Overcoming donor variability and risks associated with fecal microbiota transplants through bacteriophage-mediated treatments

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Summary: Fecal viromes depleted of enveloped viruses efficiently treat *Clostridioides difficile*-associated diarrhea in a murine model.

ABSTRACT

Fecal microbiota transplantation (FMT) and fecal virome transplantation (FVT, sterile filtrated donor feces) have proven effective in treating recurrent *Clostridioides difficile* infection (rCDI). However, variability in donor materials and costly screening, coupled with concerns (motivated by case reports) over pathogen transfer with FMT or FVT, hinders their wider clinical application in treating less acute diseases. Although FVT presents safety advantages over FMT by transferring no bacteria, the presence of eukaryotic viruses poses a risk of harmful infections. To overcome these challenges, we developed methods to broaden FVT's clinical application while maintaining efficacy and increasing safety. Namely: 1) chemostat-fermentation to reproduce the bacteriophage FVT component and remove eukaryotic viruses (FVT-ChP); 2) solvent-detergent treatment to inactivate enveloped viruses (FVT-SDT); and 3) pyronin-Y treatment to block RNA-virus replication (FVT-PyT). We evaluated the efficacy of these processed FVTs using a CDI mouse model and compared them with untreated FVT (FVT-UnT), FMT, and saline control. FVT-UnT, FVT-SDT, and FVT-ChP significantly reduced *C. difficile* colonization, limited CDI symptoms and reduced the incidence of mice reaching humane endpoints compared with the saline control. This proof-of-concept opens the possibility of developing safer and more effective phage-mediated therapies for a broad range of gut-related diseases and addresses the reproducibility issues associated with fecal donor material.

INTRODUCTION

During the past decade, it has become evident that various diseases are associated with gut microbiome (GM) dysbiosis, including recurrent *Clostridioides difficile* infection (rCDI)[1–3]. Fecal microbiota transplantation (FMT) from a healthy donor to rCDI patients has proven highly effective in curing the disease, with a success rate exceeding 90%[4]. However, FMT faces challenges such as expensive and labor-intensive donor screening[5,6], donor variability in term of treatment efficacy and reproducibility[6–8], and safety concerns since no screening methods can definitively exclude the transfer of agents that could cause adverse effects from the donor[3]. The importance of the latter was highlighted in June 2019 when two patients in the United States experienced severe bacterial infections after FMT, resulting in one fatality[9]. Subsequent safety alerts from the U.S. Food & Drug Administration have warned against potential severe adverse effects associated with the transfer of pathogenic microorganisms during FMT[10,11].

Interestingly, Ott et al.[3] and Kao et al.[12] successfully treated rCDI patients using sterile-filtered donor feces (containing viruses but no intact bacteria), a method often referred to as fecal virome transplantation (FVT). The efficacy of FVT was comparable to other clinical studies using FMT (containing bacteria, etc.), indicating that the gut virome plays a significant role in treating rCDI with FMT[3,12]. The gut virome is predominantly composed of bacteriophages (in short phages), which are host-specific bacterial viruses, but also include eukaryotic and archaeal viruses[13]. The concept of applying FVT from a healthy phenotype to a symptomatic phenotype has been further investigated in preclinical studies. For instance, Draper et al. demonstrated that FVT influenced the composition of the murine GM following initial perturbation with antibiotics[14]. Additionally, FVT treatment from lean donors alleviated symptoms of metabolic syndrome in two independent diet-induced obesity murine models[15,16], and FVT using term piglet donors prevented development of necrotizing enterocolitis in a preterm piglet model[17]. FVT has the advantage over FMT in that it transfers little to no bacteria. However, the sterile filtration process commonly used in FVT (typically using 0.45 µm filters) does not eliminate the risk of transferring eukaryotic viruses. While it is possible to screen donor feces for known pathogenic viruses, recent studies have revealed that the human gastrointestinal tract harbors hundreds of eukaryotic viruses with unknown functions[13,18,19]. Most of these viruses are likely harmless to the human host. Nevertheless, it cannot be ruled out that they may contribute to later disease development, as exemplified by the human papillomavirus, which turned out as a significant risk factor for cervical cancer years after

infection[20]. Therefore, there are good reasons to minimize the transfer of eukaryotic viruses when applying FVT to alleviate conditions associated with gut microbiome dysbiosis, particularly when treating individuals with compromised immune systems. In contrast, phages do not actively infect and replicate in eukaryotic cells and are believed to be key players in the successful treatment of gut-related diseases through FMT/FVT[3,15,17,21], and have recently been suggested to harbor immune modulating effects[22,23]. Phage therapy using defined phage cocktails has been applied for almost a century[24] and is generally considered safe since severe adverse effects are rarely reported[25–28].

FMT and FVT hold the potential to revolutionize treatments for numerous gut-related diseases, but their widespread use is unlikely due to safety concerns and donor variability. Therefore, our objective was to develop methodologies that mitigate the risk of viral infections associated with FVT by removing or inactivating the eukaryotic viral component in fecal donor material. Additionally, we aimed to address the challenge of donor variability by ensuring reproducible production of enteric donor phage communities[29]. The efficacy of these processed fecal viromes was evaluated using a murine CDI model. This proof-of-concept study represents an important first step towards developing safer and more consistent therapeutic approaches that can effectively target a wide range of gut-related diseases that are less acute than rCDI, and potentially replace FMT with phage-mediated therapies.

RESULTS

To generate fecal viromes free of eukaryotic viruses, we developed methodologies that exploit the fundamental differences in characteristics between eukaryotic viruses and phages. The majority of eukaryotic viruses are enveloped RNA viruses[30–32] and rely on eukaryotic hosts for replication. In contrast, the majority of phages are non-enveloped DNA viruses[32,33] that require bacterial hosts for replication. We employed a solvent/detergent method to inactivate enveloped viruses (FVT-SDT), utilized pyronin Y to inhibit the replication of RNA viruses (FVT-PyT), and created a chemostat-propagated virome (FVT-ChP) to dilute and remove the majority of eukaryotic viruses[29]. These differently processed fecal viromes were evaluated in a murine CDI model and compared with a saline solution (sham treatment), fecal microbiota transplantation (FMT, previously shown to be efficient in treating CDI in the used preclinical model[34,35]), and untreated donor-filtrated feces (FVT-UnT). The content of all treatments originated from the same intestinal donor material.

Effective inactivation of enveloped phages through solvent/detergent treatment and replication inhibition of RNA phages using pyronin Y

The applicability of solvent/detergent and pyronin Y treatments to inactivate eukaryotic viruses while preserving phage activity was assessed using phages with distinct characteristics. These characteristics included enveloped (phi6) versus non-enveloped (phiX174, T4, and C2) phage structures and ss/dsDNA (phiX174, T4, C2) versus ss/dsRNA (MS2, phi6) genomes (Fig. 1A & 1B). The solvent/detergent treatment completely inactivated the enveloped phage phi6, as indicated by the plaque-forming units (PFU)/mL dropping to below detection limit, while the non-enveloped phages phiX174 and T4 remained unaffected. Phage C2, on the other hand, experienced a minor decrease of 1 $\log_{10}$ in PFU/mL (Fig. 1A). Pyronin Y was used to inhibit the replication of viruses carrying RNA genomes. After exploring various combinations of pyronin Y concentrations, temperatures, and incubation times, an overnight incubation at 40°C with 100 µM pyronin Y was chosen. This treatment resulted in a 5 $\log_{10}$ reduction in PFU/mL for the ssRNA phage MS2 and complete inactivation of the dsRNA phage phi6. Phi6 demonstrated temperature sensitivity as incubation at 40°C alone was sufficient to inactivate this enveloped phage, while incubation temperature at 20°C along with 100 µM pyronin Y led to a reduction of 4 $\log_{10}$ PFU/mL of phage phi6. Unfortunately, the pyronin Y treatment at 40°C also affected the plaque-forming ability of phages C2 (dsDNA), T4 (dsDNA), and phiX174 (ssDNA), resulting in reductions of 1, 2.5, and 5 $\log_{10}$ PFU/mL, respectively (Fig. 1B).

