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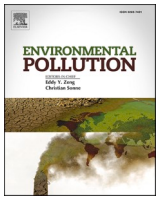

CrAss-like phages are suitable indicators of antibiotic resistance genes found in abundance in fecally polluted samples☆

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ABSTRACT

Antibiotic resistance genes (ARGs) have been extensively observed in bacterial DNA, and more recently, in phage particles from various water sources and food items. The pivotal role played by ARG transmission in the proliferation of antibiotic resistance and emergence of new resistant strains calls for a thorough understanding of the underlying mechanisms. The aim of this study was to assess the suitability of the prototypical p-crAssphage, a proposed indicator of human fecal contamination, and the recently isolated crAssBcn phages, both belonging to the Crassvirales group, as potential indicators of ARGs. These crAss-like phages were evaluated alongside specific ARGs (bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$, bla_{VIM}, $bla_{OXA\text{-}48}$, *qnrA*, *qnrS*, *tetW* and *sul1*) within the total DNA and phage DNA fractions in water and food samples containing different levels of fecal pollution. In samples with high fecal load (>10^3 CFU/g or ml of *E. coli* or somatic coliphages), such as wastewater and sludge, positive correlations were found between both types of crAss-like phages and ARGs in both DNA fractions. The strongest correlation was observed between *sul1* and crAssBcn phages (rho = 0.90) in sludge samples, followed by $bla_{CTX\text{-}M\text{-}9}$ and p-crAssphage (rho = 0.86) in sewage samples, both in the phage DNA fraction. The use of crAssphage and crAssBcn as indicators of ARGs, considered to be emerging environmental contaminants of anthropogenic origin, is supported by their close association with the human gut. Monitoring ARGs can help to mitigate their dissemination and prevent the emergence of new resistant bacterial strains, thus safeguarding public health.

1. Introduction

The growing resistance to antibiotics is a serious public health concern as it is undermining the effectiveness of treatments for bacterial diseases. Recognized as a global problem (Murray et al., 2022), antibiotic resistance is increasing the morbidity and mortality associated with bacterial infections as well as healthcare costs. The level of antimicrobial resistance, particularly multiple resistance in widely disseminated bacterial strains, has reached unprecedented levels, driven by the pressure of increased antibiotic usage (in both human and animal medicine), greater population movement, and industrialization (Prestinaci et al., 2015; Holmes et al., 2016).

Antibiotic resistance genes (ARGs) are abundant in environmental bacteria, and it has been proposed that many of them have an environmental origin. It is feasible that ARGs can transfer, via various mechanisms, from environmental strains to pathogenic bacteria found in clinical settings. Horizontal gene transfer constitutes the single most important mechanism that accelerates ARG dispersal, and the most studied mobile genetic elements involved are plasmids, transposons, and phages (Muniesa et al., 2013). Although ARGs have usually been detected in the bacterial DNA fraction, recent studies have focused on the potential role of bacteriophages as a gene transfer mechanism (Colavecchio et al., 2017; Gabashvili et al., 2020) and there is growing evidence for the occurrence of ARGs in the phage DNA fraction of environmental, food, and human samples (Anand et al., 2016; Subirats et al., 2016; Barrios et al., 2021; Calero-Cáceres and Balcázar, 2019; Colomer-Lluch et al., 2011; Sala-Comorera et al., 2021; Strange et al., 2021). Considering that bacterial cells and phages exhibit different levels of persistence in the environment (Allué-Guardia et al., 2014; Calero-Cáceres and Muniesa, 2016; Calero-Cáceres et al., 2017), it is

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plausible that ARG occurrence and persistence may differ between bacterial and phage DNA fractions, a possibility that requires further investigation.

As different environments can act as ARG reservoirs and contribute to their dissemination, potentially leading to the emergence of new resistant strains, ARGs are now considered as environmental contaminants (García et al., 2020). Measures to mitigate the introduction of ARGs into the environment would initially involve the implementation of monitoring systems, although the analysis of all ARGs is rendered unfeasible by their sheer diversity. A more practical approach would therefore be the application of a suitable indicator. Additionally, as most ARGs detected in clinical and veterinary settings and in water bodies are suspected to have an anthropogenic origin (Di Cesare et al., 2023; Wang et al., 2023) and anthropogenic pressure is recognized as a key factor in the selection and spread of ARGs (Li et al., 2020; Pruden et al., 2012), the use of a human-specific indicator would be advisable.

In recent decades, various human-specific markers of fecal contamination have been proposed (Bernhard and Field, 2000; Shanks et al., 2008; Mieszkin et al., 2009; Gómez-Doñate et al., 2012; Green et al., 2014) without a clear consensus on which are the most effective. Among them, crAssphage (cross-assembly phage), a group of highly abundant human gut bacteriophages, has been attracting growing interest (Dutilh et al., 2014). The prototypical crAssphage (p-crAssphage), the first member of the order Crassvirales, has recently been classified as belonging to the species *Carjivirus communis* within the family Intestiviridae (Turner et al., 2023). Since the discovery of p-crAssphage, other crAss-like phages with similar genomic architecture and sharing the same ancestor with p-crAssphage, have been described and grouped within the order Crassvirales ("Current ICTV Taxonomy Release | ICTV," n.d.; Guerin et al., 2018; Yutin et al., 2021), which currently comprises four families, 11 subfamilies, 42 genera, and 73 species (https://ictv.global/taxonomy). Many crAss-like phages are highly abundant in the mammalian gut, and all of them seem to infect bacteria in the phylum Bacteroidetes, specifically the *Bacteroides* genus, based on the few virions isolated to date.

p-crAssphage is a specific and abundant human fecal phage with a global distribution (Dutilh et al., 2014; Edwards et al., 2019). Although never isolated, p-crAssphage has been proposed as a suitable candidate for use in microbial source tracking (MST) to detect human fecal pollution, and several quantitative polymerase chain reaction (qPCR) assays targeting its genome have been designed for this purpose (Stachler and Bibby, 2014; Stachler et al., 2017; García-Aljaro et al., 2017). Recently, our research group isolated a new group of 25 crAss-like phages from the geographical area of Barcelona, NE Spain, named crAssBcn phages (Ramos-Barbero et al., 2023). These phages were identified as belonging to the family Steigviridae, genus *Keishivirus*, and six different species, including *Keishivirus communis,* the same species as the first isolated crAss-like phage, ϕCrAss001 (Ramos-Barbero et al., 2023). As they have a worldwide distribution and high specificity for human feces, crAssBcn phages are also suitable candidates for MST. Moreover, crAssBcn phages have an advantage over p-crAssphage in that they have all been isolated, which reinforces their suitability for application as human-specific indicators of pollution. This phage group can be analyzed using a single qPCR assay targeting common sequences of CrAssBcn and ϕCrAss001 phages (Ramos-Barbero et al., 2023).

