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Prevalence of bacterial genes in the phage fraction of food viromes

Pedro Blanco-Picazo [a], Clara Gómez-Gómez [a], Marc Tormo [b,c], Maria Dolores Ramos-Barbero [a], Lorena Rodríguez-Rubio [a,*], Maite Muniesa [a,*]

[a] Departament de Genètica, Microbiologia i Estadística, Universitat de Barcelona, Diagonal 643, Annex, Floor 0, E-08028 Barcelona, Spain
[b] Genomics Core Facility, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Parc de Recerca Biomèdica de Barcelona, 08003 Barcelona, Spain
[c] Scientific IT Core Facility, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Parc de Recerca Biomèdica de Barcelona, 08003 Barcelona, Spain

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ABSTRACT

Antibiotic resistance genes (ARGs) have been identified in viral DNA isolated from different kinds of food, but little is known about their origin. In this study, twenty-one viromes were analyzed from samples of food previously reported to carry ARGs, including meat (poultry, veal, and pork), fish (Mediterranean, Atlantic, frozen, farmed and shellfish) and vegetables (lettuce, cucumber, and spinach). Classification of the contigs by Kraken revealed a large percentage of unclassified contigs (43.7–98.2%) in all the viromes. Only 0.05–7.1% of the contigs were identified as viral and of these, more than 91% belonged to different bacteriophage families, Podophages and Siphophages being the most prevalent. According to VirSorter, the largest number of viral contigs were derived from viromes of shellfish, followed by spinach. Spinach viromes also included the largest number of phage sequences identified by PHASTER. The abundant presence of bacterial genes in the viromes, including *16S rRNA* genes, was attributed to the phage packaging of the bacterial genome fragments, as no bacterial DNA was found outside the viral capsids. The detection of *16S rRNA* genes in the different viromes allowed diverse phage bacterial hosts to be identified. The three major functional groups of genes determined were related to metabolism, detoxification/resistance, and above all, biosynthesis. Various ARGs were quantified in the viromes by qPCR, the most prevalent being β-lactamases, particularly bla_{TEM}. Analysis of ARG diversity in the viromes by Prokka and CARD revealed various resistance-related genes, whereas a more restrictive search by ResFinder identified bla_{TEM} in all the food viromes, bla_{OXA} in Atlantic fish-1 and spinach-2, *oqxB* in lettuce-1, and *dfr* in spinach-2. The presence of ARGs in the food viromes points to bacterial DNA mobilization by transduction mechanisms. Transduction of resistances by phage particles may therefore contribute to the emergence of resistant strains along the food chain and should be monitored.

1. Introduction

The study of viromes is an expanding field, and the development of more reliable shotgun sequencing techniques has provided new insights into the role of viruses in microbial communities (Norman et al., 2015; Reyes et al., 2010; Roux et al., 2016). Microbiome sequencing mainly generates data about the bacterial fraction, as most of the total DNA extracted from a microbiome is bacterial. Although progress in virome research has fed the genetic databases with new sequences, viral genomes remain under-represented (Chatterjee & Duerkop, 2018; Gregory et al., 2020; Pérez-Cataluña et al., 2021; Wang, 2020).

When analyzing a virome, purification of viral particles and removal of external non-viral DNA is essential to enrich viral sequences. Although bioinformatic analysis can help to remove non-viral sequences, the large number of unidentified viral contigs (also known as viral dark matter) implies that only a small percentage of viral sequences can be identified, particularly in complex samples, and most of the viral sequences correspond to bacteriophages (Cantalupo et al., 2011; Pérez-Cataluña et al., 2021).

Phages can mobilize a single bacterial gene inserted in the phage genome (specialized transduction) or a large fragment of bacterial DNA encapsidated during phage assembly (generalized or lateral transduction) (Chiang et al., 2019). When analyzing the extent to which transduction events in a virome are driven by bacteriophages,

* Corresponding authors.
E-mail addresses: lorenarodriguez@ub.edu (L. Rodríguez-Rubio), mmuniesa@ub.edu (M. Muniesa).

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specialized transduction can be easily detected by the presence of phage DNA flanking the transduced gene. However, this is not possible in the case of generalized or lateral transduction mechanisms, when only bacterial DNA is packaged inside the phage capsids (Fernández-Orth et al., 2019).

Recent studies have focused on the potential role of bacteriophages in the mobilization of antibiotic resistance genes (ARGs), which may cause the emergence of new resistant strains in environmental and food biomes (Colavecchio et al., 2017; Gabashvili et al., 2020). There is growing evidence for the prevalence of ARGs in the phage DNA fraction of environmental, food and human samples (Anand et al., 2016; Barrios et al., 2021; Calero-Cáceres & Balcázar, 2019; Colomer-Lluch et al., 2011; Sala-Comorera et al., 2021; Strange et al., 2021; Subirats et al., 2016), indicating a possible circular transmission of resistances via phage mobilization. The predominant strategy used by phages for ARG mobilization, however, remains unclear. There are a few reports of ARGs located in phage genomes, indicating specialized transduction, yet metagenomic studies suggest this location is infrequent (Enault et al., 2017; Kang et al., 2021). ARGs could also be mobilized by generalized or lateral transduction mechanisms (Chen et al., 2018). In this case, when only bacterial DNA is found inside the capsids, additional steps are required to remove non-packaged bacterial DNA that can potentially contaminate the virome, including stringent controls to confirm its removal.

In this study, we used a metagenomics approach to study the viromes of different foods previously reported to carry ARGs in the viral DNA fraction. After removal of non-packaged DNA, the packaged DNA was analyzed to investigate the extent of phage-mediated ARG mobilization.

2. Materials and methods

2.1. Food samples

Twenty-one samples of different types of food (vegetables, meat and fish) were analyzed. Each sample was the result of pooling the viral DNA obtained from three to five individual food items of the same type, as reported in previous studies (Blanco-Picazo, Roscales, et al., 2020; Gómez-Gómez et al., 2019; Larrañaga et al., 2018). Thus, the analyzed meat consisted of two pooled samples of veal (veal-1, veal-2), two of pork (pork-1, pork-2), and two of chicken (poultry-1, poultry-2). The fish consisted of two pooled samples of Atlantic fish (hake and sardine) (Atlantic-1, Atlantic-2), two of frozen fish (hake and monkfish) (frozen-1, frozen-2), two of aquaculture fish (salmon and trout) (farm fish-1, farm fish-2), two of shellfish (mussels and clams) (shellfish-1, shellfish-2) and one of Mediterranean fish (whiting) (Mediterranean-2). The vegetables consisted of two pooled samples of lettuce (lettuce-1, lettuce-2), two of spinach (spinach-1, spinach-2) and two of cucumber (cucumber-1, cucumber-2). All samples were purchased in local retailers in Barcelona (Spain) in 2017–2020, transported in sterile containers and kept at −80 °C until analysis.

2.2. Purification of DNA from phage particles

Fifty g of each sample was homogenized 1:5 (*w:v*) in phosphate-buffered saline (PBS) by shaking for 30 min and 50 mL of the homogenate obtained was centrifuged at 3000 × *g*. 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 viral particles in the filtrates were 20-fold concentrated using polyethylene glycol (PEG) precipitation (Sambrook & Russel, 2001) followed by incubation at 4 °C for 12 h, and centrifugation at 16000 × *g*. Two mL of the PEG concentrated suspensions were dialyzed and treated twice with chloroform 1:10 (*v:v*), mixed by vigorous vortexing for 5 min and centrifuged at 16000 × *g* for 10 min. The aim of this process was to disrupt bacterial membranes and break any DNA-containing small vesicles that might have passed through the filters. The supernatant was treated with DNase I (100 units/mL; Sigma-Aldrich. Spain) for 1 h at 37 °C to eliminate any non-packaged DNA. DNase I was inactivated by heating for five minutes at 75 °C.