By utilizing chemostat propagation, eukaryotic viruses were depleted from fecal viromes through dilution, leading to a reduction from an average of 20 to 1 eukaryotic virus that was taxonomically classified[29].

Sustained treatment efficacy of fecal viromes following inactivation of enveloped viruses

The treatment efficacy, specifically preventing the mice from reaching the humane endpoint, was assessed in a CDI model using C57BL/6J mice. The survival probability rate was evaluated using a Kaplan-Meier estimate (Fig. 2A) and compared to the mice treated with saline (2/7 mice survived). The analysis revealed a significantly improved survival rate (8/8 mice, $p = 0.004$) for mice treated with FVT-SDT. Additionally, FVT-UnT showed a tendency towards an improved survival rate (5/7 mice, $p = 0.067$) compared to the saline control. On the other hand, the FVT-PyT treated mice, and unexpectedly, the FMT treatment, showed no improvement in survival rate (1/8 and 3/8 mice survived, respectively). The effect of FVT-ChP on the survival rate showed

numerical, but non-significant, improvement (5/8 mice, $p = 0.235$). The pathological score of the cecum tissue supported the observed survival rate, as FVT-UnT, FVT-ChP, and FVT-SDT treated mice showed a significant decrease ($p < 0.05$) in pathological score compared to the saline control (Fig. 2B, Fig. S1). The concentrations of 10 pro- and anti-inflammatory cytokines were also measured in the cecum tissue to further validate the cecum histology score and the qualitative health evaluation (Fig. S2). The cytokine profiles generally reflected whether the mice survived (with low or no inflammatory response when measured at study termination) or were euthanized (with a high inflammatory response) due to *C. difficile* infection (Fig. 2C-L). Overall, the FVT-SDT treatment, which involved the inactivation of enveloped viruses, effectively prevented severe *C. difficile* infection, and increased the chances of survival. The average pathological score at 6.7 of the saline treatment was in line with the original published CDI mouse model by Chen et al. that reported a pathological score at 7.0 for *C. difficile* infected mice, compared to mice not infected with *C. difficile* that showed a score at 1.3[36]. This supported that the pathological scores of 2.5 - 3.0 observed in the FVT-UnT, FVT-ChP, and FVT-SDT treated mice corresponded to limited symptoms of *C. difficile* infection.

Successful treatments impede *C. difficile* colonization and subsequent disease development

C. difficile abundance in feces was quantified using qPCR (Fig. 3A-3C). No *C. difficile* was detected before inoculation with *C. difficile* (Fig. 3A). The FVT-SDT treated mice exhibited an average of 2 $\log_{10}$ lower *C. difficile* abundance (gene copies per gram feces) compared to the saline treated mice before the 2nd treatment, and the FVT-UnT and -ChP treatments resulted in a 1.5 $\log_{10}$ lower abundance. This suggested that these three treatments effectively impeded *C. difficile* colonization in the gut. In contrast, the FMT and FVT-PyT treated mice had similar *C. difficile* abundance as the saline treated group. A possible clearance of *C. difficile* colonization was observed at termination, as 7/8 FVT-SDT treated mice tested negative for *C. difficile*, while all other treatments showed persistent *C. difficile* presence (Fig. 3C).

The levels of the *C. difficile*-associated toxin A/B were measured using an ELISA-based assay, which showed similar patterns as the qPCR data (Fig. 3D-3F). The toxin A/B levels in the FVT-SDT treated mice were significantly lower than those in the saline group ($p < 0.05$), and only 2 mice in the FVT-SDT group exhibited detectable toxin A/B levels before the 2nd treatment. At termination, toxin A/B could not be detected in any of the FVT-SDT treated mice (Fig. 3F). The decrease in *C. difficile* abundance (Fig. 3A-3C) and the low levels of toxin A/B (Fig. 3D-3F) in

the FVT-SDT, -UnT, and -ChP treated mice support the phenotypic data of survival rate, histology, and cytokine profile (Fig. 2).

Fecal virome treated with solvent/detergent supports recovery of the bacterial community in a dysbiotic GM

There were no initial differences in bacterial and viral GM profiles at both baseline (before antibiotics) and before *C. difficile* infection between the mice that were later either euthanized or survived the *C. difficile* infection (Fig. S3, S4, S5, & S6). The antibiotic intake through the drinking water was similar across the cages (Table S1). The bacterial taxonomic profile of the mice that were euthanized due to reaching the humane endpoint was consistently dominated by *Escherichia/Shigella*, *Enterococcus*, *Clostridioides*, *Bacteroides*, and *Parabacteroides* (Fig. 4C, 4D, & Fig. S7), and the bacterial composition (Bray-Curtis dissimilarity) and diversity (Shannon diversity index) were significantly different ($q < 0.003$) from the mice that had survived the infection (Fig. 4A & 4B). In the FMT treated mice that survived the infection, bacterial taxa were observed that overlapped with those present in the FMT inoculum, suggesting potential colonization of these bacteria in the gut of the FMT treated mice (Fig. 4C & 4D). Two mice treated with FVT-PyT were colonized with 5-20% relative abundance of *Salmonella* spp., which may have contributed to increased disease severity for these mice. The bacterial taxonomic profile of the mice that had survived the infection was dominated by lactobacilli, *Bacteroides*, *Lachnospiraceae*, *Bifidobacterium*, *Akkermansia*, *Porphyromonadaceaea*, *Bacteroidales*, and *Turibacter*, which were also the dominant taxa in the mice at baseline (Fig. 4C, 4D, & Fig. S7A), suggesting partial restoration of the GM profile in mice that survived the *C. difficile* infection.

The FVT-SDT treatment improved the chances of restoring the GM composition after antibiotic treatment, thereby impacting the mice's ability to fight off the *C. difficile* infection. The viral composition and diversity also differed significantly ($p < 0.005$) between the euthanized and surviving mice (Fig. 5A & 5B). Euthanized mice showed a significant ($p < 0.05$) increase in the relative abundance of the viral order *Petitvirales* and phages predicted to have *Sutterella* as host. In contrast, mice that survived the infection until termination were characterized by a significant increase ($p < 0.05$) in the viral order *Caudovirales*, as well as phages predicted to target the genera *Parabacteroides*, *Bacteroides*, *Prevotella*, *Odorifactor*, and *Escherichia* (Fig. S7B & S7C). The eukaryotic viral profile was similar among the different FVT inoculates (Fig. S8) and constituted

0.5-2.5% of the total relative abundance (Fig. S9). However, it is important to note that the taxonomic resolution of eukaryotic viruses was insufficient to differentiate between treatments or outcomes in relation to the relative abundance of eukaryotic viruses (Fig. S8). It should also be emphasized that inactivation by dissolving the viral envelope or inhibiting replication does not exclude the detection of viruses through sequencing.

DISCUSSION

Here, we have developed methodologies to address both the risks associated with transferring pathogenic microorganisms and the variability of donors in FMT/FVT for gut-related diseases. The preservation of treatment efficacy of the modified FVTs against *C. difficile* infections (CDI)[3,12] was assessed using a CDI mouse model. The inactivation of enveloped viruses through solvent/detergent treatment emerged as the superior method to modify fecal viromes while preserving treatment efficacy against CDI, as all FVT-SDT treated mice survived without reaching the humane endpoint.

The systemic inflammatory response observed during CDI is driven by *C. difficile* toxins that increase gut tissue permeability[37,38], making the gut more susceptible to other microbial infections[39,40]. Therefore, transferring untreated fecal donor viromes (including intact viruses) may result in additional inflammation due to microbes translocating through the damaged intestinal tissue. Enveloped viruses, including those belonging to the *Herpesviridae* family, which includes major mammalian pathogens[41] were detected in both the FVT-UnT and -SDT inoculum (Fig. S8). Enveloped *Herpesviridae* were, however, expected to be inactivated by the solvent/detergent which could explain the mild symptoms and high survival rate associated with the FVT-SDT-treated mice compared to the untreated equivalent of FVT-UnT. Treating fecal viromes with solvent/detergent prior to transfer to patients may be particularly relevant for gut-associated diseases due to increased gut tissue permeability[39,40].