In the present study, possible correlations of p-crAssphage and the new crAssBcn phages with a range of ARGs were investigated by analyzing their abundances in samples with varying levels of human fecal pollution (urban wastewater, sludges, and food). Our aim was to evaluate whether crAss-like phages, besides being human-specific markers of fecal pollution, can also serve as indicators of ARGs, either in the bacterial or phage DNA fraction.

2. Materials and methods

2.1. Samples

Fifty raw influent samples were obtained from two urban wastewater treatment plants (WWTPs) in Catalonia (NE Spain). Both WWTPs (Besòs and Prat) serve a population of more than 2,000,000 inhabitants. The same plants were also the source of 40 sewage sludge samples consisting of a composite mixture of raw primary sludge (about two-thirds) and secondary sludge (about one-third), which was thickened and submitted to anaerobic-mesophilic (35 °C) digestion for 20–25 days. Additionally, different types of food (meat, fish, vegetables, and fish) were analyzed. The meat samples consisted of 20 g of veal (n = 4), pork (n = 5), and chicken (n = 5). The fish consisted of 20 g of sardine (n = 5) and shellfish (mussels (n = 5) and clams (n = 5)). The vegetables consisted of 25 g of lettuce (n = 5), chard (n = 5), and strawberries (n = 5). For the milk samples, a total of 20 ml of fresh milk (n = 5) was analyzed (see Table 1). All food samples were considered to be fresh samples as they had not undergone any packaging or freezing process and were purchased from local retailers in the Barcelona area (Spain) between 2020 and 2023.

Samples were collected in sterile containers and transported to the laboratory at 4 °C in less than 2 h. Upon arrival, samples were immediately processed for the analysis of microbial indicators and bacteriophage isolation, as described below.

Liquid samples (raw sewage and milk) were analyzed directly as described below. Solid samples, namely sludge and food, were homogenized in 1:5 (*w:v*) phosphate buffer (PBS) pH = 7.4 by shaking for 30 min. The resulting homogenate was used for the analysis of bacterial indicators and the extraction of the total DNA fraction. For phage analysis, 50 ml of the water sample or 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) that allowed viral particles to pass. The filtrate was used for the analysis of somatic coliphages and the extraction of DNA from the viral fraction as described below. In the case of sludge samples, the measurement of sludge dry weight was additionally performed to minimize variations arising from differences in water content among samples. The dry weight was determined by comparing the weight of sludge samples before and after being dried at a temperature of 105 °C for 24 h.

2.2. Bacterial and viral indicators

Samples were evaluated for the presence of the general fecal indicators *Escherichia coli* and somatic coliphages. To do this, the water samples and homogenates of solid samples were diluted 1/10 and 1/100. A volume of 0.1 ml of each dilution was plated on Chromocult Coliform Agar (Merck, Darmstadt, Germany). Incubation was initially performed for 2 h at 37 °C to allow potentially damaged microorganisms to adapt and then overnight at 44 °C.

Somatic coliphages, proposed as viral indicators of fecal pollution (Jofre, 2007), were evaluated after filtration of the liquid samples or homogenates to detect the presence of infectious fecal viruses. Decimal dilutions of the filtrates were prepared, and 1 ml volumes of each were analyzed in duplicate by the double agar layer method for the presence of somatic coliphages, following the ISO standard method (Anonymous, 2000) that uses *E. coli* strain WG5 (ATCC 700078) as the bacterial host. Plates were incubated at 37 °C for 18 h. Each homogenate was analyzed in duplicate.

2.3. Purification of total and phage DNA from phage particles

2.3.1. Total DNA fraction

Two hundred μl of raw sewage and food samples after homogenization were subjected to DNA extraction using the Qiagen DNA Blood kit (Qiagen Inc., Hilden. Germany). For the extraction of DNA from sludge, 250 mg was processed using the Power Soil kit (Qiagen Inc., Hilden.

Table 1
Origin of the analyzed samples and mean concentrations of fecal microbial indicators (*E. coli* and somatic coliphages).

	Group	Type	*E. coli* (Log_{10} CFU/ml or g ± SD)	Somatic coliphages (Log_{10} PFU/ml or g ± SD)
Urban sewage	WWTP 1	Besòs	4.5 ± 0.2	4.4 ± 0.2
	WWTP 2	Prat	4.6 ± 0.2	4.4 ± 0.2
Sludge	WWTP 1	Besòs	4.5 ± 0.3	4.6 ± 0.7
	WWTP 2	Prat	4.1 ± 0.4	4.2 ± 0.9
Food-Meat	Meat	Pork	1.3 ± 1.0	1.4 ± 0.6
	Meat	Veal	1.0 ± 0.6	0.3 ± 0.0
	Meat	Chicken	2.1 ± 0.4	0.7 ± 0.4
Food-Fish	Fish	Sardines	ND	ND
	Shellfish	Clams	0.7 ± 0.4	1.6 ± 0.6
	Shellfish	Mussels	ND	0.9
Food-Vegetable	Vegetable	Lettuce	0.6 ± 0.2	0.2
	Vegetable	Chards	0.3 ± 0.4	ND
	Fruits	Strawberry	0.5 ± 0.4	0.3 ± 0.0
Food-Milk	Dairy	Fresh Milk	ND	ND

WWTP, wastewater treatment plant; ND: Not detected; SD: standard deviation.

Germany), following the manufacturer's instructions. Total DNA was suspended in a final volume of 200 μl of ultrapure water and kept at −20 °C until further analysis.

2.3.2. Phage DNA fraction

To purify the phage fraction, the filtrated liquid samples or the filtrated homogenates of solid samples were treated with chloroform 1:10 (v:v), mixed by vigorous vortexing for 5 min, and centrifuged at 16000×*g* for 10 min. This step was performed to rule out the presence of possible vesicles containing DNA. Additionally, the samples were treated with DNase I (100 units/ml; Sigma-Aldrich. Spain) at 37 °C for 1 h to eliminate any non-packaged DNA present in the samples outside the phage particles. DNase I was then inactivated by heating for 5 min at 75 °C. Sludge samples were treated with 100 μl of DNase (10 mg/ml), while the other samples were treated with 40 μl of DNase (10 mg/ml).