At this stage of the treatment, viral capsids remain intact, no vesicles should be present, whereas any free DNA outside the viral capsids should have been removed. To confirm the absence of non-packaged bacterial DNA, an aliquot was taken and used as a template for qPCR amplification of bacterial *16S rRNA* genes (Larrañaga et al., 2018) and bla_{TEM} (Lachmayr et al., 2009) (Table S1). The protocols applied here for DNase treatment and DNase inactivation have been verified in previous studies (Colomer-Lluch et al., 2014; Fernández-Orth et al., 2019).

Packaged DNA was extracted using the QIAmp® Viral RNA Mini Kit (Hilden. Germany) and recovered in a final volume of 80 μL of bidistilled water. DNA from three to five samples of the same type was pooled and further purified using the DNA Clean&ConcentratorTM-5 Kit (Zymo Research. Irving. CA. USA) to a final volume of 50 μL.

2.3. qPCR assays

All samples were analyzed for the presence of ten ARGs conferring resistance to different groups of antibiotics (Table S1). Thus, real-time PCR (qPCR) was performed using TaqMan hydrolysis probes targeting β -lactamases (bla_{TEM}, $bla_{\mathrm{CTX\text{-}M}}$ group 1 and group 9, bla_{VIM}), and resistance to methicillin (*mecA*), sulfonamides (*sul1*), quinolones (*qnrA* and *qnrS*), aminoglycosides (*armA*), and tetracyclines (*tetW*). Finally, the absence of *16S rRNA* genes was verified by qPCR using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) and primers 338F/518R (Table S1). 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). The reaction contained 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, serial dilutions of a known concentration of gBlocks™ Gene Fragments (Integrated DNA Technologies, Coralville, IA, USA) for each ARG were used to generate the standard curves in each qPCR assay. All samples were run in triplicate (including the standards and negative controls). The number of gene copies (GC) was defined as the mean of the triplicate data obtained. To evaluate ARG abundance, the GC results were calculated with the standard curves using the last valid Ct for each ARG assay (Table S1) as the limit of quantification, when the standard curve was consistent in the different replicates. The standards were also used as positive controls.

2.4. Sequencing

The DNA concentration of each pooled sample was evaluated using a Qubit® Fluorometer (Life Technologies. CA. US) and the DNA quality was further confirmed by the 2100 Bioanalyzer system (Agilent Technologies. CA. US). DNA at a concentration of 1.5 ng/μL was used to prepare the libraries.

DNA was fragmented and used to prepare the libraries with the Nextera XT Kit (Illumina. Inc. San Diego. CA. US) according to the 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 using AmPure beads (Beckman Coulter Inc., California. USA), checked for fragment distribution and size in a 2100 Bioanalyzer and the Agilent High Sensitivity DNA Chip (DNA 1000) (Agilent Technologies. CA. US) and quantified in a Quantus™ Fluorometer (Promega. WI. US). An equimolar pool of the 21 samples was sequenced in a NextSeq (Illumina) High Output run of 300 cycles.

2.5. Bioinformatic analysis

The sequenced reads were trimmed with Trimmomatic (version

0.36) (Bolger et al., 2014) with a quality of 15 and only reads longer than 35 bp were retained. The quality of sequences was checked with FastQC (version 0.11.7) (Andrews, 2018) and *de novo* assembled with MetaSPAdes (Nurk et al., 2017), which is recommended when assembling metagenomic datasets. To evaluate the assembled bacterial and viral contigs, the contigs were subsetted according to their assigned classification (Archaea/Bacteria and Virus). For each subset of N50 contigs, the percentage of mapped reads aligned with the classified contigs of each category was calculated.

Contigs were classified with Kraken2 (version 2.1.1) (Wood & Salzberg, 2014) using the standard Kraken database that comprises NCBI taxonomic information, as well as the complete genomes in RefSeq for the bacterial, archaeal, viral and phage domains. No further additional filters were applied to results from Kraken2. Total viruses were evaluated using VirSorter (version 2 beta) (Roux et al., 2015), which classified viral sequences as full, partial, or short (those with less than 2 genes). Phages were evaluated using PHASTER (Arndt et al., 2018), which classifies prophage sequences according to the percentage of the total number of coding sequences. Sequences were assigned using specific phage-related keywords (such as 'capsid', 'head', 'integrase', 'plate', 'tail', 'fiber', 'coat', 'transposase', 'portal', 'terminase', 'protease' or 'lysin').

Taxonomic classification of bacterial *16S rRNA* genes in the contigs was based on the list of matches obtained with Kraken2 and the Greengenes database (DeSantis et al., 2006). The relative abundance of each taxonomic group in each virome was determined by the percentage of contigs carrying the corresponding *16S rRNA* gene.

Prokka was used to predict the function of the genes detected within the Bacterial/Archaeal fraction of the virome. The detected enzymes were analyzed with the Metacyc Database (Caspi et al., 2020) and the software MinPath (Ye & Doak, 2009) to determine the metabolic pathways in which they are involved (Functional Annotation — Metagenomics Workshop SciLifeLab 1.0 Documentation, n.d.). For ARG detection in the viromes, a preliminary analysis was performed using Prokka 1.13.4 (Seemann, 2014) with the terms "resist" "antibiotic" and "beta-lactam". Since searches using Prokka depend on the terms used and how the genes are described in the database, this could miss some ARGs, so ARG identification was also performed with the CARD database (McArthur et al., 2013) and ResFinder 4.1 (Zankari et al., 2012). In ResFinder, only gene identities with thresholds of 80 and 90% were considered.

Data were plotted using the Plotty Chart Studio platform (Sievert et al., 2021), heatmap was generated by Heatmapper (Babicki et al., 2016).

3. Results and discussion

High quality sequences were obtained for 20 out of 21 viromes, as

Table 1
Origin of the analyzed viromes, viral DNA concentration used, number of reads and contigs generated, length of the largest contig in each virome and distribution of contigs classified as Archaea, Bacteria, Viruses or Unclassified.

Origin	Virome code	DNA extracted (ng/μl)	# Reads	#contigs/ Total_length	Longest contig size (bp)	# Contigs (%)			
						Unclassified	Archaea	Bacteria	Viruses
Pork meat	Pork-1	18.2	1,850,044	10686/2781646	48,554	9107(85.22%)	4(0.04%)	1529 (14.31%)	39(0.36%)
Pork meat	Pork-2	4.46	1,820,774	9404/3083064	48,585	7043(74.89%)	2(0.02%)	1957 (20.81%)	389 (4.14%)
Veal meat	Veal-1	3.22	4,070,980	127948/ 34192699	48,557	109735 (85.77%)	46 (0.04%)	17865 (13.96%)	250 (0.20%)
Veal meat	Veal-2	3.26	44,530,446	729510/ 200053268	52,782	655070 (89.80%)	10 (0.00%)	70949 (9.73%)	3382 (0.46%)
Chicken meat	Poultry-1	2.28	1,686,340	9093/3594175	94,626	7555(83.09%)	0(0.00%)	810(8.91%)	644 (7.08%)
Chicken meat	Poultry-2	21.6	25,456,986	1086615/ 426834695	290,222	1064566 (97.97%)	37 (0.00%)	21410 (1.97%)	530 (0.05%)
Atlantic fish	Atlantic-1	2.29	49,719,102	84121/48152362	311,157	64265 (76.40%)	10 (0.01%)	17668 (21.00%)	2046 (2.43%)
Atlantic fish	Atlantic-2	2.30	63,714,336	89247/39354122	146,573	69823 (78.24%)	15 (0.02%)	18065 (20.24%)	1246 (1.40%)
Mediterranean Fish	Mediterran-2	43.7	13,263,780	42261/10816320	48,557	39901 (94.42%)	3(0.01%)	2099 (4.97%)	248 (0.59%)
Frozen fish	Frozen-1	21.2	21,789,254	336021/ 125259336	114,614	327431 (97.44%)	86 (0.03%)	7575 (2.25%)	804 (0.24%)
Frozen fish	Frozen-2	37.4	11,608,568	175417/ 55108478	61,172	160588 (91.55%)	24 (0.01%)	13671 (7.79%)	1003 (0.57%)
Farm fish	Farm fish-1	1.5	296	19/5084	454	17(89,47%)	0(0.00%)	2(10.53%)	0(0.00%)
Farm fish	Farm fish-2	14.5	20,439,900	125621/ 44306445	56,791	106408 (84.71%)	7(0.01%)	12217 (9.73%)	6604 (5.26%)
Shellfish	Shellfish-1	1.61	48,052,642	1135999/ 403873783	59,726	1115608 (98.21%)	146 (0.01%)	12325 (1.08%)	7652 (0.67%)
Shellfish	Shellfish-2	8.13	27,153,482	376114/ 131568150	93,484	367106 (97.60%)	44 (0.01%)	5222 (1.39%)	3601 (0.96%)
Lettuce	Lettuce-1	3.15	63,932,196	20226/12804897	372,269	8836(43.69%)	7(0.03%)	11141 (55.08%)	213 (1.05%)
Lettuce	Lettuce-2	27.7	30,533,090	44318/14330619	206,816	27127 (61.21%)	7(0.02%)	17137 (38.67%)	33(0.07%)
Spinach	Spinach-1	4.98	110,505,294	260895/ 157167615	328,163	223139 (85.53%)	131 (0.05%)	33878 (12.99%)	3296 (1.26%)
Spinach	Spinach-2	2.4	75,367,998	169641/ 112009861	242,266	130274 (76.79%)	30 (0.02%)	32615 (19.23%)	6429 (3.79%)
Cucumber	Cucumber-1	7.30	26,403,552	180953/ 59785513	95,090	159847 (88.34%)	47 (0.03%)	20768 (11.48%)	198 (0.11%)
Cucumber	Cucumber-2	24.5	1,875,514	23434/6377362	50,293	21370 (91.19%)	3(0.01%)	2034 (8.68%)	22(0.09%)