FMT is generally linked to a treatment efficacy above 90% in preclinical[34,35] and clinical CDI studies[3]. Surprisingly, the survival rate of FMT-treated mice was similar to that of the saline control group. Analysis of the inoculum by 16S rRNA gene amplicon sequencing revealed that the FMT inoculum contained approximately 20% of *Clostridium sensu stricto* spp., which has been associated with *C. difficile*-positive calves[42] and to diarrhea in pigs[43]. The relatively high abundance of *Clostridium sensu stricto* spp. in the FMT inoculum may have counteracted the curative effects typically associated with FMT[3,34,35]. While the FMT and FVT inocula originated from the same

donor material, the removal of bacteria during FVT processing may explain the higher survival rates observed with FVT-UnT, -ChP, and -SDT compared to FMT. This also suggests that even unsuitable fecal donor material for FMT could potentially be suitable for FVT. The lowest survival rate was observed with the RNA-targeting compound pyronin Y. During the initial evaluation of pyronin Y's ability to inactivate RNA phages, it became evident that DNA phages were also affected. The pyronin Y treatment may have caused a reduction in phage activity, which could have impacted the efficacy of the treatment. This finding aligns with other studies that emphasize the potential role of phages in restoring GM balance following FMT or FVT[3,14,15,17,21,44–46].

The ability of different FVT treatments to reduce the abundance of the infectious *C. difficile* strain was associated with the survival rate, histology, and cytokine profiles. Mice treated with FVT-UnT, -ChP, and -SDT showed a significant decrease in *C. difficile* abundance compared to those treated with saline, FMT, and FVT-PyT. This suggests that when fewer *C. difficile* cells are present to produce toxins associated with CDI etiology[37,38], fewer cells will reach the stationary growth phase where toxin production is highest[47]. We believe that the phages transferred along with the FVT play a role in allowing commensal bacteria associated with a healthy state to compete with the infectious *C. difficile* strain, as well as commensal bacteria that can act as opportunistic pathogens. This is supported by a significant ($p < 0.05$) increase in the relative abundance of viruses predicted to infect *Escherichia*/*Shigella*, *Parabacteroides*, and *Bacteroides*, while these same bacterial genera constituted a significantly smaller ($p < 0.05$) fraction of the bacterial relative abundance when comparing the surviving mice with the euthanized mice.

Metavirome sequencing of fecal samples only revealed ssRNA viruses (phages) belonging to the *Leviviridae* family, while the positive controls of both the dsRNA Phi6 and ssRNA MS2 phages (titer ~10^6 PFU/mL) were detected in both mock communities and spiked fecal samples (Fig. S10). This suggests that the metavirome library preparation was able to detect RNA viruses at concentrations of at least ~10^6 viral particles/mL. The limited detection of enteric RNA viruses in our study is consistent with other studies using similar techniques[48] and may be due to insufficient viral databases[49], the "too clean" nature of specific pathogen-free (SPF) mice[50,51], or the concentration of these viruses being below the detection limit (~10^6 viral particles/mL).

A major challenge of CDI is the risk of recurrent infections[52–54]. It is therefore interesting to note that 7 out of 8 FVT-SDT treated mice showed a depletion of *C. difficile* at termination, indicating

a decrease in the risk of recurrent infections when treated with a solvent/detergent modified fecal virome. Thus, the solvent/detergent treated fecal virome demonstrated superiority over other treatments against CDI in our study. Interestingly, the treatment efficacy and decrease in CDI-associated symptoms were also pronounced in mice treated with the chemostat propagated enteric virome, that in a previous study showed high reproducibility of the viral component[29]. Also Santiago-Rodriguez et al. have previously demonstrated how propagation of fecal inoculum in a chemostat-fermentation holds the potential for reproducing the enteric viral component[55].

The inherent challenges of variability and reproducibility in fecal donor material exist for both FMT and FVT[6–8]. When optimized, the chemostat fermentation-based approach has the potential for higher reproducibility, standardization, large-scale production, increased removal of eukaryotic viruses through further dilution, and relies on only a few effective donors. Therefore, it could be argued that the solvent/detergent methodology, already approved by WHO as a safe procedure for treating blood plasma[56], holds the potential to complement FMT in the treatment of CDI in the short-term perspective. In the long-term perspective, a cost-effective, standardized, and reproducible chemostat propagated enteric phageome for CDI treatment may also have tremendous potential for treating other diseases associated with gut dysbiosis.

METHODS

Study design - *Clostridioides difficile* infection model

The *C. difficile* infection model was based on Chen et al. 2008[36]. Forty-eight female C57BL/6J (JAX) mice, 8 weeks old, were obtained from Charles River Laboratories (European distributor of JAX mice) and housed at the animal facilities of the Department of Experimental Medicine (AEM, University of Copenhagen, Denmark) in Innovive disposable IVC cages. The cages provided water, food (Altromin 1324 chow diet), bedding, cardboard housing, nesting material, felt pad, and biting stem. Upon arrival, the mice were ear tagged, randomly assigned to cages with 4 mice each, and acclimatized for one week (Fig. 6). An antibiotic mixture (kanamycin 0.4 mg/mL, gentamicin 0.035 mg/mL, colistin 850 U/mL, metronidazole 0.215 mg/mL, and vancomycin 0.045 mg/mL) was prepared in the drinking water and provided to the mice through the IVCs for 3 days, and the water was replaced with clean antibiotic-free drinking water for 2 days. Subsequently, the mice received an intraperitoneal injection of clindamycin (2 mg/mL) diluted in sterile 0.9% (w/v) NaCl water (based on the average body weight of the mice, around 20 g). Twenty-four hours later, the mice were orally inoculated with 1.21 x 10^4 CFU of *C. difficile* VPI 10463 (CCUG 19126) via gavage. The mice were then divided into six different treatment groups (n = 8): saline (control), FMT, FVT-UnT (untreated FVT), FVT-ChP (chemostat propagated virome), FVT-SDT (solvent/detergent treated FVT for inactivating enveloped viruses), FVT-PyT (FVT treated with pyronin Y for inactivation of RNA viruses). The respective treatments were administered orally in two doses of 0.15 mL each, at 18 hours and 72 hours after *C. difficile* inoculation (Fig. 6). One mouse from the saline treated group was euthanized immediately after oral gavage of *C. difficile*, as the culture was accidentally administered via the trachea, reducing this group to n = 7. Unfortunately, one mouse from the FVT-UnT group was euthanized 1 week after the 2nd FVT treatment due to malocclusions that had led to malnutrition, reducing this group to n = 7. Frequent (up to every 3rd hour – day and night) blinded monitoring of the animal health status was performed after *C. difficile* infection to determine if the mice had reached the humane endpoint, leading to immediate euthanization when necessary. The monitoring was supervised by the blinded veterinarian associated with the study (co-author AVM). The following qualitative parameters were used: physical activity level (i.e., decreased spontaneous or provoked activity), consistency of feces (watery or normal), body posture (hunching or normal), and whether their fur was kept clean or not. The mice were scored on a scale of 0-2: 0 (healthy), 1 (mild symptoms), and 2 (clear

symptoms). Mice with a score of 2 that showed no improvement in the above parameters during the subsequent checkup were euthanized. Fecal pellets were sampled whenever possible at different time points until the mice were euthanized (Fig. 6). At the time of euthanization, samples of the intestinal content from the cecum and colon were taken. A portion of the cecum tissue (55) was fixed in 10% neutral-buffered formalin (Sarstedt) for histological analysis and stored at room temperature. Another part of the cecum tissue, along with the intestinal content, was preserved for cytokine analysis and stored at -80°C. All procedures involving the handling of these animals were conducted in accordance with Directive 2010/63/EU and the Danish Animal Experimentation Act, under license ID: 2021-15-0201-00836.

