (SRR35523994 - SRR35524005)**.** As sequencing data deposition requires the removal of human metagenomic reads, human reads were scrubbed from the raw read files with the NCBI's Human Read Removal Tool (https://hub.docker.com/r/ncbi/sra-human-scrubber) prior to submission. Human read count tables are archived in the GitHub and Zenodo repositories. All filtered viral sequences were deposited in GenBank and in the GitHub/Zenodo repositories for the manuscript. All R scripts involved in the processing and analysis of these data, plus summary tables used as input, have been deposited on GitHub (https:// https://github.com/LSHillary/FecalViromeOptimisation) and archived on Zenodo (DOI: 10.5281/zenodo.17527407).

Competing interests

S.H. Adams is founder and principal of XenoMed, LLC (dba XenoMet), which is focused on research and discovery in microbial metabolism. XenoMed had no part in the research design, funding, results or writing of the manuscript.

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

References

1. Luo XQ, Wang P, Li JL, Ahmad M, Duan L, Yin LZ, et al. Viral community-wide auxiliary metabolic genes differ by lifestyles, habitats, and hosts. Microbiome. 2022 Nov 5;10(1):190.

2. Shkoporov AN, Clooney AG, Sutton TDS, Ryan FJ, Daly KM, Nolan JA, et al. The Human Gut Virome Is Highly Diverse, Stable, and Individual Specific. Cell Host Microbe. 2019 Oct 9;26(4):527-541.e5.

3. Cao Z, Sugimura N, Burgermeister E, Ebert MP, Zuo T, Lan P. The gut virome: A new microbiome component in health and disease. eBioMedicine. 2022 July 1;81:104113.

4. Liang G, Cobián-Güemes AG, Albenberg L, Bushman F. The gut virome in inflammatory bowel diseases. Curr Opin Virol. 2021 Dec 1;51:190–8.

5. Ungaro F, Massimino L, Furfaro F, Rimoldi V, Peyrin-Biroulet L, D'Alessio S, et al. Metagenomic analysis of intestinal mucosa revealed a specific eukaryotic gut virome signature in early-diagnosed inflammatory bowel disease. Gut Microbes. 2019 Mar 4;10(2):149–58.

6. Zhang P, Shi H, Guo R, Li L, Guo X, Yang H, et al. Metagenomic analysis reveals altered gut virome and diagnostic potential in pancreatic cancer. J Med Virol. 2024 July 1;96(7):e29809.

7. Federici S, Kredo-Russo S, Valdés-Mas R, Kviatcovsky D, Weinstock E, Matiuhin Y, et al. Targeted suppression of human IBD-associated gut microbiota commensals by phage consortia for treatment of intestinal inflammation. Cell. 2022 Aug 4;185(16):2879-2898.e24.

8. Mao X, Larsen SB, Zachariassen LSF, Brunse A, Adamberg S, Mejia JLC, et al. Transfer of modified gut viromes improves symptoms associated with metabolic syndrome in obese male mice. Nat Commun. 2024 June 3;15(1):4704.

9. Breitbart Mya, Hewson Ian, Felts Ben, Mahaffy Joseph M., Nulton James, Salamon Peter, et al. Metagenomic Analyses of an Uncultured Viral Community from Human Feces. J Bacteriol. 2003 Oct 15;185(20):6220–3.

10. Li J, Yang F, Xiao M, Li A. Advances and challenges in cataloging the human gut virome. Cell Host Microbe. 2022 July 13;30(7):908–16.

11. Stockdale SR, Hill C. Progress and prospects of the healthy human gut virome. Curr Opin Virol. 2021 Dec 1;51:164–71.

12. Halary S, Temmam S, Raoult D, Desnues C. Viral metagenomics: are we missing the giants? Environ Microbiol Spec Sect Megaviromes. 2016 June 1;31:34–43.

13. Santos-Medellin C, Zinke LA, ter Horst AM, Gelardi DL, Parikh SJ, Emerson JB. Viromes outperform total metagenomes in revealing the spatiotemporal patterns of agricultural soil viral communities. ISME J. 2021;15:1956--1970.

