Fecal phageome of healthy individuals; presence of antibiotic resistance genes and variations caused by ciprofloxacin treatment

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Running title- Phageome and antibiotics

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Synopsis

Objectives

Antimicrobial resistance genes (ARGs) can be transferred by means of mobile genetic elements (MGEs), which play a critical role in the dissemination of resistance in the bacterial community. ARG transmission within MGEs has been reported in plasmids and transposons but less frequently in bacteriophages. Here, the bacteriophage fraction of seven human fecal samples was purified and deep-sequenced to detect the presence of ARGs in the phage particles.

Methods

Seven fecal samples (five from healthy individuals, and two from a patient before and after receiving ciprofloxacin treatment) were used to extract phage DNA, which was purified and then sequenced in a MiSeq (Illumina). Generated reads were checked for quality and assembled, and the generated contigs analyzed with Kraken, PHASTER, VirSorter and Prokka. Some genes were also validated by qPCR.

Results

Analysis of the purified phage DNA by Kraken identified from 4 to 266 viruses in the samples. The viral fraction corresponded mainly to the order *Caudovirales*, including phages from the *Siphoviridae* and *Myoviridae* families. Bacterial genes associated with antimicrobial resistance were detected in the viral DNA, as confirmed by qPCR. Higher densities of ARG-carrying phage particles were observed in the post- versus pre-ciprofloxacin treatment sample.

Conclusions

The finding of ARGs in phage particles supports the description of phages as mobile elements contributing to the dissemination of bacterial antibiotic resistance and

suggests ciprofloxacin treatment may play a role in the release of ARG-carrying particles, thereby increasing resistance.

Introduction

Descriptions of the lower gastrointestinal tract microbiome have focused chiefly on bacterial components[1,2]. However, recent research has suggested that the viral fraction also contributes to shaping the microbiome structure[3–6].

Within the virome, the phage fraction is the most abundant, with an active phageome reported to comprise 35-2800 viruses,[1,4,5] and many viral particles carry bacterial DNA. [7–9] The great abundance of phages infecting all sorts of bacterial genera suggests that phages and phage-derived particles are a mechanism of genetic exchange that may play an important role in human health and disease. [6,10]

Antibiotic treatment is associated with the rise of antimicrobial resistant bacteria [11] and affects microbiome composition and species abundance. [1] As phages constitute an important part of the microbiome, antibiotic perturbation might also affect phage populations and promote their propagation rates, [12,13] thereby increasing phage-mediated gene transfer.

Using a metagenomic approach, this study explored how phage particles may actively contribute to antimicrobial resistance through the mobilization of antibiotic resistant genes (ARGs) in human bacterial communities. When studying viromes, contamination with bacterial DNA poses a serious drawback, [14] therefore an optimized protocol was used to ensure that DNA outside the phage capsids was completely removed and that only packaged DNA was sequenced. [14] The role of phages in altering ARG patterns in an individual receiving antibiotic treatment was also assessed.

Materials and methods

Fecal samples. Samples HC6, HC9, HC10, UB6 and UB25 were obtained from five healthy individuals (aged 53, 39, 37, 27 and 26 years, respectively). The subjects did not consume antibiotics in the three months prior to sample collection. Samples PRECIP and POSTCIP were obtained from the same individual (aged 66 years): PRECIP before taking antibiotics and POSTCIP four days after starting a 5-day ciprofloxacin treatment (500 mg/12 hours) prescribed as prophylaxis before dental surgery. The same individual supplied two independent fecal samples one year and one year plus one week after the study, when no longer receiving any antibiotics. All individuals live in Barcelona (Catalonia), none were involved in a food-borne outbreak or showed any gastro-intestinal pathology or known infection, and none travelled abroad in the three months before sampling except those providing samples HC9 and HC10, who travelled to the Republic of Ecuador and Peru, respectively.

All samples were completely anonymized. The study was approved by the Clinical Ethics Committee of the Hospital de la Santa Creu i Sant Pau (12/065/1350). Informed consent was obtained for all individuals.

Purification of DNA from phage particles. Ten g of fecal samples were homogenized 1:5 (*w:v*) in PBS by shaking for 30 minutes. 50 mL of the homogenate was centrifuged at 3000 ×g and the supernatant was filtered through low protein-binding, 0.22 µm pore-size membrane filters (Millex-GP, Millipore, Bedford, MA) to remove cellular debris and bigger particles while allowing viral protein capsids, small particles (including small vesicles) and free DNA to pass. The viral capsids in the filtrates were 20-fold concentrated using protein concentrators (100 kDa Amicon Ultra centrifugal filter units, Millipore, MA, US), at 3000 ×g following the manufacturer's instructions. Two mL concentrated suspensions were treated twice with chloroform

1:10, mixed by vigorous vortexing for 5 minutes and centrifuged at 16000 ×g for 10 minutes to disrupt bacterial membranes with the aim of breaking DNA-containing small vesicles that might have passed through the 0.22 µm pore-size membrane filters. The supernatant was treated with DNase (100 units/mL; Sigma-Aldrich, Spain) using the reaction buffer provided by the manufacturer for 1 hour at 37ºC to eliminate non-packaged DNA that might have passed the filters (bacterial DNA or DNA released from vesicles after the chloroform treatment). The DNase was inactivated using the Stop Solution provided by the manufacturer.