Preparation of the *Clostridioides difficile* inoculum

Clostridioides difficile VPI 10463 (CCUG 19126), originating from a human infection, was used as infectious agent in the mouse CDI described by Chen et al.[36]. The bacteria were cultured in brain-heart-infusion supplement (BHIS) medium[57] with 0.02% (w/v) 1,4-dithiothreitol (Fisher Scientific) and 0.05% (w/v) L-cysteine (Fisher Scientific), grown at 37°C in Hungate tubes (SciQuip), and handled anaerobically as previously described[58]. For solid media 1.5% (w/v) agar (Fischer Scientific) was added. Optical density (OD_{600nm}) of bacterial cultures was measured with a Genesys30 Visible spectrophotometer (Fischer Scientific). *C. difficile* primarily synthesize its toxins during the stationary phase[47], thus the *C. difficile* culture in its early exponential phase was used as inoculum to minimize transfer of toxins. The CDI inoculum was prepared as follows: a single colony of *C. difficile* was transferred to a Hungate tube containing 10 mL BHIS medium and incubated overnight at 37°C. Then 150 µL of the *C. difficile* overnight culture was transferred to a new Hungate tube containing 10 mL BHIS medium, incubated for 3.5 hours and its OD_{600nm} was measured. A bacterial calibration curve (OD_{600nm} vs. CFU/mL) of *C. difficile* VPI 10463 was used to dilute the culture to the desired concentration (1×10^5 CFU/mL). This constituted the *C. difficile* inoculum. The cell concentration of the CDI inoculum was evaluated with CFU counts on BHIS agar plates (Fig. S11).

Propagation of host-phage pairs

The bacteria were grown in media and at a temperature suitable for each strain (Table S2). Five phages representing different characteristics (genome, size, and structure), along with their bacterial hosts, were included to assess the influence of pyronin Y and solvent/detergent treatment on phage activity; *Lactococcus* phage c2 (host: *Lactococcus lactic* MG1363), coliphage T4 (host:

Escherichia coli Luria), coliphage phiX174 (host: *E. coli* PC 0886), coliphage MS2, (host: *E. coli* W1485), and *Pseudomonas* phage phi6 (host: *Pseudomonas* sp. HER1102). Solid media were supplemented with 1.5% (w/v) agar (Thermo Fisher Scientific) for plates and 0.7% (w/v) for soft agar. An end concentration of 10 mM $MgCl_2$ and $CaCl_2$ was supplemented to media during phage propagation. Plaque activity of phages was evaluated by spot testing where 100 µL bacterial culture was mixed with 4 mL soft agar (temperature at 49°C), poured to an agar plate, and 10 µL of a phage suspension (dilution series) was deposited on the surface of the solidified soft agar, followed by incubation according to the specific bacterial strain (Table S2).

The animal origin and preparation of donor viromes and FMT

A total of 54 male C57BL/6N mice were purchased for the purpose of harvesting intestinal content for downstream FVT/FMT applications. Upon arrival, the mice were five weeks old and obtained from three vendors: 18 C57BL/6NTac mice from Taconic (Denmark), 18 C57BL/6NRj mice from Janvier (France), and 18 C57BL/6NCrl mice from Charles River (Germany). The mice were earmarked upon arrival and housed at the Section of Experimental Animal Models, University of Copenhagen, Denmark, following previously described conditions[59]. They were provided with ad libitum access to a low-fat diet (LF, Research Diets D12450J) for a period of 13 weeks until they reached 18 weeks of age, which was the planned termination point. Unfortunately, malocclusions resulted in malnutrition for two C57BL/6NRj mice, and they were euthanized before the intended termination date. All mice were euthanized by cervical dislocation, and samples of intestinal content from the cecum and colon were collected and suspended in 500 µL of autoclaved anoxic PBS buffer (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM). Subsequently, all samples were stored at -80°C. In order to preserve the viability of strict anaerobic bacteria, 6 mice from each vendor (a total of 18 mice) were sacrificed and immediately transferred to an anaerobic chamber (Coy Laboratory) containing an atmosphere of approximately 93% N_2, 2% H_2, and 5% CO_2, maintained at room temperature. The samples collected from these mice within the anaerobic chamber were used for FMT and anaerobic chemostat cultivation to produce the FVT-ChP. The intestinal content from the remaining 34 mice was sampled under aerobic conditions and used to generate the fecal virome for downstream processing of the FVT-UnT, FVT-SDT, and FVT-PyT treatments. A flow diagram illustrating the aforementioned processes is provided in Figure S12. All procedures involving the handling of donor animals were conducted in accordance with

Directive 2010/63/EU and the Danish Animal Experimentation Act, under license ID: 2012-15-2934-00256.

Untreated fecal virome (FVT-UnT)

For processing FVT solutions[15], thawed intestinal content from the cecum and colon was suspended in 29 mL autoclaved SM buffer (100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl with pH 7.5), followed by homogenization in BagPage+ 100 mL filter bags (Interscience) with a laboratory blender (Seward) at medium speed for 120 sec. The filtered and homogenized suspension was subsequently centrifuged using an Allegra 25R centrifuge (Beckman Coulter) at 5,000 x g for 30 min at 4°C. The fecal supernatant was sampled for further processing of FVT solutions, while the pellet was resuspended in PBS buffer for bacterial DNA extraction. The fecal supernatant was filtered through a 0.45 µm Minisart High Flow PES syringe filter (Sartorius) to remove bacteria and other larger particles. This filtrate was concentrated using Centriprep Ultracel YM-30K units (Millipore) that by its design of an inner and outer tube removes metabolites below the size of 30 kDa. The permeate in the inner tube was discarded several times during centrifugation at 1,500 x g at 20°C until approximately 0.5 mL was left in the outer tube, which at this point was considered as a fecal virome. The 30 kDa filter from the Centriprep Ultracel YM-30K units was removed with a sterile scalpel and added to the fecal virome to allow viral particles to diffuse. In order to trace back the origin of specific bacterial or viral taxa, the fecal viromes were mixed based on cages, taking into account the coprophagic behavior of mice[60,61]. These fecal viromes were mixed into one final mixture from mice of all three vendors representing the "untreated fecal virome", FVT-UnT, which was immediately stored at -80°C. The remaining fecal viromes were stored at 4°C prior to downstream processing to inactivate the eukaryotic viruses in the fecal viromes by either dissolving the lipid membrane of enveloped viruses with solvent/detergent treatment or inhibit replication of RNA viruses with pyronin Y.

Solvent/detergent treated fecal virome (FVT-SDT)

The solvent/detergent treatment is commonly used to inactivate enveloped viruses, as most eukaryotic viruses possess an envelope, while non-enveloped viruses, including phages, are not affected by this treatment[62–64]. Following the guidelines set by the World Health Organization (WHO)[56] and Horowitz et al.[62] for the clinical use of solvent/detergent-treated plasma, the fecal viromes were subjected to incubation in a solution containing 1% (w/v) tri(n-butyl) phosphate (TnBP) and 1% (w/v) Triton X-100 at 30°C for 4 hours. It is important to note that the majority of

inactivation typically occurs within the first 30-60 minutes of the solvent/detergent treatment[56]. The removal of TnBP and Triton X-100 was performed according to the method described by Treščec et al.[65]. The removal of the solvent/detergent agents from the fecal viromes yielded approx. 100 mL viral-flow-through from the column which was concentrated to 0.5 mL using Centriprep Ultracel YM-30K units as described in the previous section. The final product constituted the FVT-SDT treatment. The effect of the solvent/detergent treatment on phage activity was assessed with traditional plaque assay of both non-enveloped phages (C2, T4, and phiX174) and one enveloped phage (phi6).

Pyronin Y treated fecal virome (FVT-PYT)

Pyronin Y (Merck) is a strong red-colored fluorescent compound. It has been reported to exhibit efficient binding to single-stranded and double-stranded RNA (ss/dsRNA), while its binding to single-stranded and double-stranded DNA (ss/dsDNA) is less effective[66,67]. Initial screening was conducted to determine the optimal conditions for inactivation using various concentrations of pyronin Y, different incubation times, and temperatures for RNA and DNA phages. The fecal filtrate was treated with 100 µM pyronin Y and incubated at 40°C overnight to inactivate viral particles containing RNA genomes. To remove the pyronin Y molecules that were not bound, the pyronin Y-treated fecal filtrate suspensions were diluted in 50 mL SM buffer and subsequently concentrated to 0.5 mL using Centriprep Ultracel YM-30K units. This process was repeated three times, resulting in a transparent appearance of the pyronin Y-treated fecal filtrate, which constituted the FVT-PyT treatment.