14. Trubl G, Hyman P, Roux S, Abedon ST. Coming-of-age characterization of soil viruses: A user's guide to virus isolation, detection within metagenomes, and viromics. Soil Syst. 2020;4(2):1–34.

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

15. Kosmopoulos JC, Klier KM, Langwig MV, Tran PQ, Anantharaman K. Viromes vs. mixed community metagenomes: choice of method dictates interpretation of viral community ecology. Microbiome. 2024 Oct 7;12(1):195.

16. Shkoporov AN, Ryan FJ, Draper LA, Forde A, Stockdale SR, Daly KM, et al. Reproducible protocols for metagenomic analysis of human faecal phageomes. Microbiome. 2018 Apr 10;6(1):68.

17. d'Humières C, Touchon M, Dion S, Cury J, Ghozlane A, Garcia-Garcera M, et al. A simple, reproducible and cost-effective procedure to analyse gut phageome: from phage isolation to bioinformatic approach. Sci Rep. 2019 Aug 5;9(1):11331.

18. Kleiner M, Hooper LV, Duerkop BA. Evaluation of methods to purify virus-like particles for metagenomic sequencing of intestinal viromes. BMC Genomics. 2015 Jan 22;16(1):7.

19. Callanan J, Stockdale SR, Shkoporov A, Draper LA, Ross RP, Hill C. Biases in Viral Metagenomics-Based Detection, Cataloguing and Quantification of Bacteriophage Genomes in Human Faeces, a Review. Microorganisms. 2021;9(3).

20. Hsieh SY, Tariq MA, Telatin A, Ansorge R, Adriaenssens EM, Savva GM, et al. Comparison of PCR versus PCR-Free DNA Library Preparation for Characterising the Human Faecal Virome. Viruses. 2021;13(10).

21. Wang G, Li S, Yan Q, Guo R, Zhang Y, Chen F, et al. Optimization and evaluation of viral metagenomic amplification and sequencing procedures toward a genome-level resolution of the human fecal DNA virome. J Adv Res. 2023 June 1;48:75–86.

22. Zhai X, Gobbi A, Kot W, Krych L, Nielsen DS, Deng L. A single-stranded based library preparation method for virome characterization. Microbiome. 2024 Oct 24;12(1):219.

23. Binga EK, Lasken RS, Neufeld JD. Something from (almost) nothing: the impact of multiple displacement amplification on microbial ecology. ISME J. 2008 Mar 1;2(3):233–41.

24. Kim KH, Bae JW. Amplification methods bias metagenomic libraries of uncultured single-stranded and double-stranded DNA viruses. Appl Environ Microbiol. 2011;77(21):7663--7668.

25. Parras-Molto M, Rodriguez-Galet A, Suarez-Rodriguez P, Lopez-Bueno A. Evaluation of bias induced by viral enrichment and random amplification protocols in metagenomic surveys of saliva DNA viruses. Microbiome. 2018;6(1):119.

26. Emerson JB, Thomas BC, Andrade K, Allen EE, Heidelberg KB, Banfield JF. Dynamic Viral Populations in Hypersaline Systems as Revealed by Metagenomic Assembly. Appl Environ Microbiol. 2012 Sept;78(17):6309–20.

27. Geonczy SE, Hillary LS, Santos-Medellín C, Fudyma JD, Sorensen JW, Emerson JB. Virome responses to heating of a forest soil suggest that most dsDNA viral particles do not persist at 90°C. Soil Biol Biochem. 2025 Mar 1;202:109651.

28. Sorensen JW, Zinke LA, ter Horst AM, Santos-Medellin C, Schroeder A, Emerson JB. DNase Treatment Improves Viral Enrichment in Agricultural Soil Viromes. mSystems. 2021;6(5):e00614-21.

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

29. Guan, Huihui, Pu, Yanni, Liu, Chenglin, Lou, Tao, Tan, Shishang, Kong, Mengmeng, et al. Comparison of Fecal Collection Methods on Variation in Gut Metagenomics and Untargeted Metabolomics. mSphere. 2021 Sept 15;6(5):10.1128/msphere.00636-21.