In this step, viral capsids remain intact and no vesicles should be observed, whereas free DNA outside viral capsids should have been removed by the DNase treatment. To confirm the absence of non-packaged bacterial DNA, an aliquot of the phage suspension was taken at this stage and used as a template to amplify the selected ARGs described below and eubacterial 16S rDNA by qPCR (Table 1). If the protocol had successfully eliminated most of the vesicles as well as DNA outside the viral capsids, both amplifications would be negative.

The protocols applied here for DNase treatment and DNase inactivation were verified in our previous studies. [15] Briefly, the DNase performance was verified by adding known concentrations of an ARG that would not be detected by qPCR if adequately eliminated by the DNase. DNase inactivation was previously confirmed by adding known concentrations of an ARG immediately after the DNase inactivation step and by amplifying this ARG by qPCR. An incomplete DNase inactivation would have resulted in a reduced amplification of the ARG caused by DNase cleavage of the primers in the qPCR assay, which was not observed. [15]

Packaged DNA was extracted using a double extraction to achieve the required purity. Firstly, the PowerSoil® DNA isolation kit (MO Bio. CA, US) was used, which broke down the capsids and allowed the viral DNA to be recovered. Since the purity of the extracted DNA was not yet optimal, according to the Bioanalyzer system (Agilent Technologies, CA, US), further purification was carried out using the EZ1 Virus Mini Kit v2.0 (Qiagen, CA, US) following the manufacturer's instructions to a final volume of 60 μL.

Sequencing. After purification, the DNA concentration was evaluated using a Qubit® Fluorometer (Life Technologies. CA, US) and the DNA quality was further confirmed using the 2100 Bioanalyzer system (Agilent Technologies, CA, US). 0.2 ng/μL of DNA was used to prepare the libraries.

Illumina libraries were generated following the Nextera XT (Illumina, Inc., San Diego, CA, US) manufacturer's protocol for paired-end libraries (2x150 bp). For extension, 14 PCR cycles of 2.5 min were performed to increase the tagmentation process. Libraries were purified and checked in a 2100 Bioanalyzer electrophoresis instrument (Agilent Technologies, CA, US) and quantified in a Quantus™ Fluorometer (Promega, WI, US). An equimolar pool of the seven samples was sequenced in an Illumina MiSeq system with the Miseq Reagent Kit v2 (Illumina).

Bioinformatic analysis. Sequence reads were quality checked with FASTQC v0.11.2 to detect any anomalies in the sequencing process. Reads were then *de novo* assembled with default parameters using SPAdes v3.6.2. [16] Contigs were classified taxonomically with Kraken v0.10.5 [17] using the standard Kraken database that comprises NCBI taxonomic information, as well as the complete genomes in RefSeq for the bacterial, archaeal, viral as well as phage domains. In parallel, contigs were identified by comparison against the phage sequence database using PHASTER. [18]

PHASTER classifies prophage sequences according to the percentage of the total number of coding sequences (CDS) and the presence of specific phage-related words. VirSorter was used to identify viral signals in the assembled contigs.[19]

Gene annotation was performed with Prokka 1.11,[20] which establishes an e-value of 10^{-6} within the different included databases. ARGs were searched with ResFinder 2.1,[21] which establishes a percentage of identity between CDS and the ARGs. The minimum percentage of identical nucleotides shared between the best matching ARG in the database and the corresponding sequence in a contig was set to 90 %. The minimum percentage of overlapping nucleotides between a sequence and an ARG to count as a hit for that gene was set to 60 %.

Sequences flanking the ARG detected by ResFinder were examined with BLAST [22] to ascertain whether the sequences were phage or bacterial.

The search for 16S rDNA sequences in the contigs was performed with Metaxa2 software, [23] which allows detection of the 16S DNA in metagenomic samples taking into account the contigs generated in the novo assembling (done by Spades).

Standard PCR and qPCR procedures. PCRs were performed with an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Barcelona, Spain). ARGs from the control strains were amplified by conventional PCR (Table 1).

Real-time qPCR assays using TaqMan hydrolysis probes for the different ARGs (Table 1) were used under standard conditions as previously described. [24–27] Those ARGs analyzed by SYBR Green (Table 1) were quantified using PowerUp SYBR Green Master Mix (ThermoFisher Scientifics, MA, US) following the manufacturer's instructions.

Availability of data and material

The data have been deposited with links to BioProject accession number PRJNA392494 in the NCBI BioProject database (http://www.ncbi.nlm.nih.gov/bioproject/392494). BioSample metadata are available in the NCBI BioSample database (http://www.ncbi.nlm.nih.gov/biosample/) under accession number SAMN07302947, SAMN07302946, SAMN07302945, SAMN07302944, SAMN07302943, SAMN07302942 and SAMN07302940.