Fecal microbiota transplantation (FMT)

The mouse intestinal content that was sampled anaerobically (Fig. S12) was diluted 1:20 in an anoxic cryoprotectant consisting of PBS buffer and 20% (v/v) glycerol and stored at -80°C until administration.

Chemostat propagated fecal virome (FVT-ChP)

The preparation of the chemostat propagated virome was performed as described previously[29]. Briefly, anaerobic-handled mouse cecum content was utilized for chemostat propagation. The culture medium was formulated to resemble the low-fat (LF) diet (Research Diets D12450J) provided to the donor mice as their feed (Table S3), and growth conditions such as temperature and pH were set to simulate the environmental conditions present in the mouse cecum. The end cultures, which underwent fermentation with a slow dilution rate (0.05 volumes per hour),

exhibited a microbial composition that closely resembled the initial microbial composition profile of the donor[29]. These batches were combined to form the FVT-ChP treatment.

Fluorescence microscopy

Virus-like particle (VLP) counts were evaluated of all fecal viromes (FVT-UnT, FVT-SDT, FVT-ChP, and FVT-PyT, Fig. S13) by epifluorescence microscopy using SYBR Gold staining (Thermo Scientific) as described online dx.doi.org/10.17504/protocols.io.bx6cpraw.

Cytokine analysis

Pre-weighted cecum tissue was homogenized in 400µl lysis buffer (stock solution: 10ml Tris lysis buffer, 100µl phosphatase inhibitor 1, 100µl phosphatase inhibitor 2, and 200µl protease inhibitor (MSD inhibitor pack, Meso Scale Discovery, Rockville, MD) using a FastPrep Bead Beater Homogenizer (MP Biomedicals, Irvine, CA), and centrifuged (8000g; 4°C; 5 min). Samples were diluted 1:2 and analyzed for IFN-γ, GM-CSF, IL-15, IL-6, IL-10, KC/GRO, MIP-2, TNF-α IL-17A/F, and IL-22 in a customized metabolic group 1 U-PLEX (MSD) according to manufacturer's instructions. The plates were read and analyzed on a MESO QuickPlex SQ 120MM instrument using MSD's own analysis software Discovery Workbench.

Histology and cytotoxicity assay

Formalin-fixed, paraffin-embedded cecum tissue sections were stained with hematoxylin and eosin for histopathological evaluation by a blinded investigator (co-author AB). A composite score was assigned, taking into account the following pathological features: 1) immune cell infiltration, 2) submucosal edema or hemorrhage, 3) epithelial injury, each with a range of severity/extent as follows: 0: none, 1: mild, 2: moderate, 3: severe) for a cumulative pathology grade between 0 and 9 [15]. The RIDASCREEN *C. difficile* Toxin A/B ELISA kit (r-biopharm) was used to measure the toxin concentrations in the mice feces by following the instructions of the manufacturer. The OD_{450nm} was measured with a Varioskan Flash plate reader (Thermo Fisher Scientific).

Quantitative real-time PCR measuring *C. difficile* abundance

C. difficile in the fecal samples was enumerated using quantitative real-time polymerase chain reaction (qPCR) with specie specific primers (C.Diff_ToxA_Fwd: 5'-TCT ACC ACT GAA GCA TTA C-3', C.Diff_ToxA_Rev: 5'-TAG GTA CTG TAG GTT TAT TG-3'[68]) purchased from Integrated DNA Technologies. Standard curves (Table S4) were based on a dilution series of total DNA extracted from monocultures *C. difficile* VPI 10463. The qPCR results were obtained using

the CFX96 Touch Real-Time PCR Detections System (Bio-Rad Laboratories) and the reagent RealQ plus 2x Master Mix Green low Rox (Amplicon) as previously described[69].

Pre-processing of fecal samples for separation of viruses and bacteria

Fecal samples from three different time points were included to investigate gut microbiome changes over time: baseline (before antibiotic treatment), before *C. difficile* infection, and at termination. This represented in total 142 fecal samples. Separation of the viruses and bacteria from the fecal samples generated a fecal pellet and fecal supernatant by centrifugation and 0.45 µm filtering as described previously[59], except the volume of fecal homogenate was adjusted to 5 mL using SM buffer.

Bacterial DNA extraction, sequencing and pre-processing of raw data

The DNeasy PowerSoil Pro Kit (Qiagen) was used to extract bacterial DNA from the fecal pellet by following the instructions of the manufacturer. The final purified DNA was stored at -80ºC and the DNA concentration was determined using Qubit HS Assay Kit (Invitrogen) on the Qubit 4 Fluorometric Quantification device (Invitrogen). The bacterial community composition was determined by Illumina NextSeq-based high-throughput sequencing of the 16S rRNA gene V3-region, as previously described [59]. Quality control of reads, de-replicating, purging from chimeric reads and constructing zOTU was conducted with the UNOISE pipeline[70] and taxonomy assigned with Sintax [71] using the EZtaxon for 16S rRNA gene database[72]. Code describing this pipeline can be accessed in github.com/jcame/Fastq_2_zOTUtable. The average sequencing depth after quality control (Accession: PRJEB58777, available at ENA) for the fecal 16S rRNA gene amplicons was 60,719 reads (min. 11,961 reads and max. 198,197 reads).

Viral RNA/DNA extraction, sequencing and pre-processing of raw data

The sterile filtered fecal supernatant was concentrated using Centrisart centrifugal filters with a filter cut-off at 100 kDA (Sartorius) by centrifugation at 1,500 x g at 4ºC (dx.doi.org/10.17504/protocols.io.b2qaqdse). The viral DNA/RNA was extracted from the fecal supernatants using the Viral RNA mini kit (Qiagen) as previously described [59]. Reverse transcription was executed with SuperScript IV VILO Master mix by following the instructions of the manufacturer and subsequently cleaned with DNeasy blood and tissue kit (Qiagen) by only following step 3-8 in the instructions from the manufacturer. In brief, the DNA/cDNA samples were mixed with ethanol, bound to the silica filter, washed two times, and eluted with 40 µL elution buffer. Multiple displacement amplification (MDA, to include ssDNA viruses) using GenomiPhi

V3 DNA amplification kit (Cytiva) and sequencing library preparation using the Nextera XT kit (Illumina) was performed at previously described[59], and sequenced using the Illumina NovaSeq platform at the sequencing facilities of Novogene (Cambridge, UK). The average sequencing depth of raw reads (Accession: PRJEB58777, available at ENA) for the fecal viral metagenome was 17,384,372 reads (min. 53,960 reads and max. 81,642,750 reads). Pre-processing of raw sequencing data and viral contig assembly were performed as previously described[73]. Code describing this pipeline can be accessed in github.com/jcame/virome_analysis-FOOD.

Bioinformatic analysis of bacterial and viral sequences

Initially the dataset was purged for zOTU's/viral contigs, which were detected in less than 5% of the samples, but the resulting dataset still maintained >99.8% of the total reads. Cumulative sum scaling (CSS)[74] was applied for the analysis of β-diversity. CSS normalization was performed using the R software using the metagenomeSeq package[75]. R version 4.2.2 was used for subsequent analysis and presentation of data. The main packages used were phyloseq[76], vegan[77], DESeq2[78], ampvis2[79], ggpubr[80], and ggplot2[80].

Statistical analysis

α-diversity analysis was based on raw read counts for bacteriome analysis and normalized (RPKM) read counts for virome analysis, while statistics were based on ANOVA. β-diversity was represented by Bray-Curtis dissimilarity and statistics were based on PERMANOVA. Cytokine levels, toxin levels, *C. difficile* abundance, and histology data were analyzed in R using linear models with saline as control group, while log rank test was used to compare the survival distributions.

List of Supplementary Materials

This PDF file includes:

Fig. S1 to S13

Table S1 to S4

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Supervision: DSN, AKH

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Writing – review & editing: All authors critically revised and approved the final version of the manuscript.

Competing interests

All authors declare no financial or personal conflicts of interest.

Data and materials availability

All data associated with this study are present in the paper or the Supplementary Materials. All sequencing datasets are available in the ENA database under accession number PRJEB58777.