verified by FastQC. The DNA concentration, the total number of reads and contigs and the number of classified and unclassified contigs for each virome are summarized in Table 1. All samples contained a high number of contigs (Table 1) except farm fish-1, which yielded only a few. This virome is included in Fig. 1 and Table 1, but it was excluded from further analysis due to the low levels of extracted DNA. Nevertheless, it was sequenced as a control to rule out contamination during library preparation and shotgun sequencing.

The assembly of bacterial and viral contigs was evaluated by calculating N50 values (Table S2). Reads aligned to the contigs classified in the Archaea/Bacteria or Virus groups are also shown in Table S2.

3.1. Bacteriophage families in the viromes

In each virome, the lengths of all the contigs (Table 1) were within the range that can fit in *Caudovirales* or *Inoviridae* capsids, which can package DNA from 18 to 735 kb and 5.5 to 10.6 kb, respectively (Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses, 2011). Kraken analysis showed a large percentage of unclassified contigs (43.7–98.2%) in all the viromes (Table 1), reflecting the low proportion of available viral data in comparison with other groups, which is responsible for the so-called "viral dark matter" (Blanco-Picazo, Fernández-Orth, et al., 2020; Cantalupo et al., 2011; Fernández-Orth et al., 2019). The percentage of contigs identified as viral was very low (0.05–7.1%; Table 1, Fig. 1), considering that only viral (packaged) DNA was analyzed. These results are in line with previous studies analyzing viromes, which report that viral contigs in complex samples correspond to no more than 1–4% of the total DNA (Cantalupo et al., 2011; Jebri et al., 2019; Pérez-Cataluña et al., 2021), with a slightly higher percentage in certain environments such as aquaculture pools (Colombo et al., 2016) and desert ponds (Fancello et al., 2013).

A recent comparison of different pipelines for virome composition analysis concluded that BLAST (BLASTn, Megablast and tBLASTx) is the most reliable tool for the assignment of viral sequences (Tangherlini et al., 2016). BLAST comparison against viral databases showed that more than 91% of all identified viruses belonged to different bacteriophage families, with a clear predominance of short-tailed phages (*Podoviridae* and *Autographviridae*) (37.0%) and Siphophages (*Siphoviridae* and *Drexlerviridae*) (35.2%), followed by Myophages (*Myoviridae*, *Ackermannviridae, Herelleviridae* and *Chaseviridae*) (28.3%). 5.1% of the identified viruses corresponded to filamentous phages (*Inoviridae*) and only 6.7% to eukaryotic viruses, which were practically only detected in meat samples (*Herpesviridae*, *Parvoviridae* and *Retroviridae* were the most frequent). 3.7% of the recognized viral sequences corresponded to very minor groups or to non-classified viruses (Fig. 2).

3.2. Viral sequences in the viromes

Using VirSorter, the lowest number of viral sequences, as an absolute value, was found in meat viromes, and the highest in fish, particularly in shellfish-1 (mussels) (Fig. 3A). Among vegetables, the highest number of viral contigs was detected in spinach viromes (Fig. 3A). The high abundance in shellfish-1 could be expected, as bivalves are prone to accumulating viruses and other microorganisms in their hepatopancreas, even though viral contig abundance cannot be directly correlated with viral abundance. In addition, shellfish samples analyzed in a previous study (Blanco-Picazo, Roscales, et al., 2020) revealed a relatively high number of ampicillin-resistant aerobic bacteria (4.7×10^7 CFU/25 g) and somatic coliphages (1.1×10^3 PFU/25 g), despite the shellfish being suitable for consumption according to EU regulations (Blanco-Picazo, Roscales, et al., 2020).

In a more selective screening of phages using PHASTER, a lower number of phage contigs were recognized compared to VirSorter, which is a more general viral database (Fig. 3). The highest number of contigs with phage sequences, as an absolute value, was found in spinach, followed by Atlantic fish-2 and shellfish-2 viromes (Fig. 3B). Considering the number of reads and contigs in the viromes (Table 1), the differences in absolute abundance of viral and phage sequences do not seem attributable to differing sequence coverage but are more likely due to the variable characteristics and microbiota of the food samples. The most common phage genes identified in the viromes (Table S3) corresponded to those encoding tail proteins, followed by head, capsid, terminase and portal proteins. Among all the identified genes, only 3.8% corresponded to integrase genes and 1.1% to recombinase genes, both indicative of temperate phages. The results reflect a lower proportion of temperate phages compared with previous reports (Reyes et al., 2010; Stern et al., 2012).

3.3. Bacterial genes in the viromes

As mentioned, contigs identified as bacterial constituted the largest group among the known sequences (Table 1; Fig. 1). The presence of bacterial DNA in the viral DNA fraction has previously been reported in virion-enriched samples after purification and has been attributed to different causes, such as a low availability of phage sequences in public databases in comparison with bacterial entries, misannotation of prophage genes within bacterial genomes that 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. In addition, it has been attributed to the presence of gene transfer agents or to generalized or lateral transducing events (Cantalupo et al., 2011; Enault et al., 2017; Navarro & Muniesa, 2017;

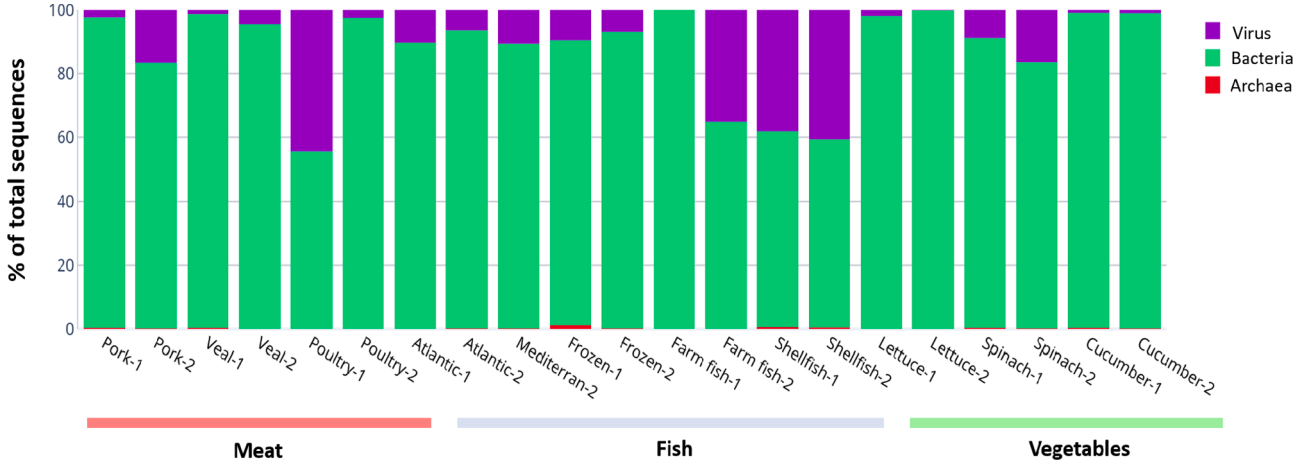

Fig. 1. Percentages of the contigs identified within the groups Archaea, Bacteria and Virus in the viromes of food samples.