Results

Identification of DNA from phage particles

Study of DNA from phage particles gave a total of 11754439 reads (Table 2), with an average depth of 1.68±0.18 Mb per sample and a sufficiently high quality for our purposes. Trimming was not carried out, as the QC performed by FastQC showed high quality reads, even in the last read positions. Sequence adaptors were also not detected. The results obtained when trimming was performed were almost the same (analysis not shown). Reads were first assembled with SPAdes and then taxonomically classified with the Kraken Standard Database (Table 2). In addition, the assembled contigs were used to perform a search for phage sequences with PHASTER [18] (Table 2 and Supplementary File 1), which discriminates between sequences with a high certainty of belonging to phages and those that are questionable or incomplete. PHASTER identified a different number of phage-related contigs in the DNA from phage particles compared to Kraken (Table 2).

The generated contigs were annotated using Prokka v1.11, [20] which allowed several phage genes to be identified (Table 2 and raw data in Supplementary File 2). However, since genes in phage genomes can be shared among several phage taxa,

198 Prokka cannot determine if a given gene belongs to a specific phage. Data analysis
199 revealed phage structural genes (encoding capsid and tail), terminase and hydrolases
200 (Supplementary File 2). Also, Prokka detected ARG sequences in UB25, HC9, UB6,
201 PRECIP and POSTCIP (Supplementary File 2, blue).

202 Besides phage genes, Kraken analysis revealed numerous genes from different
203 bacterial species in the phage DNA fraction of the samples (Supplementary File 3).
204 Some genes were clearly of bacterial origin, while others, such as integrases or
205 ribonucleotide reductases, can be present in either phages or bacteria. [27,28]

206 Contamination with non-packaged DNA was excluded by the controls, which
207 showed an absence of 16S rDNA and ARGs in the phage suspensions before
208 desencapsidation. Efficient DNA removal by DNase activity was confirmed and DNase
209 was completely inactivated by thermal treatment.

210 Additional information was obtained with VirSorter, which classified matches in
211 three categories. (i) Category 1 ("*most confident*" predictions) regions have significant
212 enrichment in viral-like genes and at least one hallmark viral gene detected. A hallmark
213 viral gene is typically of viral origin annotated as such (capsid gene, portal, terminase,
214 etc.). (ii) Category 2 regions correspond to "likely" predictions and are either enriched
215 in viral-like or non-*Caudovirales* genes, or have a viral hallmark gene detected.
216 Category 3 ("*possible*" prediction) was not considered.

217

218 **Viral identification**

219 According to the Kraken output, a high percentage of sequences in the viromes
220 (hereby understood as DNA extracted from the viral fraction, mostly phage particles)
221 belonged to bacterial or archaeal DNA (4.8%-29%), while the percentage of viral
222 sequences among the samples varied from 0.5% to 23.9% (Table 2 and Supplementary

File 3). The remaining sequences were not identified, or classed as uncharacterized viruses (viral dark matter). [29]

The distribution of the Kraken-identified viral sequences in the DNA from phage particles revealed that the order *Caudovirales*, which includes most bacteriophages, was the most abundant (Fig. 1, Table 3). Within this group, *Siphoviridae* was the most prominent family, within which phages of lactic acid bacteria, including Skunalikevirus, [30] were the most abundantly identified by comparison against the databases. All the sequences found in samples UB6 and UB25 belonged to the *Siphoviridae*. Sequences of phages belonging to the *Myoviridae* family were only found in the PRECIP sample, particularly those infecting *Clostridium* and *Bacillus*. The POSTCIP sample contained a small percentage of *Escherichia coli* phages of the *Podoviridae* family. Based on database sequences, HC6 showed a contig with 100 % identity with the phage ΦX174 from the family *Microviridae*, which infects *E. coli*, and HC9 contained *Rhodococcus* phages. In addition, 50% of the viral sequences in HC9 belonged to the family *Mimiviridae,* giant viruses that use *Amoebae* as hosts. [31]

VirSorter also identified numerous phage genes belonging to *Caudovirales* in several contigs of each sample (Table 2), particularly when categories 1 and 2 were considered together. However, this is not indicative of the number of phages because it is possible that several contigs belonged to the same phage. Data are available upon request.

Detection of ARGs in the phage particles

The DNA from phage particles was searched for ARGs using ResFinder 2.1 and Prokka 1.11 (Table 4). ResFinder identifies only a limited number of acquired ARGs,

while Prokka identifies any gene possibly involved in antibiotic or heavy metal resistance, including those not exclusively involved in antibiotic resistance. The DNA from phage particles was also searched for bacterial 16S rDNA using Metaxa2, and it was detected in several contigs from all samples (Table 4). In contrast, 16S rDNA was not detected in the samples before the capsids were broken down, as shown above.