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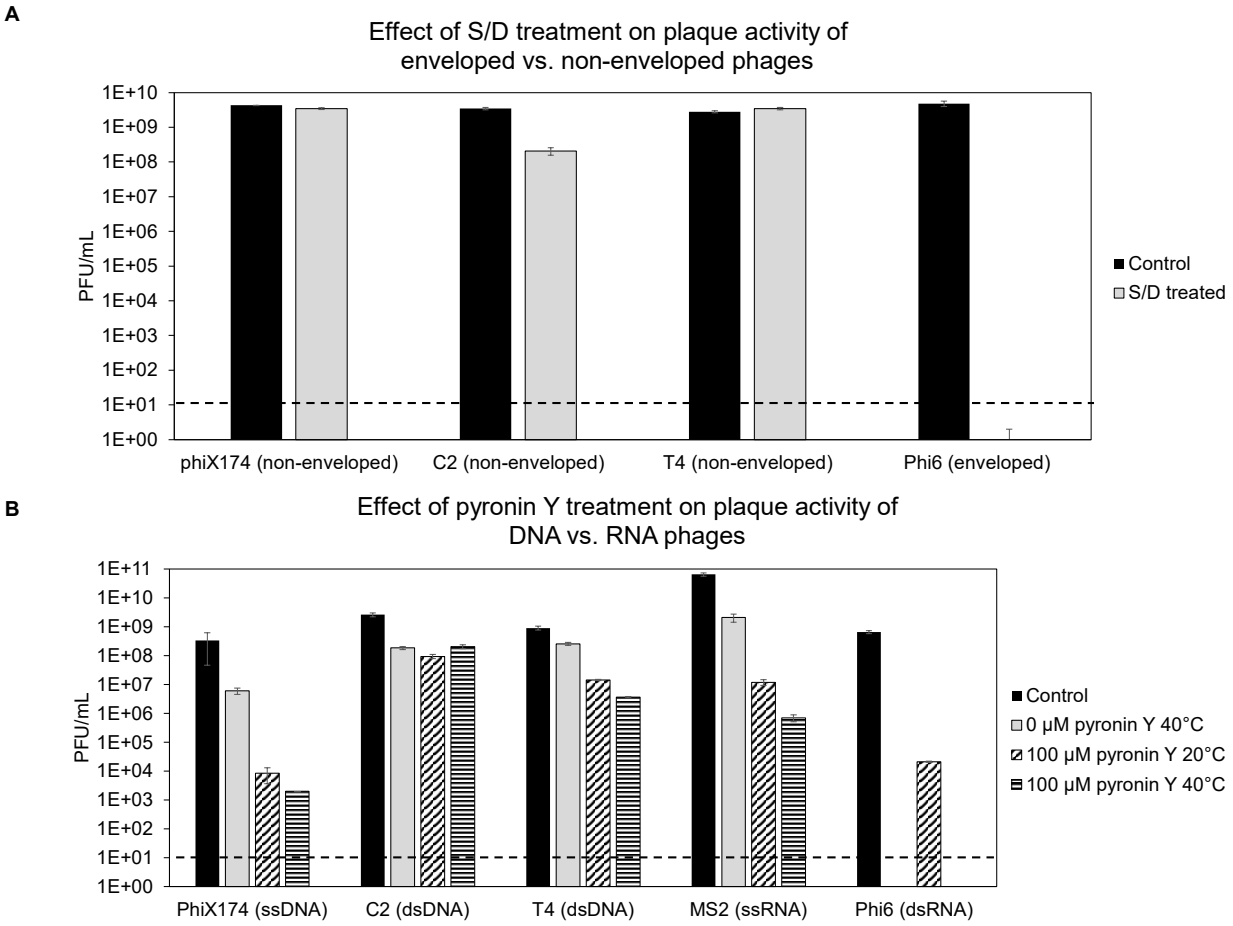

Fig. 1: Evaluation of inactivation of phage activity with solvent/detergent (S/D) or pyronin Y treatment. A) Three non-enveloped phages (phiX174, C2, and T4) and one enveloped phage (phi6) were treated with solvent/detergent, and their plaque activity (plaque forming units per mL) was evaluated on their respective bacterial hosts. B) Phages representing ssDNA (phiX174), dsDNA (C2 and T4), ssRNA (MS2), and dsRNA (phi6) were treated with pyronin Y, and their plaque activity (PFU/mL) at different incubation conditions was evaluated on their respective bacterial hosts. Dashed lines mark the detection limit of the applied assay.

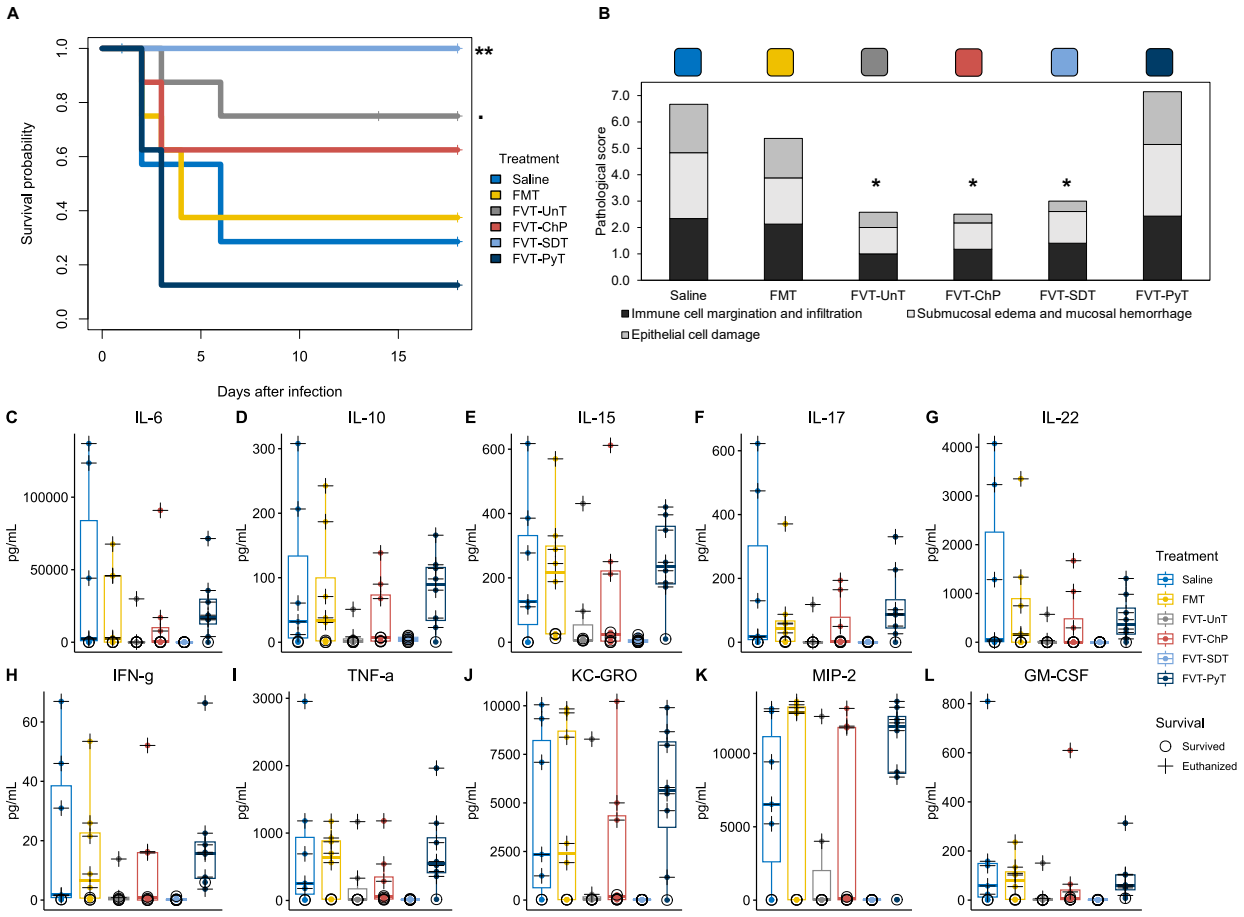

Fig. 2: Overview of mouse phenotypic characteristics. A) Kaplan-Meier curve showing the survival probability of the mice that was associated to the different treatments. B) Pathological score of cecum tissue evaluating the effect of the treatments' ability to prevent *C. difficile* associated damage of the cecum tissue. C) to L) Showing the overall cytokine profile in the mouse cecum tissue of the different treatments. The euthanized (cross) mice are differentiated from the mice that survived (circle) the *C. difficile* infection. "**" = $p < 0.005$, "*" = $p < 0.05$, "." = $p < 0.1$.