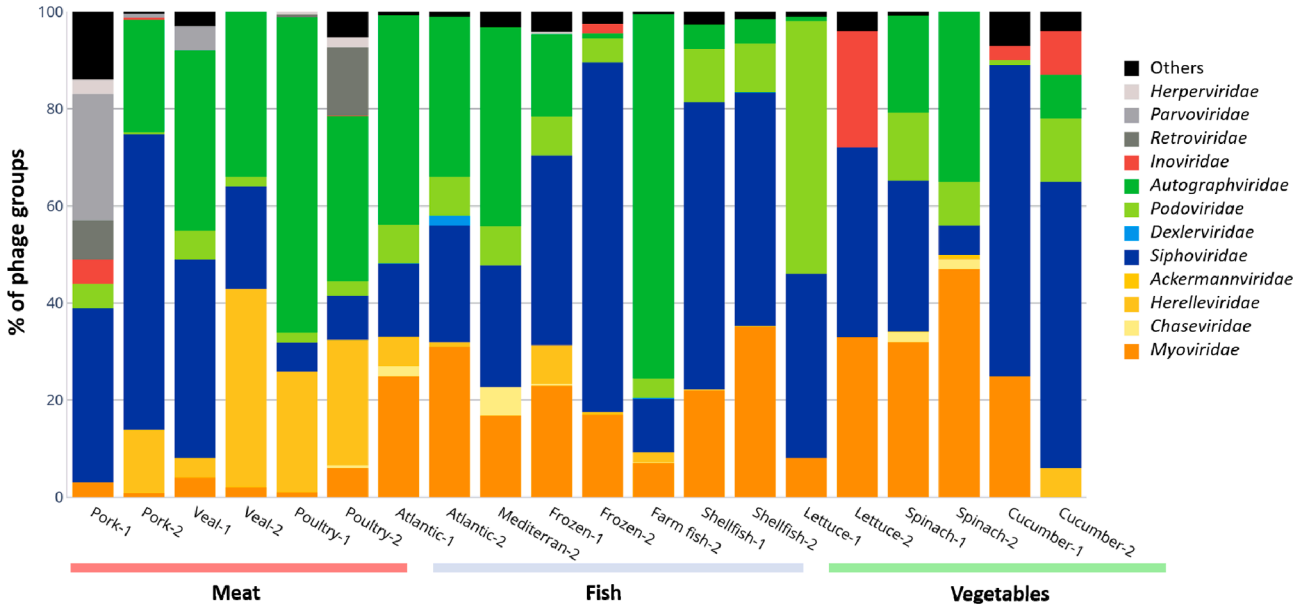

Fig. 2. Distribution of phage groups in the viromes of food samples. Siphophages (blue bars), short-tailed phages (green bars), Myophages (orange bars), filamentous phages (red bars), eukaryotic viruses (grey bars), and non-classified viruses/others (black bars). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

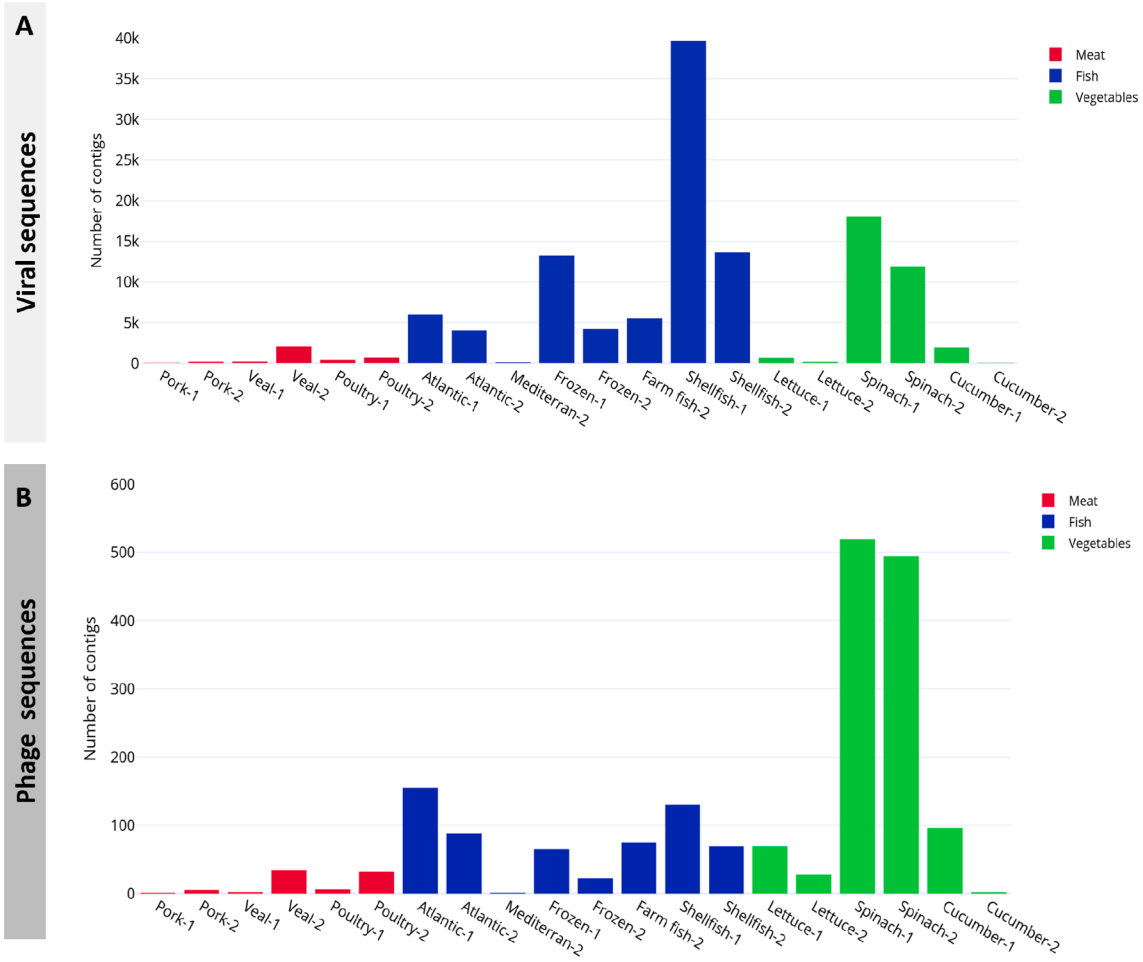

Fig. 3. Analysis of viral sequences in the viromes. A) Number of contigs corresponding to viral sequences analyzed by VirSorter. B) Number of contigs containing phage sequences analyzed by PHASTER. Meat viromes are indicated by red bars, fish viromes by blue bars and vegetable viromes by green bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Humphrey et al., 2021). However, to ensure that the analysis of DNA inside the phage particles was not contaminated by external DNA during the extraction and library preparation, several controls were performed. First, the efficiency of the DNAse test was checked, as was the absence of inhibitors in the sample (Colomer-Lluch et al., 2014). Additionally, the potential presence of *16S rRNA* and bla_{TEM} genes in the samples was studied by qPCR before disrupting the capsids (Brown-Jaque et al., 2016). Their absence outside the capsids indicated that non-encapsidated DNA (plasmidic or chromosomal) had been correctly removed during the extraction process. The *16S rRNA* gene was targeted because of its ubiquity and multicopy presence in many bacterial species and also because its absence in viromes has been proposed as a control to rule out contamination by bacterial DNA. However, our results question the utility of the *16S rRNA* gene for this function once the capsids are broken, as its detection after the application of various removal methods (Cantalupo et al., 2011; Enault et al., 2017; Göller et al., 2020) indicates it can be packaged inside the virion capsids.