30. Conceição-Neto N, Zeller M, Lefrère H, De Bruyn P, Beller L, Deboutte W, et al. Modular approach to customise sample preparation procedures for viral metagenomics: a reproducible protocol for virome analysis. Sci Rep. 2015 Nov 12;5(1):16532.

31. Gregory AC, Zablocki O, Zayed AA, Howell A, Bolduc B, Sullivan MB. The Gut Virome Database Reveals Age-Dependent Patterns of Virome Diversity in the Human Gut. Cell Host Microbe. 2020 Nov 11;28(5):724-740.e8.

32. Pavlopoulos GA, Baltoumas FA, Liu S, Selvitopi O, Camargo AP, Nayfach S, et al. Unraveling the functional dark matter through global metagenomics. Nature. 2023 Oct 1;622(7983):594–602.

33. Zhang L, Chen F, Zeng Z, Xu M, Sun F, Yang L, et al. Advances in Metagenomics and Its Application in Environmental Microorganisms. Front Microbiol. 2021;12:766364.

34. World Medical Association. World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Participants. JAMA. 2025 Jan 7;333(1):71–4.

35. Santos-Medellín C, Blazewicz SJ, Pett-Ridge J, Firestone MK, Emerson JB. Viral but not bacterial community successional patterns reflect extreme turnover shortly after rewetting dry soils. Nat Ecol Evol. 2023 Nov;7(11):1809–22.

36. Crusoe MR, Alameldin HF, Awad S, Boucher E, Caldwell A, Cartwright R, et al. The khmer software package: enabling efficient nucleotide sequence analysis [Internet]. F1000Research; 2015 [cited 2024 Feb 23]. Available from: https://f1000research.com/articles/4-900

37. Kopylova E, Noe L, Touzet H. SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. Bioinformatics. 2012;28(24):3211--3217.

38. Woodcroft BJ, Aroney STN, Zhao R, Cunningham M, Mitchell JAM, Nurdiansyah R, et al. Comprehensive taxonomic identification of microbial species in metagenomic data using SingleM and Sandpiper. Nat Biotechnol [Internet]. 2025 July 16; Available from: https://doi.org/10.1038/s41587-025-02738-1

39. Nurk S, Koren S, Rhie A, Rautiainen M, Bzikadze AV, Mikheenko A, et al. The complete sequence of a human genome. Science. 2022 Apr;376(6588):44–53.

40. Li H. Minimap2: pairwise alignment for nucleotide sequences. Bioinformatics. 2018 Sept 15;34(18):3094–100.

41. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009 Aug 15;25(16):2078–9.

42. Aroney STN, Newell RJP, Nissen JN, Camargo AP, Tyson GW, Woodcroft BJ. CoverM: read alignment statistics for metagenomics. Bioinformatics. 2025 Apr 1;41(4):btaf147.

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

43. Li D, Liu CM, Luo R, Sadakane K, Lam TW. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics. 2015;31(10):1674--1676.

44. Mikheenko A, Prjibelski A, Saveliev V, Antipov D, Gurevich A. Versatile genome assembly evaluation with QUAST-LG. Bioinformatics. 2018 July 1;34(13):i142–50.

45. Camargo AP, Roux S, Schulz F, Babinski M, Xu Y, Hu B, et al. Identification of mobile genetic elements with geNomad. Nat Biotechnol. 2024 Aug;42:1303–12.

46. Roux S, Adriaenssens EM, Dutilh BE, Koonin EV, Kropinski AM, Krupovic M, et al. Minimum Information about an Uncultivated Virus Genome (MIUViG). Nat Biotechnol. 2018;37(1):29–37.

47. Krupovic M. Networks of evolutionary interactions underlying the polyphyletic origin of ssDNA viruses. Curr Opin Virol. 2013 Oct 1;3(5):578–86.

48. Morgulis A, Coulouris G, Raytselis Y, Madden TL, Agarwala R, Schäffer AA. Database indexing for production MegaBLAST searches. Bioinformatics. 2008 Aug 15;24(16):1757–64.