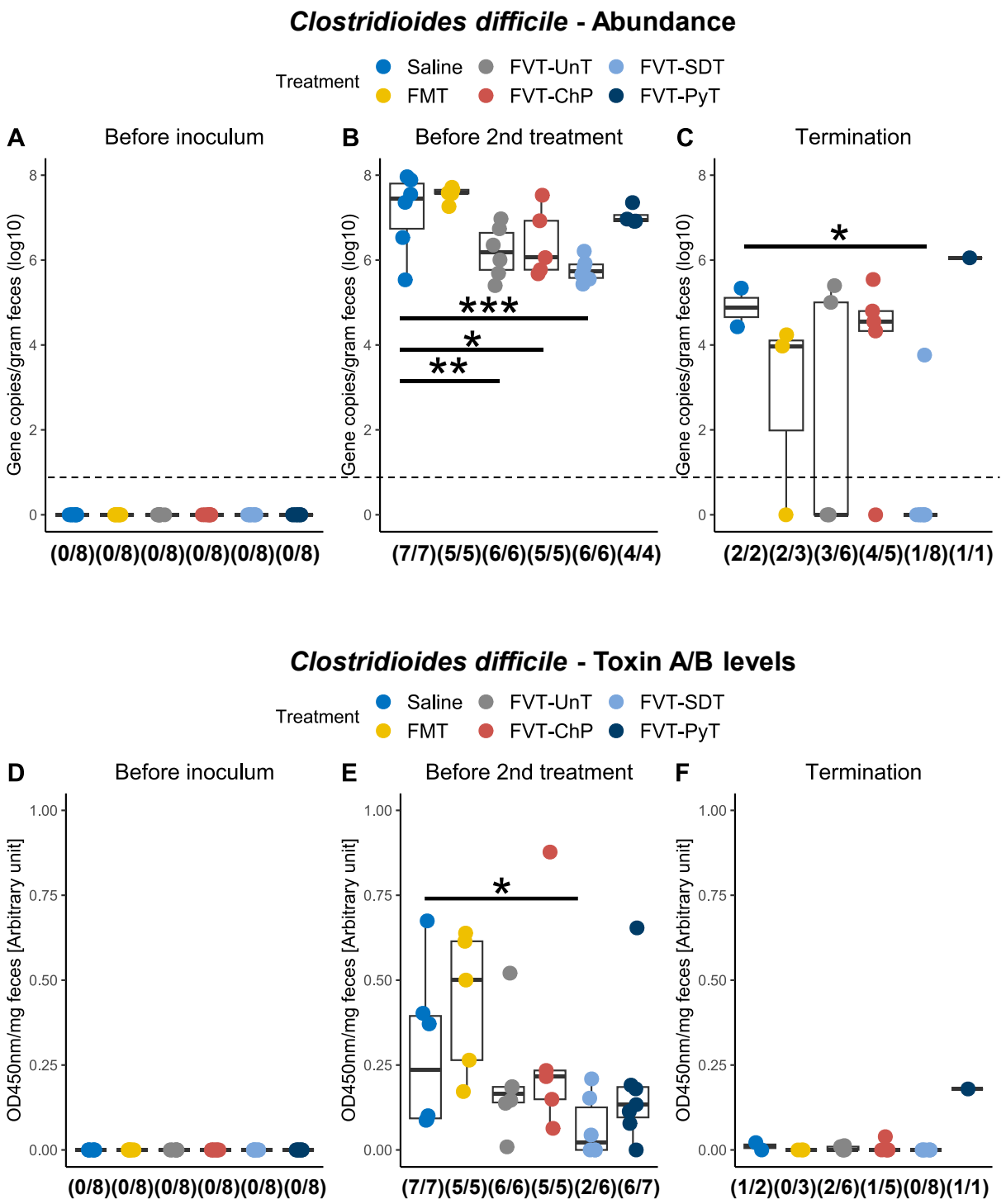

Fig. 3: Evaluation of *C. difficile* abundance A) to C) by qPCR targeting the *toxA* gene and D) to F) the associated toxin A/B levels measured with ELISA on feces samples from three different time points: before *C. difficile* inoculum, before 2nd treatment, and at study termination. The fraction below the bar plots highlights the number of mice that were detected positive of either *C. difficile* or toxin A/B. Dashed line marks the detection limit of the applied assay. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$.

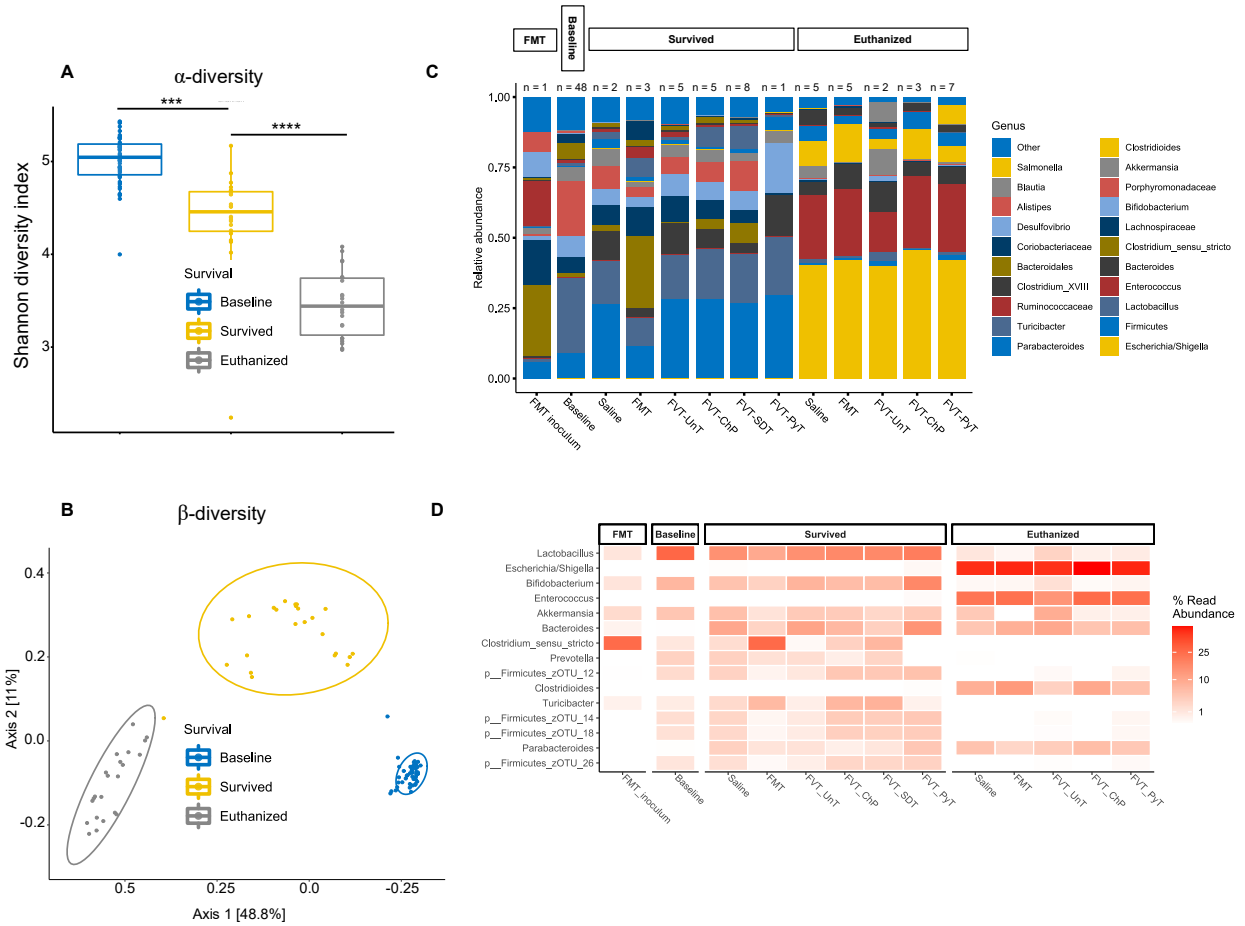

Fig.4: Bacteriome analysis based on 16S rRNA gene amplicon sequencing. A) The general bacterial Shannon diversity index (α-diversity) and B) Bray-Curtis dissimilarity based PCoA plot (β-diversity) at baseline (before antibiotic treatment), that was compared with the mice that had survived *C. difficile* infection regardless of the treatment and the euthanized mice. C) Barplot and D) heatmap illustrating the bacterial relative abundance of the dominating bacterial taxa that was associated to the different mice that either survived the infection or were euthanized. The "n" above the bar plots highlights the number of mice of which the taxonomical average was based on. *** = $p < 0.005$. **** = $p < 0.0005$.