At this stage of the treatment, phage capsids remain intact, so no vesicles should have been released, whereas any free DNA outside the phage capsids should have been removed. To confirm the absence of non-packaged ARGs, an aliquot was taken and used as a template for qPCR amplification (Table S1). The protocols applied here for DNase treatment and DNase inactivation have been extensively verified in previous studies (Colomer-Lluch et al., 2014; Fernández-Orth et al., 2019; Blanco-Picazo et al., 2023).

To break the capsids and release the genetic material, the samples were treated with proteinase K (20 mg/ml) in 250 μl of proteinase K buffer and incubated for 1 h at 55 °C. Encapsidated DNA was extracted with phenol-chloroform (1:1) (v:v) treatment, and the aqueous phase was further treated with chloroform (1:1) (v:v), centrifuging at the same speed and time as in the previous step. The remaining phenol/chloroform was removed by adding the mixture to Phase Lock Gel Tubes (5-Prime, Hucoa Erlöss, Madrid. Spain) and centrifuging following the manufacturer's instructions. DNA was precipitated using 100 % ethanol and 3 M sodium acetate, then resuspended in 200 μl of ultrapure water. DNA was quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientifics, Wilmington, DE. US).

2.4. qPCR assays

2.4.1. ARG quantification

Samples were analyzed for the presence of nine ARGs conferring resistance to different groups of antibiotics (Table S1) in the total DNA and phage DNA fractions of the samples. The ARGs were selected for conferring resistance to different antibiotic groups relevant in clinical settings, abundant in the environment or used in veterinary medicine. These included β-lactamase genes that confer resistance to β-lactam antibiotics, some of which detect different gene variants (bla_{TEM}, $bla_{CTX\text{-}M}$ group 1 and group 9, $bla_{OXA\text{-}48}$ and bla_{VIM}), two quinolone resistance genes (*qnrA* and *qnrS*), *sul1*, which confers resistance to sulfonamides and is frequently found in environmental and clinical bacterial populations, and *tetW*, which is commonly found in the gastrointestinal tracts of animals and humans as well as in soil and water environments (Colomer-Lluch et al., 2011; Pruden et al., 2012; Mehdi et al., 2018). Quantitative real-time PCR (qPCR) was performed using TaqMan hydrolysis probes targeting beta-lactamases (bla_{TEM}, $bla_{CTX\text{-}M}$ group 1 and group 9, bla_{VIM} and $bla_{OXA\text{-}48}$), sulfonamides (*sul1)*, quinolones (*qnrA* and *qnrS*), and tetracyclines (*tetW*). In food samples, with lower levels of fecal pollution, the analysis was restricted to four of the most abundant ARGs (*sul1*, bla_{TEM}, *tetW*, and *qnrS*).

2.4.2. CrAss-like phage quantification

Samples were analyzed for the presence of crAss-like phages in the total DNA and phage DNA fractions of the samples. For this purpose, two qPCR assays were used, one detecting p-crAssphage (García-Aljaro et al., 2017), and one designed to detect the new group of crAssBcn phages (Ramos-Barbero et al., 2023).

2.4.3. Amplification conditions

Amplification was performed using the standard run of the StepOne Real Time PCR System (Applied Biosystems, Foster City, US) in a 20 μl reaction mixture with TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Foster City, US). The reaction included 9 μl of the sample DNA or standards with known DNA concentration. The results were analyzed with the Applied Biosystems StepOne Instrument program.

For quantification of crAss-like phages and ARGs, serial dilutions of a known concentration of gBlocks Gene Fragments (Integrated DNA Technologies, Coralville, IA, US) were used for each ARG to generate the standard curves in each qPCR assay. Standard curves were prepared using serial decimal dilutions of known concentrations of gene copies (GC)/μl with double-distilled water of commercial gBlocks, as indicated by the manufacturer. Each dilution was amplified in triplicate in at least five independent runs, and the average cycle threshold (Ct) values and GC number for each dilution were used to calculate the abundance of each gene in the volume tested. A duplicate of each dilution of the standard curve, each sample, and the negative control (nuclease-free water) was run in each plate. To evaluate crAss-like phages and ARG abundance, the GC results were calculated with the standard curves using the last valid Ct for each ARG assay as the limit of quantification (LOQ) (Table S1), when the standard curve was consistent in the different replicates. Each GC detected, corresponds respectively to one crAss-like phage or to one phage particle containing an ARG. The standards were also used as positive controls. To screen for PCR inhibitors, samples were diluted 1/10 in nuclease-free water and analyzed in parallel.

2.5. Statistical analysis

Data computation, statistical tests, and chart generation were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, US). The data corresponding to the different samples and ARGs are presented as scatter plots displaying the mean values of all samples with values above the LOQ. Data from all sample types were compared using the Kruskal–Wallis test. Spearman's ranks correlation test was used to detect relationships between the individual ARGs and the two types of crAss-like phages, as well as with the sum of all ARGs (total ARGs).

3. Results and discussion

3.1. Levels of fecal pollution in the samples

The samples were classified according to the level of fecal pollution. The concentration of general fecal indicators has been shown to be relevant for determining the origin of fecal pollution using molecular markers by qPCR (Casanovas-Massana et al., 2015). In fact, a threshold concentration of 3 $\log_{10}$ colony forming units (CFU)/100 ml was stablished for *E. coli*, below which some molecular markers used in microbial source tracking were not suitable because their concentrations decreased below the detection limit. This threshold was used for classifying the samples of our study in two groups according to the concentration of fecal indicators: high (urban raw sewage and sludge) or low (food).

The abundance of the fecal indicators (*E. coli* and somatic coliphages) differed significantly ($p < 0.05$) between the two sample groups. In urban raw sewage and sludge, the mean concentration of both indicators was approximately 4.5 $\log_{10}$ units per ml or g of dry weight, except in sludge from WWTP 2, where slightly lower values were observed, although without statistical significance ($p > 0.05$) (Table 1).