Phage DNA in the samples was also analyzed by real-time qPCR targeting specific ARGs (β-lactamases *bla*$_{\text{TEM}}$, *bla*$_{\text{CTX-M-1}}$ group and *bla*$_{\text{CTX-M-9}}$ group, *bla*$_{\text{OXA-48}}$, methicillin resistance *mecA*, sulfonamide resistance *sul1* and quinolone resistance *qnrA* and *qnrS*). [24,25] These were selected for their clinical significance and because they have previously been detected in human fecal viromes. [24,25] We also included most of the ARGs identified in the metagenomic analysis (tetracycline resistance *tetW*, β-lactamase *bla*$_{\text{CFXA6}}$, and macrolide resistance *ermF* and *mefA*). qPCR amplified at least one ARG in all the samples except PRECIP (Table 4). Controls confirmed that the Taq polymerase used was not contaminated with any ARG, as was previously reported for Taq polymerase contaminated with *bla*$_{\text{TEM}}$. [32] The results obtained by metagenomic analysis and qPCR did not always coincide. In general, metagenomics only detected the most abundant ARGs (those showing a lower threshold cycle (Ct) by qPCR) (Table 4). To shed light on the origin of the ARGs detected by ResFinder, the flanking regions were analyzed by BLAST. The length of the flanking regions of each ARG varied depending on the contig length, ranging from 127 to 5050 bp. Sequences showing more than 95% identity with the fragment analyzed corresponded mostly to *Bacteroides fragilis* or in a few cases other *Bacteroides* and bacterial species (Table 4). All sequences corresponded with mobile genetic elements such as transposons or insertion sequence elements, and specific phage sequences were not identified.

Comparison of samples from the same individual before (PRECIP) and after (POSTCIP) ciprofloxacin treatment

PRECIP and POSTCIP samples showed differences in the metagenome structure and viral content (Table 2). With a similar number of reads, the bacterial sequences detected in POSTCIP did not change remarkably in terms of species. However, a greater prevalence of genes from the Bacteroidetes group, lower prevalence of Actinobacteria and higher viral prevalence and diversity was observed in the POSTCIP sample (Fig. 2). All identified sequences in both samples corresponded to *Caudovirales*; PRECIP presented a higher prevalence of *Myoviridae,* whereas in POSTCIP almost all were *Siphoviridae* (Table 2; Fig. 2).

The PRECIP sample did not show any ARGs, and only Prokka identified two efflux pump genes (involved in antimicrobial resistance) (Table 4). In contrast, ARGs were detected in the POSTCIP sample by both metagenomic analysis and qPCR (Table 4). Notably, after the treatment with ciprofloxacin, POSTCIP showed low but detectable levels of quinolone resistance genes (*qnrA* and *qnrS*).

To confirm that the higher ARG prevalence was caused by ciprofloxacin and to gain insight into the background ARG levels in this individual, two additional samples were taken one year and one year plus one week after the treatment. No ARGs were detected by qPCR in phage DNA from these two samples.

Discussion

Studies of the phage community in human biomes have identified at least 1000 phage and prophage sequences, the majority belonging to double-stranded DNA viruses of the *Caudovirales* order (*Siphoviridae*, *Myoviridae*, *Podoviridae*) and others to single-

298 stranded DNA viruses such as the *Microviridae* family. [33,34] An accurate analysis of the
299 DNA from phage particles greatly depends on the methods used, including the
300 extraction technique. In our study, we avoided concentration steps that could bias
301 species detection in the samples, [35] and phenol was not used, as it negatively affects the
302 purity of the extracted DNA. [25]

303 Results also depend on the depth of sequencing. Illumina recommends starting
304 with a minimum of 100k reads, but here, as in similar studies, the number was increased
305 from 1 to 3 Mb [36] to minimize the loss of sequences belonging to rare species. [35]
306 Additionally, precise separation of the phage fraction is critical for avoiding chimeras,
307 which can be generated when analyzing total DNA in a metagenomic study. Shotgun
308 metagenomics thus provides a functional and detailed taxonomical classification of the
309 species composing the virome. [37]

310

311 As no perfect tool exists for the detection of phage sequences or ARGs, it is
312 advisable to use a combination of different software, allowing contigs to be grouped
313 from different standpoints. We used SPAdes for the metagenomic assembly since it has
314 been favorably described compared with other options like Soap, Metavelvet, Omega or
315 Metahit.[38] Different software tools will yield different results. For example, Kraken
316 includes any virus (not only phages, although these are the most abundant) and phage
317 sequence annotated as such in the databases. In contrast, VirSorter is more restrictive,
318 particularly for category 1 regions, and will only identify genes with a clear phage
319 origin. However, VirSorter allows the prediction of viral sequences outside the host
320 genome, even from short fragments. [19]