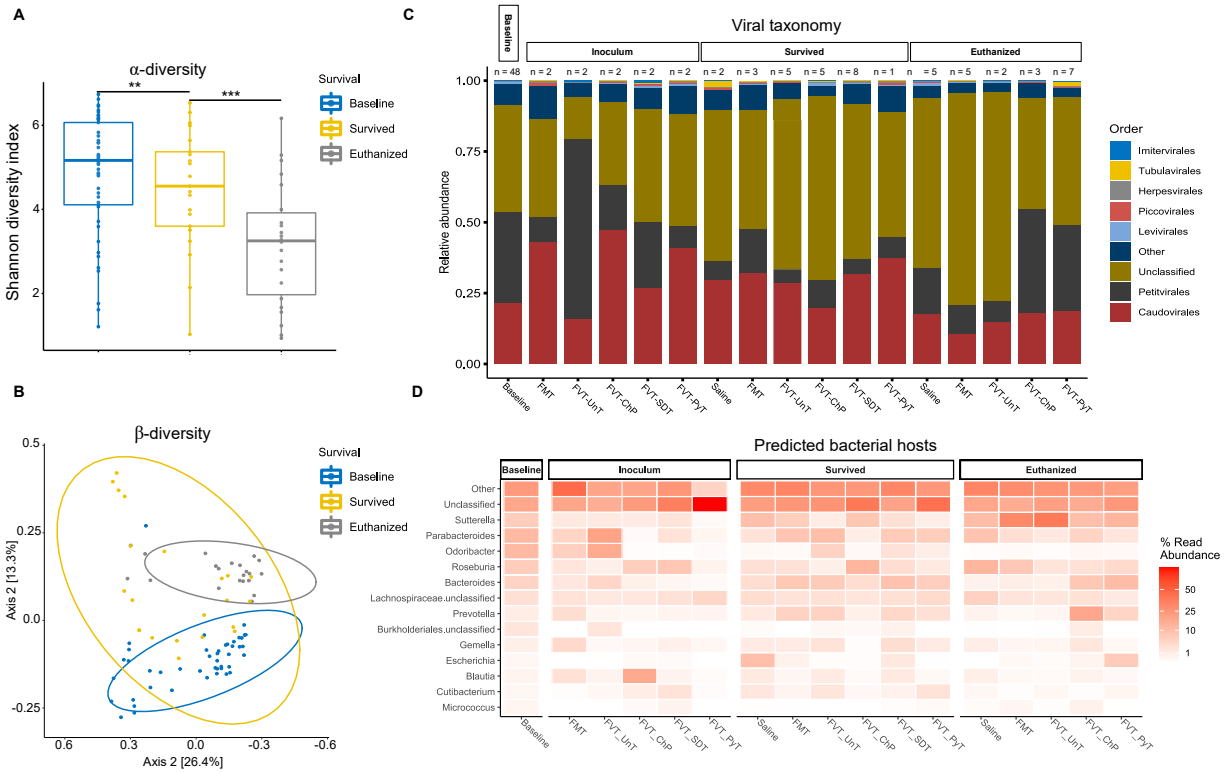

Fig. 5: Metavirome analysis based on whole-genome sequencing. A) The viral Shannon diversity index (α-diversity) and B) Bray-Curtis dissimilarity based PCoA plot (β-diversity) at baseline (before antibiotic treatment), that was compared with the mice that had survived *C. difficile* infection regardless of the treatment and the euthanized mice. C) Barplot showing the relative abundance of the viral taxonomy, and D) heatmap illustrating the relative abundance of the bacterial hosts that are predicted on the basis of the viral sequences. The "n" above the bar plots highlights the number of mice of which the taxonomical average was based on. ** = $p < 0.01$, *** = $p < 0.005$.

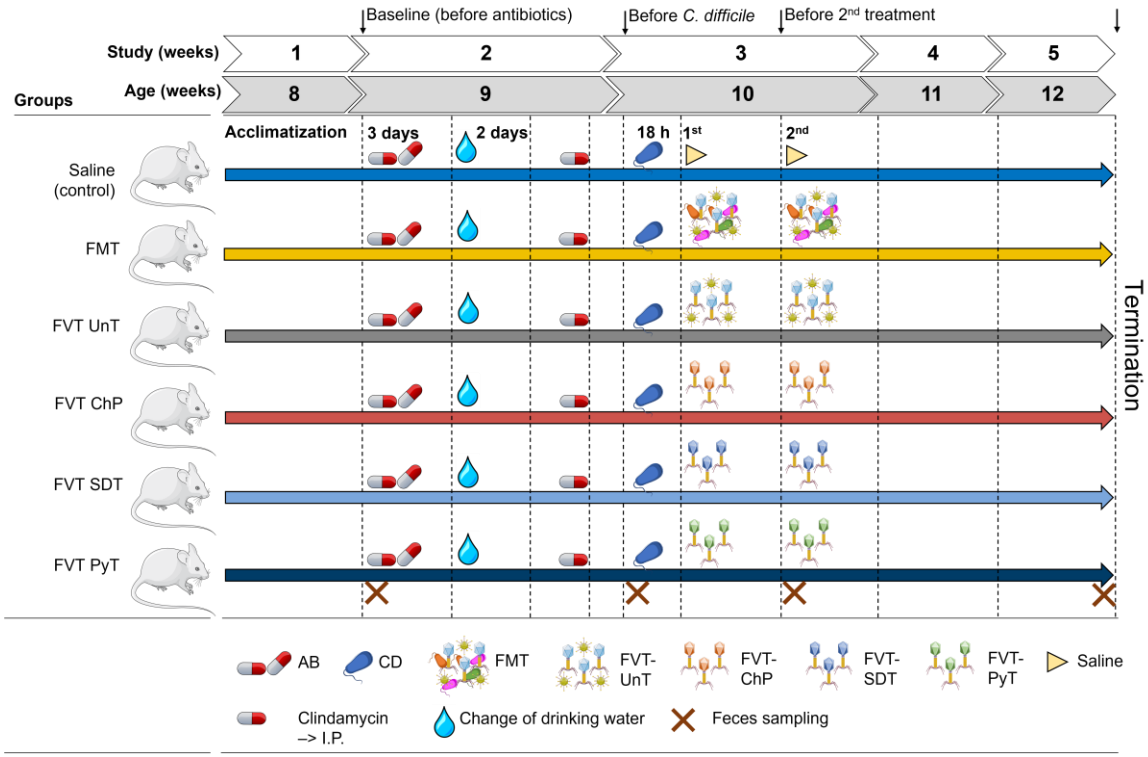

Fig. 6: Overview of the animal model. The mice were initially treated with an antibiotic mixture in their drinking water, intraperitoneal (I.P.) injection of clindamycin, and then inoculated with *C. difficile* (~10^4 CFU). Eighteen hours after the mice were treated with either saline (as control), FMT (fecal microbiota transplantation), FVT-UnT (fecal virome transplantation – Untreated, e.i. sterile filtered donor feces), FVT-ChP (FVT-chemostat propagated fecal donor virome to remove eukaryotic viruses by dilution), FVT-SDT (FVT-solvent/detergent treated to inactivate enveloped viruses), and FVT-PyT (FVT-pyronin Y treated to inactivate RNA viruses). Crosses marks time points of feces sampling.