In the food samples, the concentration of both fecal indicators was highest in meat. Values for *E. coli* ranged from 2.1 to 1 $\log_{10}$ CFU/g in chicken, veal, and pork. The mean concentration of somatic coliphages in pork was in the same order of magnitude as *E. coli* while in chicken and veal it was approximately 1 $\log_{10}$ units lower. In the shellfish samples, the concentration of *E. coli* was below 1 $\log_{10}$ CFU/g, whereas that of somatic coliphages was approximately 1 $\log_{10}$ higher. No fecal indicators were observed in sardines or milk. On the other hand, the concentration of *E. coli* in strawberries and leafy vegetables was very low, the mean values being below 1 $\log_{10}$ CFU/g in both sample types; the values obtained for somatic coliphages were similar.

The obtained values for the fecal indicators in the sewage and sludge samples are in accordance with previous studies (see García-Aljaro et al., 2019; Martín-Díaz et al., 2020, for review). In the case of food samples, the concentrations found in the literature vary depending on the sample origin as well as the sources of contamination. In the case of meat samples, fecal contamination could have occurred through direct contact with the gastrointestinal tract contents during processing or through cross-contamination from food handlers or contaminated surfaces. For vegetables, contamination could have resulted from irrigation with contaminated water or the use of organic fertilizers. Meanwhile, shellfish may have become contaminated because of the presence of fecally polluted water in their growing areas (Doyle et al., 2020). It has to be noted that all the food samples of this study, considering the microbiological parameters evaluated, were suitable for consumption according to EU regulations (Anonymous, 2005). Based on these results, the samples were further analyzed as indicated below.

3.2. Abundance of ARGs, p-crAssphage, and crAssBcn phages in samples with a high human fecal load

All the analyzed ARGs were detected in the total DNA fraction of urban sewage samples from both treatment plants with a prevalence higher than 80 % except for *qnrA*, which was detected in 56 % of samples in WWTP 1 and 77 % in WWTP 2. As no statistically significant differences in ARG concentration were observed between the two WWTPs ($p > 0.05$), the results for wastewater and sludge from both plants are presented together (Fig. 1 A and B). The most abundant ARGs in the total DNA and phage DNA fractions in wastewater were *sul1*, *qnrS*, and *tetW* with median concentrations of ~6 $\log_{10}$ GC/ml (Figs. 1 and 2). In the phage DNA fraction, the median concentration of *sul1*, *qnrS*, and *tetW* was around ~3 $\log_{10}$ units lower than in the total DNA fraction.

In the case of sludge, once again, no statistically significant differences were observed in ARG concentrations between samples from WWTP 1 and 2 ($p > 0.05$), and hence the results from both plants for each gene and fraction are presented together in Figs. 1 and 2. All the targeted ARGs were detected in the total DNA fraction with a prevalence higher than 75 %, the most abundant gene being *sul1*, with a median concentration of 8.6 $\log_{10}$ GC/g dry weight (dw), followed by *qnrS* and *tetW* (7.5 and 6.8 $\log_{10}$ GC/g dw, respectively). The median concentrations of bla_{TEM} and *qnrA* were ~6 $\log_{10}$ GC/g, whereas those of $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}9}$ were lower by 1 $\log_{10}$ unit.

The ARGs observed in the phage fraction showed a different prevalence: *sul1* (90%), *tetW* (80%), *qnrS* (55%), and $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}9}$ (2 % and 9 %, respectively). All of them were detected at a median concentration of ~2 $\log_{10}$ GC/g dw, differing between ~3 and ~6 $\log_{10}$ units with respect to the total DNA fraction, which is in accordance with previous findings (Calero-Cáceres et al., 2014). All other ARGs were below the detection limit.

p-crAssphage and the newly described crAssBcn phages were detected in 100 % of urban wastewater and sludge samples in both the total and phage DNA fractions. In urban wastewater, their median concentrations ranged from 4.7 to 4.9 $\log_{10}$ GC/ml in the total DNA fraction, being 1 to 0.5 $\log_{10}$ units lower in the corresponding phage fraction. In sludge, the median concentration of p-crAssphage was 6.3 $\log_{10}$ GC/g in the total DNA fraction and 3 $\log_{10}$ units lower in the phage fraction. Compared to p-crAssphage, the concentration of crAssBcn phages in the total DNA was 1 $\log_{10}$ higher (7.4 $\log_{10}$ units, $p < 0.05$) and the difference in concentration between the two fractions was around 4 $\log_{10}$ units ($p < 0.05$).

The differences between the total and the phage DNA fractions could be attributed to the different DNA extraction methods used for the different fractions. The procedure for phage DNA extraction is the strictest, involving various pre-treatment steps of the samples, including sample filtration, and purification of the bacteriophages by chloroform, as well as treatment with DNAse before DNA extraction using phenol-chloroform. All these steps were introduced to ensure that only phage DNA (encapsidated DNA) is extracted, and that not bacterial or free DNA or vesicles containing DNA could be responsible of the results. This protocol was extensively validated in previous studies (Blanco-Picazo et al., 2022a; Blanco-Picazo et al., 2023). While this protocol minimizes the presence of non-encapsidated DNA, the successive steps may cause losses of DNA that may vary in each extraction procedure. On the contrary, total DNA extraction involved only the use of a DNA extraction column-based commercial kit of the whole sample, which is a more reproducible procedure. In addition to the different protocols used, the phage DNA extract only contains encapsidated DNA, whereas the total DNA extract contains free DNA, cellular (bacterial) DNA, as well as phage DNA outside or inside bacterial cells and vesicles.

The concentrations of crAssBcn and p-crAssphage differed significantly in the total DNA but not the phage DNA fraction. It is possible that crAssBcn phages and p-crAssphage display different replication cycles and burst sizes. The order Crassvirales is highly diverse, with very few phages cultured so far (Shkoporov et al., 2018; Hryckowian et al., 2020; Guerin et al., 2021; Papudeshi et al., 2023; Ramos-Barbero et al., 2023), including crAssBcn phages, in contrast to the prototypical p-crAssphage, which has only been described *in silico*. crAssBcn phages have been established as virulent phages with a relatively large (100 Kb) double-stranded circular DNA genome, but studies on their replication suggest they do not follow a common lytic cycle, that shows a sequence