321

One of the problems encountered when analyzing viromes is the possibility of bacterial DNA contamination. The methods used were designed to prevent contamination of the DNA extracted from phage particles. This contamination could have three sources: bacterial or eukaryotic cells, free-cell DNA or DNA inside outer membrane vesicles (OMV). [39] Filtration eliminated bacterial or eukaryotic cells and many OMV. The chloroform treatment, not always included in other virome studies, was used to minimize the impact of DNA-containing OMV. [39] The residual presence of vesicles containing DNA that passed through the filter and were not eliminated by the chloroform treatment cannot be completely ruled out. However, their contribution to the DNA pool analyzed should not be significant, based on previous studies using electron microscopy [24] in which the amount of phage particles visualized coincided with the number of ARGs in the DNA extracted from these particles and no OMV were observed. Finally, free DNA was removed using a strong DNase treatment, demonstrated to efficiently eliminate non-packaged DNA. [15,40,41] Absence of bacterial DNA contamination was confirmed by the controls, which showed that free, non-packaged DNA had been removed to the extent that 16S rDNA and ARGs were undetectable by qPCR or metagenomics before the viral capsids were broken, whereas these genes were later detected in the DNA from disrupted phage particles.

After rigorous efforts to eliminate all DNA outside the viral capsids, our results still showed a wide fraction of bacterial DNA in phage particles, as previously observed in viromes.[35] Up to 70-80% of reads of viral metagenomes yield no significant matches, [3,42] possibly because few studies have focused specifically on phage sequencing, resulting in a lack of specific phage databases and a low availability of phage sequences in public databases in comparison with bacterial entries. [43,44] In addition, there is a bias

toward the more studied human viruses, and a large fraction of virus sequences in the databases correspond to uncharacterized viruses (viral dark matter) [29]. Moreover, when analyzing our sequences, we found that a number of prophage sequences within bacterial genomes are erroneously annotated as bacterial, do not appear as phages when searched in general databases, and cannot be identified in comparisons against the viral database subsets.

Thus, assuming the absence of a significant proportion of bacterial DNA contamination (but not completely ruled out), our hypothesis is that a high proportion of bacterial DNA found within viral particles in this and other studies is the consequence of generalized transduction or similar mechanisms able to package and spread fragments of the bacterial genome in phage capsids. [14,45–47] We recently showed that phage particles harboring ARGs likely originate from bacterial strains in the microbiome, and proposed that bacteria use this mechanism to spread their genome.[24] This idea is supported by a recent study demonstrating that phage packaging in *Staphylococcus aureus* frequently contributes to the mobilization of enormous fragments of the genome of a single bacterial cell with great frequency. This new mechanism, named "co-lateral transduction", shows not only that phages can package and mobilize bacterial genes, but that it is not a random process and that certain regions of the *S. aureus* chromosome are preferentially hypermobile platforms of gene transfer. [47]

If this mechanism also explains our observations, then using 16S rDNA as a measure of contamination has its limitations, because like other bacterial genes, 16S rDNA could also be packaged in the phage capsids and detected within the viral DNA. Supporting our hypothesis, 16S rDNA was detected in the DNA from the phage

372 particles, and in more contigs than those showing ARGs. This was to be expected
373 considering that 16S rDNA should be more abundant in the bacterial population than
374 ARGs, perhaps with the exception of cells carrying ARGs located in multicopy
375 plasmids. Thus, monitoring 16S rDNA before breaking the capsids provides an accurate
376 way of verifying the absence of bacterial DNA contamination.
377
378 Special care should be taken when annotating ARGs. It has been repeatedly
379 demonstrated that a sequence showing homology with an ARG does not necessarily
380 indicate a true gene conferring antibiotic resistance.[14,48] Therefore, the real extent of
381 ARGs in microbiomes and the associated risk of resistance transfer can be
382 overestimated. Nevertheless, the phageome is known to harbor ARGs,[13,25,49] as shown
383 by the few studies on human phageomes [41,50] and environmental samples.[51]
384 The metagenomic data from the viral particles confirmed the presence of ARGs
385 in most samples. Parallel qPCR assays of the same DNA detected most of the ARGs
386 identified by metagenomic analysis, but the latter did not find all the ARGs detected by
387 qPCR. Although the Ct value of the qPCR is only indicative and varies among qPCR
388 assays, the output of the two methods largely coincided in the most abundant ARGs
389 (lower Ct), which suggests that high ARG densities are required for coverage by our
390 libraries. An exception was *bla*$_{\text{TEM}}$, which was not detected by metagenomics despite a
391 Ct indicating higher abundance than other ARGs, perhaps because it was still close to
392 the coverage limit.
393 In one of the few studies measuring the efficiency of viral metagenomics in
394 comparison with PCR, the two approaches produced divergent results in different
395 matrices spiked with murine norovirus.[35] When sequencing the virome, it is quite
396 difficult to estimate the level of coverage needed to detect less abundant genes. [52]

Again, different software such as Prokka and ResFinder gave non-matching results. Prokka combines the output of different tools that predict coding sequences, ribosomal RNA, transfer RNA, signal leader peptides and non-coding RNA, whereas ResFinder uses BLAST to identify ARGs. As each approach analyzes sequences from a different standpoint, their results are not comparable, but both can provide valuable insights.