49. Camargo AP, Nayfach S, Chen IMA, Palaniappan K, Ratner A, Chu K, et al. IMG/VR v4: an expanded database of uncultivated virus genomes within a framework of extensive functional, taxonomic, and ecological metadata. Nucleic Acids Res. 2023 Jan 6;51(D1):D733–43.

50. Nayfach S, Camargo AP, Schulz F, Eloe-Fadrosh E, Roux S, Kyrpides NC. CheckV assesses the quality and completeness of metagenome-assembled viral genomes. Nat Biotechnol. 2020;39(5):578--585.

51. Benler S, Yutin N, Antipov D, Rayko M, Shmakov S, Gussow AB, et al. Thousands of previously unknown phages discovered in whole-community human gut metagenomes. Microbiome. 2021 Mar 29;9(1):1–17.

52. Camarillo-Guerrero LF, Almeida A, Rangel-Pineros G, Finn RD, Lawley TD. Massive expansion of human gut bacteriophage diversity. Cell. 2021 Feb 18;184(4):1098-1109.e9.

53. Garmaeva S, Gulyaeva A, Sinha T, Shkoporov AN, Clooney AG, Stockdale SR, et al. Stability of the human gut virome and effect of gluten-free diet. 2021 May 18;34:109132.

54. Lai S, Jia L, Subramanian B, Pan S, Zhang J, Dong Y, et al. mMGE: a database for human metagenomic extrachromosomal mobile genetic elements. Nucleic Acids Res. 2021 Jan 8;49(D1):D783–91.

55. Carter MM, Olm MR, Merrill BD, Dahan D, Tripathi S, Spencer SP, et al. Ultra-deep sequencing of Hadza hunter-gatherers recovers vanishing gut microbes. Cell. 2023 July 6;186(14):3111-3124.e13.

56. Nayfach S, Páez-Espino D, Call L, Low SJ, Sberro H, Ivanova NN, et al. Metagenomic compendium of 189,680 DNA viruses from the human gut microbiome. Nat Microbiol. 2021 July 1;6(7):960–70.

57. Shah SA, Deng L, Thorsen J, Pedersen AG, Dion MB, Castro-Mejía JL, et al. Expanding known viral diversity in the healthy infant gut. Nat Microbiol. 2023 May 1;8(5):986–98.

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

58. Soto-Perez P, Bisanz JE, Berry JD, Lam KN, Bondy-Denomy J, Turnbaugh PJ. CRISPR-Cas System of a Prevalent Human Gut Bacterium Reveals Hyper-targeting against Phages in a Human Virome Catalog. 2019 Sept 11;26:325–35.

59. Tisza MJ, Buck CB. A catalog of tens of thousands of viruses from human metagenomes reveals hidden associations with chronic diseases. Proc Natl Acad Sci. 2021 June 8;118(23):e2023202118.

60. Van Espen L, Bak EG, Beller L, Close L, Deboutte W, Juel HB, et al. A Previously Undescribed Highly Prevalent Phage Identified in a Danish Enteric Virome Catalog. mSystems. 2021 Oct 19;6(5):10.1128/msystems.00382-21.

61. Bouras G, Nepal R, Houtak G, Psaltis AJ, Wormald PJ, Vreugde S. Pharokka: a fast scalable bacteriophage annotation tool. Bioinformatics. 2023 Jan 1;39(1):btac776.

62. Hockenberry AJ, Wilke CO. BACPHLIP: predicting bacteriophage lifestyle from conserved protein domains. Breitbart M, editor. PeerJ. 2021 May 6;9:e11396.

63. Roux S, Camargo AP, Coutinho FH, Dabdoub SM, Dutilh BE, Nayfach S, et al. iPHoP: An integrated machine learning framework to maximize host prediction for metagenome-derived viruses of archaea and bacteria. PLOS Biol. 2023 Apr 21;21(4):e3002083.

64. Kassambara A. ggpubr: 'ggplot2' Based Publication Ready Plots [Internet]. 2020. Available from: https://CRAN.R-project.org/package=ggpubr

65. Wickham H, Averick M, Bryan J, Chang W, McGowan LD, François R, et al. Welcome to the tidyverse. J Open Source Softw. 2019;4(43):1686.