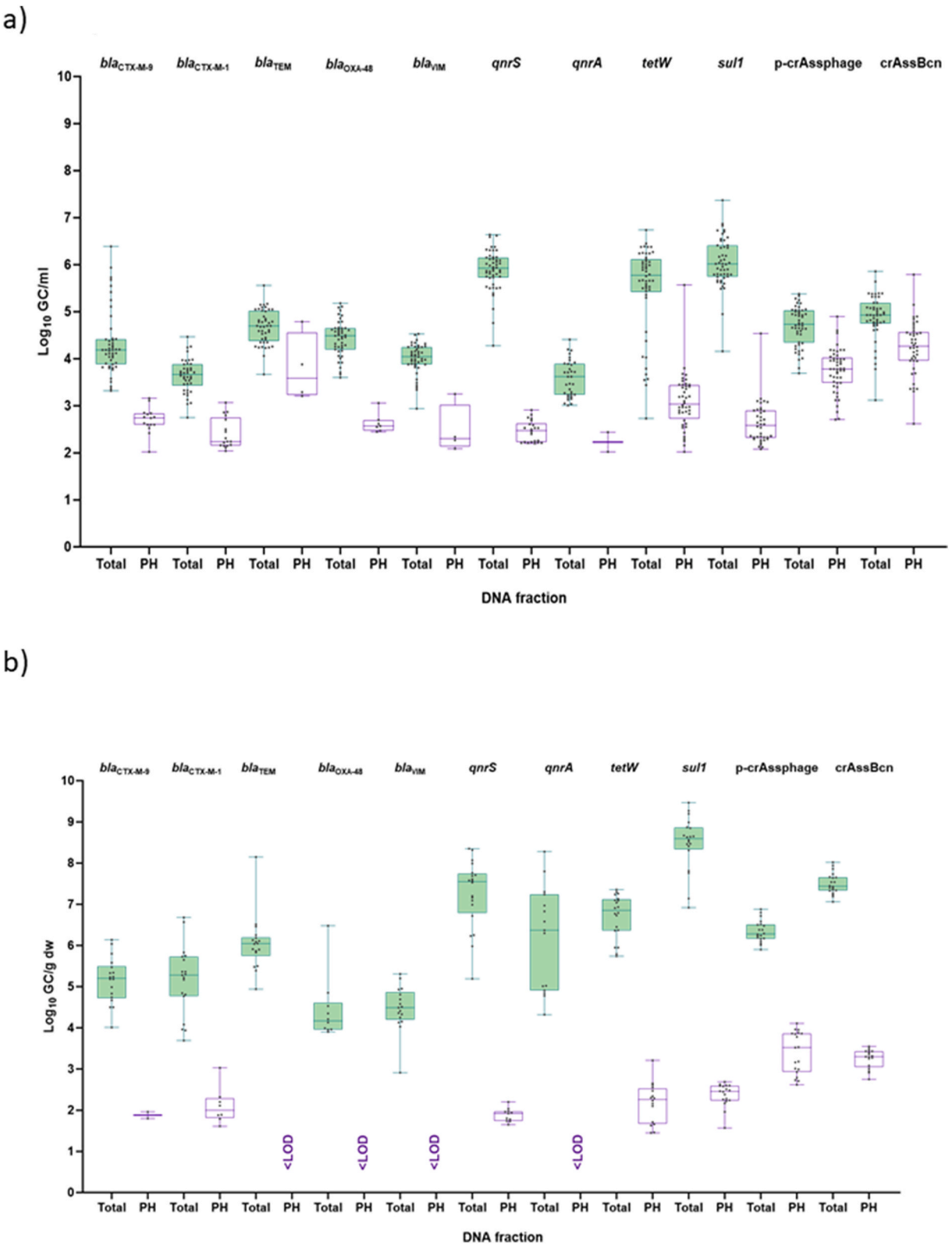

Fig. 1. Box plot representation of antibiotic resistance genes and crAss-like phages in (A) wastewater and (B) sludge samples from WWTP1 and 2 in the total (Total) and phage (PH) DNA fractions. The upper and lower boxes denote the 75th and 25th percentiles. The upper and lower bars show the maximum and minimum values of each gene. <LOD: values below the limit of detection.

of smaller bursts (Ramos-Barbero et al., 2023). However, there is no information about the replication cycle of non-culturable phages as p-crAssphage. Some studies suggest that some crAss-like phages can be found as free phage particles outside the bacterial cell, but also into the bacterial cell, either in lysogenic or pseudolysogenic states or as a plasmid-like elements (Shkoporov et al., 2018; Schmidtke et al., 2024). If the biological cycle of both phages is different, it may account for the different concentrations found in the total DNA fraction (if