No specific phage sequences were found in the ARG flanking regions but notably ARGs were located next to MGEs such as transposons or insertion sequences (IS). This prevents the identification of the origin of the DNA (whether bacterial or phage). As these elements are also present in the bacterial genomes, they could provide platforms for ARG mobilization. The flanking regions were mostly identified as belonging to *Bacteroides fragilis*. Since Bacteroidetes is the phylum most extensively represented in our samples, this could explain why metagenomics detected more ARGs related to these bacterial groups (Table 4). In contrast, β-lactamase genes (excluding those from Bacteroidetes such as bla_{CFXA6}) are mostly associated with Enterobacteriaceae, [53] present in the gut microbiome to a lesser extent than Bacteroidetes. [54] Thus, β-lactamase genes or *qnr* genes belonging mostly to the less abundant Enterobacteriaceae were only detected by the more sensitive qPCR assays and not by metagenomics.

The samples analyzed before and after the ciprofloxacin treatment gave different results. The PRECIP sample belongs to the 20% of fecal samples negative for ARGs in phage DNA by qPCR described in previous work, [25,55] whereas the POSTCIP sample showed different mechanisms of resistance to various drugs. Although the absence of ARGs in the normal state has not been explained, the presence of bacterial genes other than ARGs (including 16S rDNA) in the viral DNA of the PRECIP sample indicates

that genomic DNA packaging occurred, although to a lesser extent than in POSTCIP. It can be speculated that in this case some individual conditions repress the induction of phage particles in general, or specifically those particles containing ARGs from the microbiota, considering the microbiota as the main source of ARG-carrying phage particles. [24] Thus, ciprofloxacin might have reverted this repression or most probably caused an increase in the induction of phage particles, which were detectable for a period after the treatment. Absence of ARGs in phage DNA in samples taken one year later suggests the microbiota of the individual had returned to the PRECIP values.

This observation supports the hypothesis that the presence of ARGs in the DNA from phage capsids of POSTCIP was due to the antibiotic treatment. Stable maintenance of ARGs and their elements in the absence of antibiotic selection has been reported, [56] but this is attributed to other genes in the same element conferring a different kind of selective advantage. In contrast, some antibiotics are known to exacerbate the transfer of their own resistance by the mobilization of genetic elements. [56] Higher rates of phage induction and recombination [57] and greater abundance and diversity have been reported in the presence of antibiotics in the swine gut microbiome. [58] Ciprofloxacin, one of these antibiotics, is reported to be an activator of the SOS response and one of the most effective inducers of MGEs, including phages and derived particles.[12,59,60]

In metagenomic studies, accurate methods and the use of a combination of different software are strongly recommended to increase data output. Despite its limitations, the metagenomic approach confirmed the presence of phage particles containing several sequences of bacterial origin, including ARGs. The packaging mechanisms are unknown but could involve transduction or other strategies by which bacterial DNA is packaged, protected and spread. However, the association of ARGs

with phage particles points to the latter as a likely system of resistance dissemination among the bacterial community. Future studies could elucidate the impact of phages on the regulation of microbiota and the spread of multi-resistant bacteria as a problem of global health.

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Transparency of declaration

All authors have none to declare

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641

642 **Table 1.** Oligonucleotides used in this study

ARG	Assay	Oligonucleotide	Sequence	Amplimer (bp)	LOQ of qPCR (Ct)	Reference
bla$_{TEM}$	qPCR-T	UP	CACTATTCTCAGAATGACTTGGT	85	30.5	61
		LP	TGCATAATTCTCTTACTGTCATG			
		probe TEM	FAM-CCAGTCACAGAAAAGCATCTTACGG-MGBNFQ			
bla$_{CTX-M-1}$-group	qPCR-T	UP	ACCAACGATATCGCGGTGAT	101	31.3	62
		LP	ACATCGCGACGGCTTTCT			
		probe CTX-M-1	FAM–TCGTGCGCCGCTG- MGBNFQ			
bla$_{CTX-M-9}$-group	qPCR-T	UP	ACCAATGATATTGCGGTGAT	85	30.5	26
		LP	CTGCGTTCTGTTGCGGCT			
		probe CTX-M-9	FAM – TCGTGCGCCGCTG- MGBNFQ			
bla$_{OXA-48}$	qPCR-T	UP	CGGTAGCAAAGGAATGGCAA	133	32.4	24
		LP	TGGTTCGCCCGTTTAAGATT			
		probe OXA-48	FAM – CGTAGTTGTGCTCTGGA- MGBNFQ			
bla$_{CfxA6}$	qPCR-S	UP	GCGCAAATCCTCCTTTAACAA	802	31.5	63
		LP	ACCGCCACACCAATTTCG			
ermF	PCR-S	UP	CGACACAGCTTTGGTTGAAC	309	34.0	64
		LP	GGACCTACCTCATAGACAAG			
mecA	qPCR-T	UP	CGCAACGTTCAATTTAATTTTGTTAA	92	33.8	65
		LP	TGGTCTTTCTGCATTCCTGGA			
		probe mecA	FAM-AATGACGCTATGATCCCAATCTAACTTCCACA-MGBNFQ			
mefA	qPCR-S	UP	AGTATCATTAATCACTAGTGC	348	33.7	66
		LP	TTCTTCTGGTACTAAAAGTGG			
qnrA	qPCR-T	UP	AGGATTGCAGTTTCATTGAAAGC	138	34.0	13
		LP	TGAACTCTATGCCAAAGCAGTTG			