66. Graves S, Piepho HP, Selzer ML. Package 'multcompView'. Vis Paired Comp. 2015;451:452.

67. Gao CH, Chen C, Akyol T, Dusa A, Yu G, Cao B, et al. ggVennDiagram: Intuitive Venn diagram software extended. iMeta. 2024;3(1):e177.

68. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, et al. vegan: Community Ecology Package. 2019; Available from: https://cran.r-project.org/package=vegan

69. Santos-Medellín C, Estera-Molina K, Yuan M, Pett-Ridge J, Firestone MK, Emerson JB. Spatial turnover of soil viral populations and genotypes overlain by cohesive responses to moisture in grasslands. Proc Natl Acad Sci. 2022 Nov 8;119(45):e2209132119.

70. Shimada O, Ishikawa H, Tosaka-Shimada H, Yasuda T, Kishi K, Suzuki S. Detection of deoxyribonuclease I along the secretory pathway in paneth cells of human small intestine. J Histochem Cytochem. 1998;46(7):833–40.

71. Yan A, Butcher J, Mack D, Stintzi A. Virome Sequencing of the Human Intestinal Mucosal–Luminal Interface. Front Cell Infect Microbiol. 2020 Oct 22;10:582187.

72. Zeng S, Almeida A, Li S, Ying J, Wang H, Qu Y, et al. A metagenomic catalog of the early-life human gut virome. Nat Commun. 2024 Feb 29;15(1):1864.

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

73. Smith L, Goldobina E, Govi B, Shkoporov AN. Bacteriophages of the Order Crassvirales: What Do We Currently Know about This Keystone Component of the Human Gut Virome? Biomolecules. 2023 Mar 24;13(4):584.

74. Guerin E, Shkoporov A, Stockdale SR, Clooney AG, Ryan FJ, Sutton TDS, et al. Biology and Taxonomy of crAss-like Bacteriophages, the Most Abundant Virus in the Human Gut. Cell Host Microbe. 2018 Nov 14;24(5):653-664.e6.

75. Yutin N, Benler S, Shmakov SA, Wolf YI, Tolstoy I, Rayko M, et al. Analysis of metagenome-assembled viral genomes from the human gut reveals diverse putative CrAss-like phages with unique genomic features. Nat Commun. 2021 Feb 16;12(1):1044.

76. Bokulich NA. Integrating sequence composition information into microbial diversity analyses with k-mer frequency counting. mSystems. 2025 Feb 20;10(3):e01550-24.

77. Dubinkina VB, Ischenko DS, Ulyantsev VI, Tyakht AV, Alexeev DG. Assessment of k-mer spectrum applicability for metagenomic dissimilarity analysis. BMC Bioinformatics. 2016 Jan 16;17(1):1–11.

78. Jenike KM, Campos-Domínguez L, Boddé M, Cerca J, Hodson CN, Schatz MC, et al. k-mer approaches for biodiversity genomics. Genome Res. 2025 Jan 2;35(2):219–30.

79. Kim MS, Bae JW. Lysogeny is prevalent and widely distributed in the murine gut microbiota. ISME J. 2018 Apr 1;12(4):1127–41.

80. Sbardellati DL, Vannette RL. Targeted viromes and total metagenomes capture distinct components of bee gut phage communities. Microbiome. 2024 Aug 23;12(1):155.

81. Sommers P, Chatterjee A, Varsani A, Trubl G. Integrating Viral Metagenomics into an Ecological Framework. Annu Rev Virol. 2021;8(1):133--158.

82. Krupovic M, Forterre P. Single-stranded DNA viruses employ a variety of mechanisms for integration into host genomes. Ann N Y Acad Sci. 2015;1341(1):41–53.

83. Oren A, Arahal DR, Göker M, Moore ERB, Rossello-Mora R, Sutcliffe IC. International Code of Nomenclature of Prokaryotes. Prokaryotic Code (2022 Revision). Int J Syst Evol Microbiol. 2023;73(5a):005585.