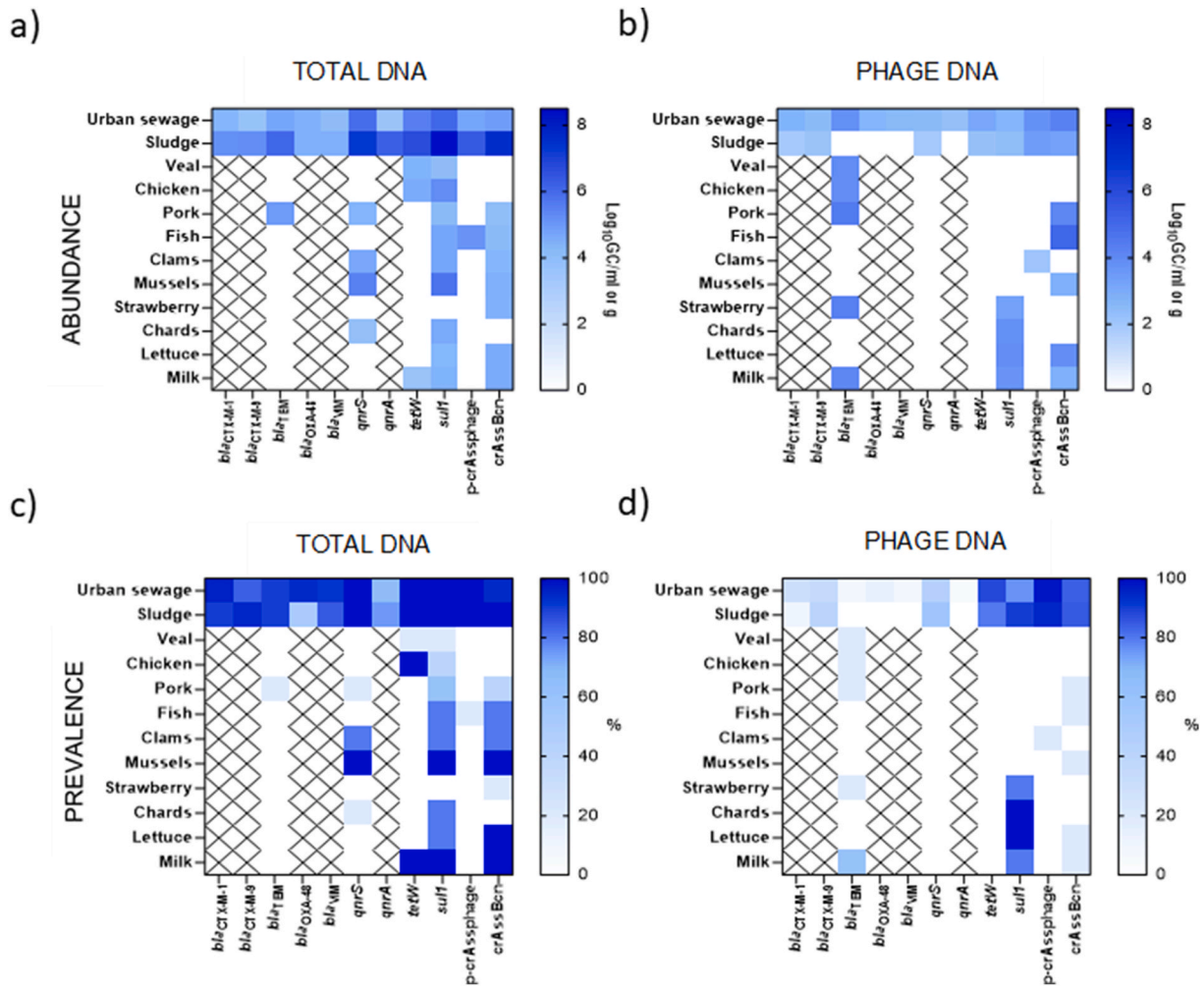

Fig. 2. Heatmap representation of the abundance (A, B) and prevalence (C, D) of different antibiotic resistance genes and crAss-like phages in the total (left) and phage (right) DNA fraction. X, genes not analyzed due to their low concentration.

predominates the phage genome as a plasmid-like state) or in phage DNA fraction (if predominates as free phage particles).

The values obtained for the p-crAssphage marker in this study are similar to those reported for the p-crAssphage markers in previous studies in Spain (García-Aljaro et al., 2017; Ballesté et al., 2019), Japan (Malla et al., 2019), Italy (Crank et al., 2020), Thailand (Kongprajug et al., 2019), US (Wu et al., 2020), and Australia (Ahmed et al., 2018). Similar results have also been reported for the CPQ056 and CPQ06 markers in sludge (Wang et al., 2022). CrAssBcn phages have been detected in human fecal metagenomes worldwide (Ramos-Barbero et al., 2023) but to the best of our knowledge, the present study is the first one to quantify crAssBcn phages in wastewater and sludge.

3.3. *Abundance of ARGs, p-crAssphage, and crAssBcn phages in samples with a low human fecal load*

The prevalence of ARGs in the food samples was highly variable depending on the food type, although *sul1* was the most prevalent gene in both fractions (see Supplementary Table S2). Regarding the total DNA fraction of meat samples, three ARGs (*sul1*, *qnrS* and bla_{TEM}) were detected in pork in at least one sample, the most prevalent being *sul1* (Fig. 2) with a median concentration of 4.1 $\log_{10}$ GC/g (detected in 3/5 samples). A different pattern was observed for chicken, the most prevalent gene being *tetW* (Fig. 2), detected in all samples with a median concentration of 4.6 $\log_{10}$ GC/g, followed by *sul1*, which was observed in two samples. In the total DNA fraction of veal, ARGs were found only in one sample, namely *sul1* and *tetW,* with a concentration of around 4 $\log_{10}$ GC/g.

All milk samples were positive for *sul1* and *tetW*, with a median concentration of 4.4 and 3.8 $\log_{10}$ GC/ml, respectively, while no other ARGs were detected. In vegetables, the most prevalent ARG in the total DNA fraction was *sul1*, its median concentration being approximately 4.7 $\log_{10}$ GC/ml in chard and 4.2 $\log_{10}$ GC/ml in lettuce. Additionally, *qnrS* was detected in a single sample of chard. Strawberries were negative for all the tested ARGs. In fish, *sul1* was the only ARG detected, whereas *sul1* and *qnrS* were detected in 90% of shellfish samples at a concentration of around 5 $\log_{10}$ GC/g (Supplementary Table S2).

In the phage DNA fraction of food samples, only *sul1* and bla_{TEM} were detected, their prevalence differing according to the food type at concentrations ranging between 3 and 4.1 $\log_{10}$ GC/g units for *sul1* and 3.9–4.4 $\log_{10}$ GC/g units for bla_{TEM}. In meat, bla_{TEM} was found in only one sample each of veal, chicken, and pork at a concentration of approximately 4 $\log_{10}$ GC/g units. In vegetables, phages carrying *sul1* were detected at a concentration of around 4 $\log_{10}$ GC/g units (Table S3) and 1 log lower in strawberries. Additionally, one strawberry sample was positive for bla_{TEM}. Phages carrying *sul1* and bla_{TEM} were also detected in milk samples at similar concentrations. The ARG values in the different food samples, all suitable for consumption, in the total and in the phage fraction were in accordance with previous reports analyzing ARGs in food in the bacterial and the phage fraction (Larrañaga et al., 2018; Gómez-Gómez et al., 2019; Blanco-Picazo et al., 2020, 2022a; 2022b, 2023).

CrAssBcn was the most prevalent crAss-like phage detected in the total DNA fraction in food samples, being found only in pork, vegetables, milk, fish, and filter-feeding mollusks at a concentration ranging from 3.7 $\log_{10}$ GC/g in pork to 4.8 $\log_{10}$ GC/g in a filter feeding sample (Table S2). CrAssBcn was also detected occasionally in a strawberry sample. In contrast, p-crAssphage was only detected in a single fish sample.

Regarding the phage DNA fraction, crAssBcn was detected occasionally in pork, fish, mussels, lettuce, and milk, with variable concentrations (Fig. 2B and C), whereas p-crAssphage was only detected in a single sample of clams (Table S3).