The addition of extra purification steps to avoid bacterial DNA contamination and obtain virus-like particles of greater purity is often at the expense of losing viral material. Thus, a reduction of *16S rRNA* genes in the viromes can be due not only to a more efficient virion purification but also to an overall loss of DNA arising from a more rigorous process. The presence of *16S rRNA* genes within the capsids, indicative of the capacity of phage particles to package bacterial DNA (Blanco-Picazo, Fernández-Orth, et al., 2020; Cantalupo et al., 2011; Fernández-Orth et al., 2019), supports the frequent involvement of transduction mechanisms (Chen et al., 2018; Del Casale et al., 2011).

Another control step introduced in the study was the amplification of bla_{TEM} before viral capsid disruption. Its absence suggested that non-packaged plasmidic DNA had been removed by DNAse digestion, as this gene was later found in all the viromes after breaking the capsids.

3.3.1. Analysis of 16S rRNA genes

Presuming that the presence of *16S rRNA* genes in the capsids was the result of packaging of the bacterial genome during particle assembly, we proceeded to identify the bacterial species hosting the phage particles in the viromes (Fig. 4). Taxonomic classification (Fig. 4) using virome *16S rRNA* genes was obtained at different levels (from genus to family), and the percentage of contigs harboring *16S rRNA* genes of each group is presented as a heatmap in Fig. 4. In a few cases, the matching data found in the databases were attributed to unclassified bacteria. The predominant group in the viromes was Flavobacteriales, followed by

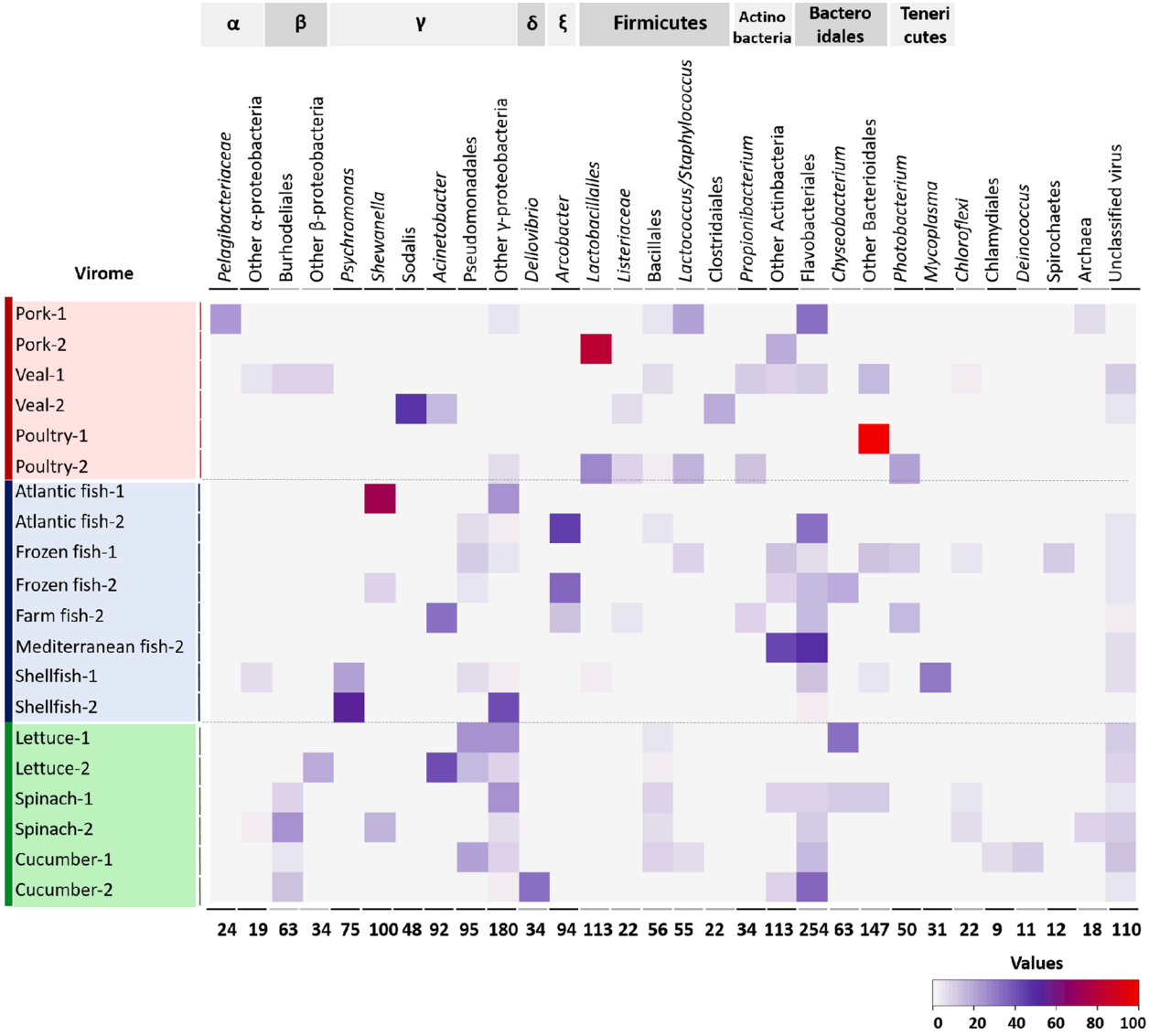

Fig. 4. Heatmap of the percentage of host bacterial species in the viromes identified by analysis of *16S rRNA* genes in the phage particles. α, β, γ, δ and ε correspond to classes of the phylum Proteobacteria. Total counts are shown in the bottom of the heatmap.

γ-Proteobacteria, Actinobacteria and other Bacteroidales. Firmicutes was more prevalent in meat than in the other food types, and within this phylum, Lactobacillales was the most represented order. Some taxonomic groups were abundant but only found in a few samples, such as *Pelagibacteriaceae* in pork or *Arcobacter* in fish. A result that initially seemed surprising was the presence of the *Chlamydiae 16S rRNA* gene in cucumber and veal viromes. However, interactions between plants and *Chlamydiae*, which have a broad environmental distribution, are supported by the discovery of plant genes in Chlamydial genomes (Collingro et al., 2020), and could explain the presence of *Chlamydiae* genes in vegetable viromes and consequently in ruminants.

3.3.2. Gene functions

On the assumption that bacterial DNA was present within the particles, enzymes identified by Prokka in the bacterial and archaeal fractions of the viromes were analyzed using MinPath to identify the pathways in which these enzymes are involved. Gene functionality was determined to explore if genes with specific functions were packaged at different frequencies. MinPath is a parsimony approach for the reconstruction of biological pathways based on protein family predictions, and the estimations, although conservative, have a high degree of accuracy and are more faithful for a query dataset (Ye & Doak, 2009).

Phages are known to play an important role in the metabolism, biosynthesis, and stress responses of their hosts (Fernández et al., 2018) and the genes identified in the phage particles correspond to these three major functions (Fig. 5). Genes encoding biosynthetic enzymes (involved in lipid, nucleotide, amino acid, cofactor, and secondary metabolism) were the most frequently detected. Correspondingly, the impact of phages on the biosynthesis of cell walls and biofilm has been widely reported (Fernández et al., 2018). In Cyanobacteria, phage presence affects photosynthesis and other biosynthetic pathways (Lindell et al., 2005; Thompson et al., 2011), and phages carry biosynthetic gene clusters that provide the host with competitive advantages (Dragoš et al., 2021; Du Toit, 2021).