84. Cumby N, Davidson AR, Maxwell KL. The moron comes of age. Bacteriophage. 2012 Oct 1;2(4):e23146.

85. Martin C, Emerson JB, Roux S, Anantharaman K. A call for caution in the biological interpretation of viral auxiliary metabolic genes. Nat Microbiol. 2025 Aug 27;1–8.

86. Pratama AA, Bolduc B, Zayed AA, Zhong ZP, Guo J, Vik DR, et al. Expanding standards in viromics: in silico evaluation of dsDNA viral genome identification, classification, and auxiliary metabolic gene curation. PeerJ. 2021;9:e11447.

87. Fudyma JD, ter Horst AM, Santos-Medellín C, Sorensen JW, Gogul GG, Hillary LS, et al. Exploring viral particle, soil, and extraction buffer physicochemical characteristics and their impacts on extractable viral communities. Soil Biol Biochem. 2024;194:109419.

bioRxiv preprint doi: https://doi.org/10.1101/2025.11.25.690293; this version posted November 26, 2025. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

88. Geonczy SE, Hillary LS, Santos-Medellín, Christian, Sorensen JW, Emerson JB. Patchy burn severity explains heterogeneous soil viral and prokaryotic responses to fire in a mixed conifer forest. mSystems. 2025 May 14;10(6):e01749-24.

89. ter Horst AM, Santos-Medellín C, Sorensen JW, Zinke LA, Wilson RM, Johnston ER, et al. Minnesota peat viromes reveal terrestrial and aquatic niche partitioning for local and global viral populations. Microbiome. 2021 Nov 26;9(1):233.

90. Andrade-Martínez JS, Camelo Valera LC, Chica Cárdenas LA, Forero-Junco L, López-Leal G, Moreno-Gallego JL, et al. Computational Tools for the Analysis of Uncultivated Phage Genomes. Microbiol Mol Biol Rev. 2022 Mar 21;86(2):e00004-21.

91. Olm MR, Crits-Christoph A, Bouma-Gregson K, Firek BA, Morowitz MJ, Banfield JF. inStrain profiles population microdiversity from metagenomic data and sensitively detects shared microbial strains. Nat Biotechnol. 2021 June;39(6):727–36.

92. Marine R, McCarren C, Vorrasane V, Nasko D, Crowgey E, Polson SW, et al. Caught in the middle with multiple displacement amplification: the myth of pooling for avoiding multiple displacement amplification bias in a metagenome. Microbiome. 2014 Jan 30;2(1):3.

93. Ospino MC, Engel K, Ruiz-Navas S, Binns WJ, Doxey AC, Neufeld JD. Evaluation of multiple displacement amplification for metagenomic analysis of low biomass samples. ISME Commun. 2024 Feb 12;4(1):ycae024.

94. Roux S, Solonenko NE, Dang VT, Poulos BT, Schwenck SM, Goldsmith DB, et al. Towards quantitative viromics for both double-stranded and single-stranded DNA viruses. PeerJ. 2016;4:e2777.

95. Yilmaz S, Allgaier M, Hugenholtz P. Multiple displacement amplification compromises quantitative analysis of metagenomes. Nat Methods. 2010 Dec;7(12):943–4.

96. Jansen D, Matthijnssens J. The Emerging Role of the Gut Virome in Health and Inflammatory Bowel Disease: Challenges, Covariates and a Viral Imbalance. Viruses. 2023 Jan 6;15(1):173.

97. Chang WS, Harvey E, Mahar JE, Firth C, Shi M, Simon-Loriere E, et al. Improving the reporting of metagenomic virome-scale data. Commun Biol. 2024 Dec 20;7(1):1687.

98. Castro-Mejía JL, Muhammed MK, Kot W, Neve H, Franz CMAP, Hansen LH, et al. Optimizing protocols for extraction of bacteriophages prior to metagenomic analyses of phage communities in the human gut. Microbiome. 2015 Nov 17;3(1):1–14.

99. Haagmans R, Charity OJ, Baker D, Telatin A, Savva GM, Adriaenssens EM, et al. Assessing Bias and Reproducibility of Viral Metagenomics Methods for the Combined Detection of Faecal RNA and DNA Viruses. Viruses. 2025 Jan 23;17(2):155.