3.4. Correlations between crAss-like phages and ARGs in the total and phage DNA fractions

For a more accurate assessment of the relationship between ARGs and crAss-like phages, considering they were found in a context of fecal contamination, the data were normalized with respect to bacterial and phage fecal indicator levels. In this way, fluctuations in fecal load that may affect the correlation analyses would be accounted for. Thus, the concentrations of ARGs in the total DNA fraction were normalized with respect to the fecal bacterial indicator *E. coli,* whereas in the phage DNA fraction they were normalized with respect to somatic coliphage counts. The correlation between ARGs and crAss-like phages varied depending on the type of phage (p-crAssphage or crAssBcn), sample (urban wastewater, sludge, or food) and DNA fraction (total or phage DNA) (see supplementary information, Tables S4–S7).

In the total DNA fraction of urban wastewater samples (Table S6), the normalized concentrations of ARGs of high and intermediate abundance ($bla_{\text{CTX-M-1}}$, bla_{VIM}, *qnrS* and *sul1*) showed statistically significant moderate to strong positive correlations with p-crAssphage (rho 0.46 for *qnrS* and 0.63 for the other ARGs). Also, a strong correlation was found between the total ARGs and p-crAssphage (rho = 0.61). Regarding crAssBcn, the correlation with individual ARGs (in this case, $bla_{\text{CTX-M-9}}$, bla_{TEM}, and $bla_{\text{OXA-48}}$) was moderate (rho between 0.47 and 0.53). In the phage DNA fraction of urban wastewater (Table S7), $bla_{\text{CTX-M-9}}$ was highly strongly positively correlated with p-crAssphage (rho = 0.86).

The differences observed between p-crAssphage and crAssBcn, particularly in the total DNA fractions, could be explained by the presence of different bacterial hosts in the samples and variations in phage replication cycles. As mentioned before, very little is known about the replication cycle and hosts of the highly diverse group. The bacterial host of p-crAssphage is predicted to be a *Bacteroides* species, although this phage has never been isolated (Dutilh et al., 2014). In the case of crAssBcn, the host is *Bacteroides intestinalis* (Ramos-Barbero et al., 2023). These differences might lead to stochastic fluctuations in phage concentrations in the total DNA fraction, depending on the replication stage of each phage. Such factors, together with the high diversity of the bacterial populations present in sewage, carrying a wide range of different ARGs, can make it challenging to establish better correlations with the targeted phages. Strong positive correlations between p-crAssphage and ARGs through metagenomic studies as well as qPCR analysis of the total DNA fraction have been previously reported in polluted urban environments, including an impacted urban watershed (Stachler et al., 2019) and agricultural soil (Li et al., 2021), in a peri-urban river (Chen et al., 2019), and in watersheds after hurricanes (Davis et al., 2020). In river biofilm samples the analysis of gene families encoding for beta-lactamases showed positive associations with crAssphage in 50 % of the samples analyzed (Kneis et al., 2022). In contrast, other authors have confirmed that crAssphage could be used as a fecal pollution marker globally but did not observe a correlation between the quantity of ARGs in the metagenomes and the quantity of crAss-like phage sequences in human fecal samples (Karkman et al., 2019). This lack of correlation is attributable to the lower abundance of p-crAssphage in sewage from Asia and Africa compared to Europe and US populations. The same study described a high correlation between ARGs and crAssphage in environments with high fecal pollution from anthropogenic origin, suggesting that ARG abundance is primarily due to human fecal contamination, for which crAssphage is a good indicator (Karkman et al., 2019).

Discrepancies between qPCR results and metagenome analysis may be explained by the different limit of detection of the different techniques. Compared to PCR-based methods, the limit of detection of metagenomics may be a couple of orders of magnitude higher, or even more, depending on DNA yields and sequencing efforts (Lindner et al., 2024). Metagenomics can be a useful tool for providing a wider range of ARGs than when targeting ARGs using qPCR like in this study. However, it can fail to detect those targets present at lower concentrations, hence reducing the correlation between indicators and ARGs. In our study, and taking into account that for routinary analysis crAss-like phage markers have to be monitored using qPCR techniques, the use of qPCR to detect both, crAss-like phages and ARGs, was considered the best strategy to obtain the more reliable correlations. Otherwise, correlation can be affected if different techniques are used for monitoring different targets (Pruden et al., 2012).

In sludge, the highest rho values in the total DNA fraction (Table S6) were observed for $bla_{\text{CTX-M-1}}$ and bla_{TEM} (rho $\sim$ 0.6), showing strong correlation for both crAss-like phages, whereas in the phage DNA fraction strong to very strong correlations were observed with the highly abundant genes *qnrS, tetW* and *sul1* (rho $\geq$0.68). The total ARGs were also very strongly correlated with the DNA from both phages (rho = 0.79 for p-crAssphage and 0.93 for crAssBcn) (Table S7).

We have observed a higher correlation in the phage DNA fraction of sludge between ARGs and crAss-like phages compared to either the total or phage DNA in urban wastewater. The observed differences are not well understood and warrants further investigation. One plausible explanation could be that sludge treatment selects for certain bacteriophages carrying different ARGs, which would persist similarly to crAssphages, indicating that crAssphages would be good indicators of ARGs carried by phages in sludge.

We have also observed that the ARGs with significant correlations differed between urban wastewater and sludge, which could be attributed to a different diversity of bacterial populations and bacteriophages in these matrices due to the selection of certain bacteria during sludge processing. The alteration of the relative abundances of bacterial populations in urban wastewater and sludge is reflected in the different relative abundances of ARGs in both the total and phage DNA fractions (Calero-Cáceres et al., 2014). In a similar way, changes in the abundances of different bacterial populations in wastewater and sludge and in both DNA fractions are likely to affect the ARG correlations with crAss-like phages, particularly considering that not only ARGs may fluctuate, but also the different prevalence of crAss-like phages in both DNA fractions as explained above.

Sludge has gained attention in recent years for its potential role in the dissemination of ARGs. As sludge is known to be a reservoir of ARGs found in both bacteria and phages (Calero-Cáceres et al., 2014), its release into the environment, for example, as an agricultural fertilizer, risks transferring ARGs to soil, water, and ultimately to human and animal populations. Bacteriophages, which have higher environmental persistence than bacteria, could play a role in maintaining the ARG reservoir and mobilizing the genes (Calero-Cáceres and Muniesa, 2016; Calero-Cáceres et al., 2017). Therefore, crAss-like phages could serve as an indicator of ARG pollution in sludge (Karkman et al., 2019), especially those in the phage fraction.