Although phages are also associated with metabolic functions (Enav et al., 2014; Mara et al., 2020), a lower proportion of genes involved in metabolic pathways (N, C, and S cycles, aerobic and anaerobic respiration, fermentation, and metabolite degradation) was observed in the viromes. This finding contrasts with the results of genomic analysis in bacteria and other organisms, in which metabolic pathways appear to be predominant (Koonin & Wolf, 2008; Turnbaugh et al., 2007; Zhao & Eun, 2020). If phage packaging of bacterial genes is a random event, it could be expected that the most abundant genes in bacterial genomes are packaged at higher frequencies, and genes involved in biosynthesis and metabolism should be found at similar proportions in the viromes, a tendency not observed in the present study. This raises the question of whether some genes are preferentially selected for transduction because they confer an advantage for the bacteria. In turn, phages would be indirectly favored, as bacteria might tolerate phage infection as long as they obtain benefits.

The least frequently identified genes corresponded to pathways of detoxification and resistance to metals, antibiotics (particularly vancomycin) and other toxic compounds. They were most prevalent in vegetable viromes, with only a fraction found in meat and a residual presence in fish (Fig. 5). Vegetable viromes are highly influenced by the phages infecting plant-associated and soil microorganisms, such as *Burkhordelia* (Spain et al., 2009) or *Bacillus* (Liu et al., 2019) (Figs. 4 and 5). Soil microorganisms are a reservoir of resistance genes (Riesenfeld et al., 2004) and detoxification functions of symbiotic bacteria associated with plants have been described (Werren, 2012).

When comparing the different food types, the most marked differences in the relative abundance of functions were observed in fish viromes, which had more biosynthetic and fewer metabolic genes compared to meat and vegetable samples (Fig. 5). However, the causes and consequences of these observations remain to be elucidated.

Detoxification genes may confer an advantage for the cells in the presence of the toxic substance, while the acquisition of genes of biosynthesis or metabolism may confer advantages for the bacteria in many situations. In addition, in comparison to genes involved in biosynthesis or metabolism, genes related to detoxification are less abundant in bacterial genomes and consequently should be mobilized less frequently by phage particles.

3.3.3. Antibiotic resistance genes

In previous studies on the role of phages in ARG mobilization, our group analyzed the same type of food samples as here (Blanco-Picazo, Roscales, et al., 2020; Gómez-Gómez et al., 2019; Larrañaga et al., 2018) and reported that the ARGs found in the phage particles are of bacterial origin. The average abundances of ARGs in the viromes of the pooled samples are presented in Table 2.

Prokka and CARD were also used to search for resistance-encoding genes in the viromes. This non-restrictive search allowed the inclusion

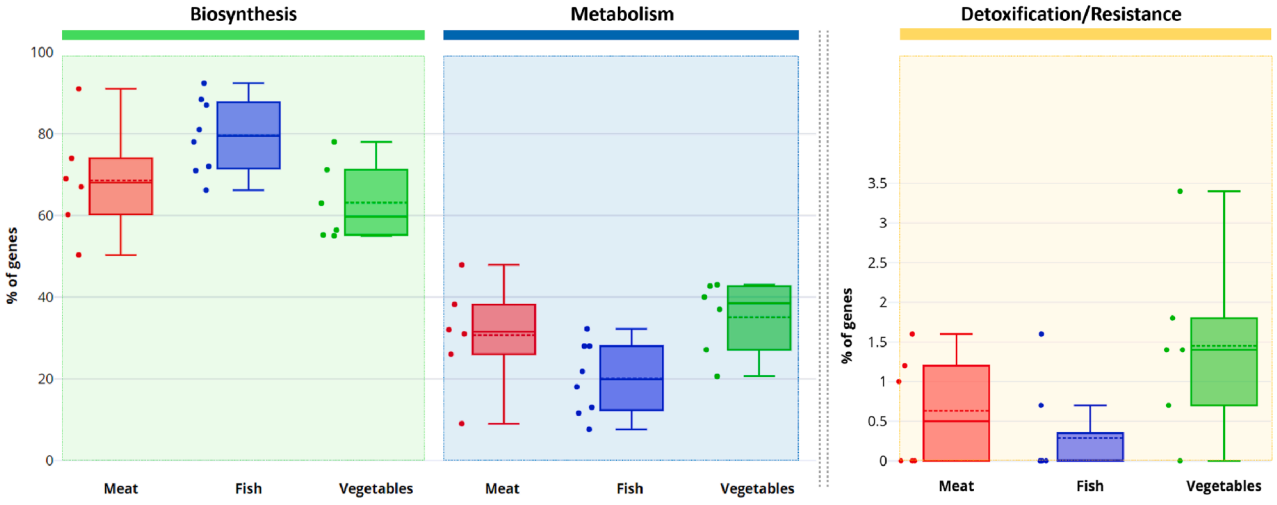

Fig. 5. Percentages of genes belonging to metabolic or biosynthetic pathways and related to detoxification/resistance functions among samples of vegetables (green), meat (red) and fish (blue). The cross-pieces of each box represent (from top to bottom) the maximum, upper-quartile, median, lower-quartile and minimum values. The dashed line shows the mean value. The upper boxes in the box plot include samples showing values within the 75th percentile and lower box samples show values within the 25th percentile. Dots outside the box indicate the value for each sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
List of ARGs quantified by qPCR and identified by ResFinder with their % of identity and by CARD and Prokka, including a description of the resistance conferred by each gene.

Virome	qPCR (GC/100 g)	Resistance to	ResFinder** (% identity)	Prokka/CARD Gene	Resistance to
Pork-1	*bla*$_{TEM}$ (nq*)	β-lactams	*bla*$_{TEM}$ (100)	*bla*$_{TEM}$	β-lactams
	bla$_{CTX-M-1}$(nq)	β-lactams		*marA, marR*	Multiple antibiotics
	sul1(nq)	sulfonamides			
	qnrA(nq)	quinolones			
	qnrS (nq)	quinolones			
Pork-2	*bla*$_{CTX-M-1}$(nq)	β-lactams	*bla*$_{TEM}$ (100)	*bla*$_{TE}$	β-lactams
	bla$_{VIM}$ (nq)	β-lactams		*ehpR*	Phenazine
	sul1(nq)	sulfonamides			
	qnrA(nq)	quinolones			
	qnrS (nq)	quinolones			
Veal-1			*bla*$_{TEM}$ (100)	*bla*$_{TEM}$	β-lactams
	sul1(nq)	sulfonamides		*blh*	β-lactams
	qnrA(nq)	quinolones		*mdtA, mdtB*	Multiple antibiotics
	qnrS (nq)	quinolones		*czcB*	Cobalt-zinc-cadmium
				acr3	Arsenic
				sugE	Quaternary ammonium
Veal-2	*bla*$_{CTX-M-9}$ (nq)	β-lactams	*bla*$_{TEM}$ (100)	*bla*$_{TEM}$	β-lactams
	bla$_{VIM}$ (6.82)	β-lactams		*ampC*	β-lactams
	sul1(nq)	sulfonamides		*arnA*	Bifunctional polymyxin
	qnrA(nq)	quinolones		*fsr*	Fosmidomycin
				bmr3, bmrA, emrY, ermK, marR, mdtA, mdtB, mdtC, mdtD, mdtE, mdtG, mdtH, mdtK, mexA, mexB, norM, stp, mrR,	Multiple antibiotics
				czcA, czcB	Cobalt-zinc-cadmium
				copB, pcoC	Copper
				merR	Mercury
				smvA	Methyl viologen
				cnrA	Nickel and cobalt
				ohrA	Organic hydroperoxide
				sugE	Quaternary ammonium
				arsR	Arsenic
Poultry-1	*bla*$_{TEM}$ (nq)	β-lactams	*bla*$_{TEM}$ (100)	*bla*$_{TEM}$	β-lactams
	bla$_{CTX-M-1}$(nq)	β-lactams			
	sul1(nq)	sulfonamides			
Poultry-2	*bla*$_{TEM}$ (nq)	β-lactams	*bla*$_{TEM}$ (100)	*bla*$_{TEM}$	β-lactams
	bla$_{CTX-M-9}$ (nq)	β-lactams		*bmr3, emrD, mdtA, mdtC, mdtN, mezB, norM,, mdtL*	Multiple antibiotics
	bla$_{VIM}$ (nq)	β-lactams		*cnrA*	Nickel and cobalt-
	arma (nq)	aminoglyco-sides		*czcA*	Cobalt-zinc-cadmium
	sul1(nq)	sulfonamides			
Atlantic-1	*bla*$_{TEM}$ (5.93)	β-lactams	*bla*$_{TEM}$ (99.9)	*bla*$_{TEM}$	β-lactams
	bla$_{CTX-M-1}$(4.94)	β-lactams	*bla*$_{OXA}$ (96.2)		
	bla$_{CTX-M-9}$(5.46)	β-lactams		*mdtA, mdtC, mdtL, mdtK, emrD, bmra, mdtN, mexB, norM, mdtC*	Multiple antibiotics
	sul1(5.23)	sulfonamides		*cnrA*	Nickel and cobalt
	tetW (5.85)	tetracyclines		*czcA*	Cobalt-zinc-cadmium
				sugE	Quaternary ammonium
Atlantic-2	*bla*$_{TEM}$ (7.70)	β-lactams	*bla*$_{TEM}$ (100)	*merR*	Mercury
	bla$_{CTX-M-1}$ (4.89)	β-lactams		*arpC, bmrA, mdtA, mdtC, mdtK, mdtL, yheH, norM, marA, marR, bmr3,*	Multiple antibiotics
	bla$_{CTX-M-9}$ (6.03)	β-lactams		*copA, copD*	Copper resistance
	sul1 (6.00)	sulfonamides			
	tetW (5.86)	tetracyclines			
Frozen-1	*bla*$_{TEM}$ (6.30)	β-lactams	*bla*$_{TEM}$ (100)	*bla*$_{TEM}$	β-lactams
	bla$_{CTX-M-1}$ (5.07)	β-lactams		*ohrA*	Organic hydroperoxide
	bla$_{CTX-M-9}$ (5.41)	β-lactams			
	sul1 (5.42)	sulfonamides			
	tetW (6.02)	tetracyclines			
Frozen-2	*bla*$_{TEM}$ (6.51)	β-lactams	*bla*$_{TEM}$ (100)	*bla*$_{TEM}$	β-lactams
	bla$_{CTX-M-1}$ (5.17)	β-lactams		*mdtC, norM, marA, marR, ykkD*	Multiple antibiotics
		β-lactams		*czcB*	Cobalt-zinc-cadmium