		probe qnrA	FAM-TATGCCGATCTGCGCGA- MGBNFQ			
qnrS	qPCR-T	UP	CGACGTGCTAACTTGCGTGA	118	34.6	[13]
		LP	GGCATTGTTGGAAACTTGCA			
		probe qnrS	FAM-AGTTCATTGAACAGGGTGA-MGBNFQ			
sul1	qPCR-T	UP	CCGTTGGCCTTCCTGTAAAG	67	34.5	[67]
		LP	TTGCCGATCGCGTGAAGT			
		probe sul1	FAM-CGAGCCTTGCGGCGG-MGBNFQ			
tetW	qPCR-T	UP	GACGGACACCATGTTTTTGGA	62	33.7	This study
		LP	AGGAAGTGACTGCCGCTTGA			
		probe tetW	AGCGTGGGATTACCA			
16S rDNA	qPCR-T	UP	TCCTACGGGAGGCAGCAGT	467	32.2	[68]
		LP	GGACTACCAGGGTATCTAATCCTGTT			
		probe 16S	FAM-CGTATTACCGCGGCTGCTGGCAC-MGBNFQ			

qPCR-T: quantitative real time PCR using TaqMan hydrolysis probes.
qPCR-S: quantitative real time PCR using a SybrGreen assay.
UP: upper primer, LP: lower primer, FAM: 6-carboxyflouorescein reporter, MGBNFQ: Minor groove binding non-fluorescent quencher.
LOQ. Limit of quantification determined with the standard curve used in this study for each qPCR assay.

Table 2. Bioinformatic analysis of DNA from phage particles**.**

Sample	# Reads	#Contigs	Kraken		PHASTER	Prokka		VirSorter			
			Nº taxons[a] (%)	Nº viruses[b] (%)	Nº of phages	Nº of phage genes	CDS	Category 1[c]		Category 2[d]	
								Nº phage contigs[e]	Nº phage genes[f]	Nº phage contigs[e]	Nº phage genes[f]
PRECIP	1653277	11100	532 (4.8)	11 (2.0)	17	40	7162	7	159	51	1379
POSTCIP	1612020	18469	1689 (9.1)	182 (10.8)	12	41	10769	4	28	61	1014
HC6	1758529	6015	842 (14.0)	4 (0.5)	12	25	2297	2	7	13	391
HC9	1799296	4604	440 (9.6)	4 (0.9)	10	39	4050	1	21	31	968
HC10	1372547	3838	1113 (29.0)	266 (23.9)	7	31	2415	0	0	34	739
UB6	1954802	17814	3035 (17.0)	56 (1.8)	12	35	10419	6	79	48	1298
UB25	1603968	4949	781 (15.8)	24 (3.0)	8	21	6457	4	69	28	1022

[a] All microorganisms: archaea, bacteria and viruses.
[b] viruses including phages.
[c] "most confident prediction": high probability of phage identification.
[d] "likely": good probability of phage identification.
[e] Nº of contigs where genes of phages have been identified.
[f] Nº of genes of phages identified in all contigs.
CDS: Coding regions.

Table 3. DNA from phage particles **of the samples**. Percentages of sequences identified by Kraken as different phages.

Phages	**Hosts**	**PRECIP**	**POSTCIP**	**HC6**	**HC9[a]**	**HC10[a]**	**UB6**	**UB25**
Caudovirales		**91**	**100**	**75**	**50**	**99.2**	**100**	**100**
Siphoviridae		63.7	99.5	75	25	99.2	100	100
Skunalikevirus	*Lactococcus*	27	31	50		31	21	29
C2likevirus	*Lactococcus*					9		
Unclassified	*Lactococcus*	27	57	25		55.4	41	66
	Lactobacillus	9						5
	Leuconostoc		2				33	
	Enterococcus						2	
	Propionibacterium						2	
	Rhodococcus				25			
	Others	0.7	10		25	3.8	1	
Myoviridae		27.3						
	Clostridium	9						
	Bacillus	9						
	Others	9.3						
Podoviridae			0.5					
Microviridae	*Enterobacteria*			**25**				
Mimiviridae	*Unclassified*				**50**			
Unassigned		**9**				**0.8**		

[a] subject HC9 travelled to the Republic of Ecuador; subject HC10 travelled to Peru

659 **Table 4**. **ARGs, ARG flanking regions and 16S rDNA in the DNA from phage particles**