In food samples, correlation analysis was hampered by the low abundance of both fecal indicators and ARGs. Nonetheless, in the total DNA fraction (Table S6), crAssBcn phages showed a strong correlation with *sul1* (rho = 0.54), which was one of the most abundant ARGs, as well as with the total ARGs (rho = 0.68) but probably due to the low number of positive samples, not statistically significant. In the phage DNA fraction of food (Table S7), correlations could not be established due to the absence of positive samples and the low abundance of ARGs,

when present.

One of the limitations of this study is the number of samples analyzed, as well as the use of samples from different sources, that may bias some of the results obtained. While increasing the number of samples would led to more robust results, our study shows that the main difference obtained is caused by the fecal load of the samples rather than their source. When using source point samples (wastewater and sludge), higher and more robust correlations were obtained. In low-polluted samples, both ARGs and indicators decrease their numbers, variability increases and correlations cannot be found. Therefore, the use of other samples falling in either of both levels of fecal pollution (high or low) would have displayed similar results. As many other MST indicators have shown, they perform as expected in highly polluted samples, but when samples are diluted or aged, the utility of MST indicators decreases accordingly (Ballesté et al., 2020).

The majority of the ARGs were less correlated with the culturable fecal indicators than with crAss-like phages (Table S4). This is not surprising since ARGs and crAss-like phages were both analyzed by qPCR, whereas the fecal indicators were analyzed by culture techniques. Correlation has been shown to be greatly affected by the technique used to measure the parameters to be analyzed (Pruden et al., 2012). An exception was *sul1* in the total DNA fraction of urban sewage, which showed similar strong correlations with somatic coliphages (rho = 0.67). In the phage DNA fraction (Table S5), *tetW and sul1* were more strongly correlated with somatic coliphages (rho = 0.54 and 0.53, respectively) than crAss-like phages. In sludge, the ARG correlation with the fecal indicators was lower than with crAss-like phages, showing statistically significant correlation between bla_{TEM} and somatic coliphages (rho = 0.55) in the total DNA fraction and *qnrS* and somatic coliphages (rho = 0.79).

If we compare ARG correlations with crAss-like phages of non-normalized data *vs* normalized data, the Spearman correlation coefficients were significantly lower without normalization, suggesting that the detected ARGs can be explained by the degree of fecal pollution. This is in accordance with Karkman et al. (2019) (Karkman et al., 2019), who reported that ARG abundance in anthropogenically impacted environments could be largely explained by the content of fecal pollution in the samples.

Since its first discovery, crAssphage has been proposed as a potential useful marker for tracking human fecal pollution, considering it is a prevalent and specific resident of the human gut. Recently, new members of the Crassvirales group, the crAssBcn phages infecting *Bacteroides intestinalis* (Ramos-Barbero et al., 2023) have been isolated and also showed to be highly prevalent in human fecal and urban wastewater samples, as well as geographically widespread. In fact, the putative bacterial host of all crAss-like phages are bacteria from the *Bacteroides* genus, and some *Bacteroides* species are highly specific inhabitants of the human intestinal tract. The specificity between the *Bacteroides* group and their hosts (Xu et al., 2003; Payan et al., 2005) enables the selection of phages specifically present in humans by using a human-specific strain. Antibiotics are emerging contaminants of anthropogenic origin, and elevated levels of antibiotic resistant bacteria and ARGs have been widely reported in anthropogenic impacted environments (Berendonk et al., 2015; Rowe et al., 2017). Considering the origin of many ARGs is the human fecal pollution (Stachler and Bibby, 2014; Karkman et al., 2019), the use of highly specific human fecal indicator could be a useful tool to determine their potential prevalence. Moreover, the reduction of such indicators could help to evaluate the performance of water treatments for the removal of ARGs. In the last years, it has been described that many ARGs in the environment can be mobilized by phages, and it has been reported that the role of phages in the mobilization of ARGs particularly in the environment can be more relevant as previously thought (Colomer-Lluch et al., 2011; Lekunberri et al., 2017; Zhang et al., 2022). This is because phages, including those phages carrying ARGs, are generally more persistent to different natural inactivation and disinfection factors than their bacterial hosts (Calero-Cáceres and Muniesa, 2016). This suggests that the use of bacteriophages as indicators could be more appropriate for ARG-carrying phages than the use of bacterial indicators (Costán-Longares et al., 2008). Moreover, crAss-like phages show a similar decay than other phages used as indicators in waters (Ballesté et al., 2019). Consequently, crAss-like phages can be used as general MST indicators, suitable to monitor the presence of resistant bacteria from human origin, but additionally, they can potentially better represent and indicate the dynamics of ARG-carrying phage particles in human fecal-polluted environments.

Our results underscore the effectiveness of crAss-like phages as indicators not only of human fecal pollution but also as a potential tool for monitoring ARGs in the environment, particularly when ARGs are abundant in the samples.

4. Conclusions

- In this study, we confirmed the utility of two crAss-like phages as indicators of ARGs. We screened different sample types and a range of ARGs conferring resistance to different classes of antibiotics commonly used in human medicine and veterinary practice.
- Both crAss-like phages differed in concentration according to the matrix and in their correlations with the most abundant ARGs found in environmental samples. Analyzing the total DNA fraction of sewage and sludge, which is more straightforward than targeting the phage DNA fraction, revealed a correlation between the total ARGs and crAss-like phages. However, analysis of crAss-like phages in the phage fraction of sludge may provide a more reliable indication of ARG occurrence in bacteriophages, which represent a more persistent reservoir of ARGs in the environment.
- The use of crAss-like phages to monitor ARGs may help to provide a more comprehensive understanding of their presence in different environments, particularly those with high fecal loads. This approach avoids the individual analysis of each gene, which would be expensive and time-consuming.
- In future studies, the persistence of crAss-like phages and ARGs in different environmental conditions should be compared to evaluate if they exhibit a similar rate of decay, which would reinforce the potential usefulness of crAss-like phages as indicators of ARGs.

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CRediT authorship contribution statement

Sara Morales-Cortés: Writing – original draft, Investigation. **Laura Sala-Comorera:** Investigation, Formal analysis. **Clara Gómez-Gómez:** Investigation. **Maite Muniesa:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition. **Cristina García-Aljaro:** Writing – review & editing, Writing – original draft, Funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.124713.

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