(*continued on next page*)

Table 2 (*continued*)

Virome	qPCR (GC/100 g)	Resistance to	ResFinder** (% identity)	Prokka/CARD Gene	Resistance to
	$bla_{CTX-M-9}$ (6.34)				
	sul1 (5.33)	sulfonamides			
	tetW (6.06)	tetracyclin			
Farm fish-2	bla_{TEM} (7.81)	β-lactams	bla_{TEM} (100)	bla_{TEM}	β-lactams
	$bla_{CTX-M-1}$ (5.27)	β-lactams		*mdtH, mdtC, yheH*	Multiple antibiotics
	$bla_{CTX-M-9}$ (5.34)	β-lactams		*czcA*	Cobalt-zinc-cadmium
	sul1 (5.60)	sulfonamides		*ohrA*	Organic hydroperoxide
	tetW (6.04)	tetracyclines		*sugE*	Quaternary ammonium
Mediterran-2	bla_{TEM} (7.32)	β-lactams	bla_{TEM} (100)	bla_{TEM}	β-lactams
	$bla_{CTX-M-1}$ (5.88)	β-lactams		*MarA, marR*	Multiple antibiotics
	$bla_{CTX-M-9}$ (7.09)	β-lactams			
	tetW (5.93)	tetracyclin			
Shellfish-1	bla_{TEM} (7.35)	β-lactams	bla_{TEM} (99.9)	bla_{TEM}	β-lactams
	$bla_{CTX-M-1}$ (5.45)	β-lactams		*arnA*	Polymyxin
	$bla_{CTX-M-9}$ (6.04)	β-lactams		*mdtC*	Multiple antibiotics
	sul1 (5.01)	sulfonamides			
	tetW (6.23)	tetracyclines			
Shellfish _2	bla_{TEM} (7.13)	β-lactams	bla_{TEM} (100)	bla_{TEM}	β-lactams
	$bla_{CTX-M-1}$ (4.91)	β-lactams		*arnA*	Polymyxin
	$bla_{CTX-M-9}$ (6.11)	β-lactams		*yheH, mdtC*	Multiple antibiotics
	sul1 (5.01)	sulfonamides			
	tetW (5.71)	tetracyclin			
Lettuce-1	bla_{TEM} (10.26)	β-lactams	bla_{TEM} (100)	bla_{TEM}	β-lactams
	$bla_{CTX-M-1}$ (6.94)	β-lactams		*ampC*	β-lactams
	$bla_{CTX-M-9}$ (10.28)	β-lactams		*fsr*	Fosmidomycin
	bla_{VIM} (8.73)	β-lactams		*bcr*	Bicyclomycin
	mecA (5.80)	β-lactams			
	qnrA (7.83)	quinolones		*Bmr, marA, marR mdlB, mdtC, mdtB, mdtC, mdtH, mdtK, mdtL, stp, yheI*	Multiple antibiotics
	qnrS (4.42)	quinolones		*yddG*	Methyl viologen
	sul1 (7.73)	sulfonamides	*oqxB* (82)**	*ohrA*	Organic hydroperoxide
	armA (6.27)	aminoglycosides			
Lettuce-2	bla_{TEM} (9.72)	β-lactams	bla_{TEM} (100)	bla_{TEM}	β-lactams
	$bla_{CTX-M-1}$ (6.51)	β-lactams			
	$bla_{CTX-M-9}$ (8.98)	β-lactams			
	bla_{VIM} (7.75)	β-lactams			
	armA (5.89)	aminoglycosides		*fsr*	Fosmidomycin
	sul1 (6.62)	sulfonamides		*marA, ermrK, mdtA, mdtC*	Multiple antibiotics
	qnrA (4.82)	quinolones		*cnrA*	Nickel and cobalt
	qnrS (6.23)	quinolones			
Spinach-1	bla_{TEM} (8.55)	β-lactams	bla_{TEM} (99.9)	bla_{TEM}	β-lactam
	bla_{VIM} (8.35)	β-lactams		*pbp*	β-lactam
	qnrA (5.09)	quinolones		*ble*	Bleomycin
				bmrA, mexB	Multiple antibiotics
				copA	Copper
				ohrR	Organic hydroperoxide
Spinach-2	bla_{TEM} (9.39)	β-lactams	bla_{TEM} (99.9)	bla_{TEM}	β-lactams
	bla_{VIM} (8.52)	β-lactams	bla_{OXA} (96.7)	bla_{OXA-10}	β-lactams
				ampC	β-lactams
	qnrA (5.19)	quinolones	*dfr* (83.2)**	*drrA*	Thrimetroprim Daunorubicin/doxorubicin
				bhsA, bmrA, mexA, mdtA, mdtB, mdtD, mdtK, mdtL, mdtN, mexB, ermK, mdtA	Multiple antibiotics
				ohrR	

(*continued on next page*)

Table 2 (*continued*)

Virome	qPCR (GC/100 g)	Resistance to	ResFinder** (% identity)	Prokka/CARD Gene	Resistance to
					Organic hydroperoxide
				arsR, acr3	Arsenic
				czcA	Cobalt-Zinc-Cadmium
				cnrA	Nickel and cobalt
				sugE	Quaternary ammonium
				merR1	Mercury
Cucumber-1	bla_{TEM} (9.84)	β-lactams	bla_{TEM} (100)	bla_{TEM}	β-lactams
	$bla_{CTX\text{-}M\text{-}1}$ (7.63)	β-lactams		*drrA*	Daunorubicin/ doxorubicin
	bla_{VIM} (8.80)	β-lactams		*emrK, mdtA, mdtC, yheI*	Multiple antibiotics
	qnrA (5.76)	quinolones		*acr3*	Arsenic
				pcoC	Copper
Cucumber-2	bla_{TEM} (9.44)	β-lactams	bla_{TEM} (100)	bla_{TEM}	β-lactams
	bla_{VIM} (8.31)	β-lactams		*marA, marR, mdtc*	Multiple antibiotic
	qnrA (5.10)	quinolones		*cadC*	Cadmium