	ARG			ARG Flanking regions	16S rDNA
	qPCR(Ct)	**Prokka** [b]	**ResFinder**	**(length of the flanking fragment)** (GenBank Accession #)	**# contigs**
PRECIP	---	MdtH, MdtK	---	---	7
POSTCIP	*mecA* (32.76), *qnrA* (32.09), *qnrS* (32.94), ***tet(W)*** **(25.35)**	Aluminium[c], Bicyclomycin, CzcA[c], DrrA, Glyoxalase/Bleomycin, operon repressor, MdtA, MdtB, MdtC, MdtE, MdtK, MexA, MexB, NorM, MarA, YheH, EmrK, SugE, TerB, TetO, TelA, Trypsin-resistant surface T6 protein, VanW	*bla*$_{CfxA5}$	*Bacteroides fragilis* insertion sequence IS*614B* **(93 bp)** (AY769934)	16
			tet(W)	*Faecalibacterium prausnitzii* plasmid mobilization relaxosome protein MobC **(5050 bp)** (CP026548.1)	
HC6	*bla*$_{TEM}$ (28.45), *bla*$_{CTX-M-1}$ group (32.09) [a], *bla*$_{CfxA6}$ (26.74), ***erm(F)*** **(30.17),** *qnrA* (34.29) [a], *qnrS* (34.59) [a], *tet(W)* (29.99)	---	*erm(F)*	*No flanking regions, contig aligns totally*	3
HC9	*bla*$_{TEM}$ (28.26), *bla*$_{CTX-M-1}$ group (32.97)[a], *bla*$_{CTX-M-9}$ group (32.74) [a], *bla*$_{CfxA6}$ (28.88)	CzcA[c], TerB	---	---	6
HC10	*bla*$_{CTX-M-1}$ group (32.86) [a], *qnrS* (30.12), *tet(W)* (31.94)	---	---	---	4
UB6	*bla*$_{TEM}$ (28.43), *bla*$_{CTX-M-9}$ group (33.41) [a], ***bla***$_{CfxA6}$ **(22.43),** ***mef(A)*** **(30.15),** *sul1* (32.79), *tet(W)*(27.09)	BmrA, CnrB[c], CzcA[c], DrrA, Fosmidomycin, Lincosamide, MarA, MecR1, MdtE, MdtK, TetM	*aadE*	*No flanking region, contig aligns totally*	13

			bla$_{CfxA}$	*B. fragilis* transposon Tn*4555* TnpA, integrase, TnpC, excisionase, mobilization protein and unknown genes (**1118 bp**) (U75371.3)	
			bla$_{CfxA6}$	*No flanking regions, contig aligns totally*	
			mef(A)	*B. fragilis* strain HMW 615 transposon CTnHyb **(304 bp)** (KJ816753.1)	
			tet(Q)	HMW615 transposon CTnHyb (**1836 bp**)(KJ816753.1)	
			tet(X)	*B. fragilis* transposon Tn*4351* (**1836 bp**)(M37699.1)	
UB25	*bla*$_{TEM}$ (28.21), *bla*$_{OXA-48}$ (34.10)[a], *bla*$_{CTX-M-9}$ group (29.81), ***bla*$_{CfxA6}$ (27.73), *mef(A)* (26.21),** *qnrS* (30.85), *qnrA*(34.25) [a] , *sul1* (32.58), *tet(W)* (24.37)	Fosmidomycin, CnrB[c] CzcA[c], Lincosamide, MdtE, MdtK, TetM	***bla*$_{CfxA}$**	*Bacteroides ovatus* insertion sequence IS*4*-like **(127 bp)** (HE999703.1)	16
			mef(A)	*B. fragilis* transposon CTnHyb**, (277 bp)** (KJ816753.1)	
			tet(Q)	*B. fragilis* transposon Tn*4351* (**390 bp**)(M37699.1)	
			tet(X)	*B. fragilis* transposon Tn*4351* (**390 bp**)(M37699.1)	

[a]Beyond the limit of quantification of the standard curve of each qPCR assay but still detectable.

[b]The annotation of specific proteins responsible for antibiotic resistance or the elements to which resistance is conferred are indicated.

[c] Proteins conferring resistance to metals.

Ct: Threshold cycle.

ARGs in bold indicate coincidence between qPCR and ResFinder results.

Figure legends

Figure 1. Pie Graphs of Kraken data for samples HC6, HC9, PRE-CIP and POSTCIP from healthy individuals. Distribution and relative abundance of different bacteriophages identified in the viromes with regard to the total viral sequences identified. Interactive pie graphs available upon request. This figure appears in colour in the online version of *JAC* and in black and white in the printed version of *JAC.*

Figure 2. Pie Graphs of Kraken data showing metagenomic comparison of the virome of the two samples from the same individual taken before (PRECIP) and after (POSTCIP) ciprofloxacin treatment. This figure appears in colour in the online version of *JAC* and in black and white in the printed version of *JAC.*