* not quantified; propagation in an *Escherichia coli* host strain allowed ARGS to be identified in these samples, but not their absolute abundance (Gómez-Gómez et al., 2019).
** All ARGs were detected by ResFinder using an identity threshold of 90% except *oqxB* and *dfr*, which were detected using 80%.

of genes encoding resistance to specific antibiotics as well as to metals (Co, Zn, Hg, and Cd) or other substances (quaternary ammonium, methyl viologen, organic hydroperoxide) and multiple antibiotic resistance proteins (mainly transporters and efflux pumps) (Table 2). The number of identified resistance genes varied among the different viromes, and none were associated with a particular food type. The largest diversity of resistance genes was observed in a veal sample (veal-2), which could not be explained by significant bacterial contamination, as the meat samples complied with EU regulations for fecal indicators, as confirmed in a previous study (Gómez-Gómez et al., 2019). However, this finding may indicate that the monitoring of bacterial indicators does not always accurately predict viral contaminants (Jofre, 2007).

When using ResFinder, which is a more specific software for acquired ARGs, with an identity threshold of 90%, the β-lactamase gene bla_{TEM} was found in all the viromes with 100% of coverage and a high % of identity, except in farm fish-1 (not included). Using a 90% threshold, bla_{OXA} was also detected in Atlantic fish-1 and spinach-2. In addition, an efflux pump membrane transporter *oqxB* was found in lettuce, and *dfr*, which confers resistance to Trimethoprim, in spinach, although both were only detected with an identity threshold of 80% (Table 2). Previous studies using qPCR have established that bla_{TEM} is one of the most prevalent and abundant ARGs in the phage fraction of fish, meat, and vegetable samples (Blanco-Picazo, Roscales, et al., 2020; Gómez-Gómez et al., 2019; Larrañaga et al., 2018) Whereas bla_{TEM} was the most abundant ARG in our samples (Table 2), other ARGs detected by qPCR were not found in the metagenomic analysis, suggesting that this approach requires higher gene concentrations. When sequencing the virome, it can be difficult to estimate the level of coverage needed to detect less abundant genes (Hjelmsø et al., 2017; Sims et al., 2014), and our results show that the efficiency of shotgun sequencing can decrease below the coverage threshold. On the other hand, although qPCR is a more sensitive technique, metagenomics can target a wider variety of genes.

ARGs in the viral DNA fraction can be packaged in phage capsids regardless of whether they are located in chromosomes or, as occurs more frequently, in plasmids. There is substantial evidence that plasmids can also be efficiently packaged in phage capsids by means of lateral transduction or similar mechanisms (Fišarová et al., 2021; Mann & Slauch, 1997; Rodríguez-Rubio et al., 2020).

In fact, it is possible that all ARG transfer systems occur simultaneously. In addition to vertical transfer, horizontal gene transfer plays a predominant role in the spread of resistance (Lerminiaux & Cameron, 2019). Probably the most explored horizontal gene transfer mechanism is conjugation, which depends on close contact between donor and recipient cells. Natural transformation, which has been less investigated (Lerminiaux & Cameron, 2019), may be less frequent, because the integrity of naked DNA is at risk in the environment and the recipient cells must be able to carry out natural transformation (Lerminiaux & Cameron, 2019). Finally, specialized, generalized, or lateral transduction could play a role in environments where the protection conferred by the viral capsid allows the transferred DNA to persist for longer; these strategies may have been underestimated, with potential evidence attributed to bacterial DNA contamination instead.

Transduction in the food matrix, during the food production process or in the intestinal tract of humans and animals after food ingestion could contribute to increasing the number of ARG-carrying phage particles in the intestinal microbiota. Fortunately, these particles alone do not seem to pose a direct threat for human health. Moreover, transduction cannot be completely avoided, as it is a natural part of phage/bacterial interactions. However, the presence of antibiotic residues in the environment as well as in human and animal bodies increases the rate of transduction events and promotes the persistence of new resistances (Kraemer et al., 2019; Ross & Topp, 2015). The presence of certain antimicrobials, such as β-lactam antibiotics or quinolones, can activate DNA SOS responses, and trigger horizontal gene transfer events, including transduction (Beaber et al., 2004; Maiques et al., 2006; Modi et al., 2013). Once acquired, maintenance of the ARG is mandatory for the survival of the recipient host if the antibiotic is present in the biome. To assess if ARG-carrying phage particles in food constitute emerging contaminants that are contributing to the growing global problem of antimicrobial resistance, the first step is their identification and monitoring.

ARG detection in viral fractions by qPCR indicates that phage capsids containing ARGs are abundant in the environment and food (Colomer-Lluch et al., 2011; Gunathilaka et al., 2017; Marti et al., 2014). However, the relatively low number of ARGs identified in temperate phage genomes suggests specialized transduction might not be the most common method of transmission (Enault et al., 2017; Kang et al., 2021). Instead, phages may incorporate ARGs by transposition or insertion events (Goh et al., 2013; Willi et al., 1997; Yang et al., 2017). More likely, however, is the involvement of generalized, lateral or related packaging mechanisms, as that would support the high amounts of bacterial DNA detected in the capsids apart from the ARGs. In fact, transduction is described as a very effective mobilization mechanism of bacterial DNA, plasmids and chromosomes, both in Gram positive and Gram negative bacteria, playing an important role in the evolution of the bacterial hosts (Fillol-Salom et al., 2021; Humphrey et al., 2021). Moreover, autotransduction also produces transducing particles, generated from the

temperate phage progeny, and has been proposed as an efficient strategy for ARG transfer in *Staphylococcus aureus* (Haaber et al., 2016). These and similar mechanisms might be used by bacteria to spread their own DNA content to their relatives.

4. Conclusions

The results of this study reflect the abundance of phages and transducing particles containing bacterial DNA in the viral fraction of food samples. The packaging of bacterial DNA in phage capsids allows genes to be mobilized between cells in food microbiomes or incorporated into our intestinal microbiota after ingestion of food items. Among the genes packaged in phage particles, *16S rRNA* genes were quite abundant, and provided clues about the bacterial hosts that generated these particles. It has been proposed that an absence of *16S rRNA* genes in viromes may rule out contamination by bacterial DNA, but while it is useful for the detection of bacterial DNA before disrupting the capsids, our results question its usefulness as a control of bacterial DNA contamination once the capsids are broken. The higher abundance of bacterial genes in the viromes involved in biosynthesis as opposed to other functions opens the question of whether the transduction of certain genes might be favored if they provide advantages for the recipient bacteria. As confirmed in previous studies, ARGs are also mobilized in phage particles, although their abundance is better monitored by qPCR than metagenomic analysis. In contrast, shotgun sequencing detects a broader spectrum of ARGs in viromes but fails to recognize those present in lower quantities. The presence of ARGs in the food chain is a subject of concern addressed by public policies (Koutsoumanis et al., 2021). ARG-carrying phage particles in food may be an unexplored route by which new resistances are introduced to humans and animals and should therefore be monitored to evaluate if they pose a health threat.

Availability of data and material

The metagenomic data set generated was deposited and is publicly available in the in the NCBI BioProject PRJNA731626 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA731626).

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CRediT authorship contribution statement

Pedro Blanco-Picazo: Resources, Investigation, Validation, Writing – original draft, Visualization. **Clara Gómez-Gómez:** Resources, Investigation, Validation, Writing – original draft, Visualization. **Marc Tormo:** Investigation, Validation, Data curation. **Maria Dolores Ramos-Barbero:** Data curation, Writing – review & editing. **Lorena Rodríguez-Rubio:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Maite Muniesa:** Conceptualization, Methodology, Data curation, Writing – review & editing, Supervision, Project administration, 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.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111342.

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