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Virome-associated antibiotic-resistance genes in experimental aquaculture plant

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ABSTRACT

We report a comprehensive characterization of viral and microbial communities of an aquaculture sample by a shotgun and a *16S rRNA* gene profiling metagenomic approach. *Caudovirales* represented the largest viral component, with over 50% of the total taxonomic abundance while around 30% of the total open reading frames (ORFs) identified were from eukaryotic viruses (*Mimiviridae* and *Phycodnaviridae*). ARGs in virome accounted for 0.85% of the total viral ORFs and they showed a similar distribution both in virome and in microbiome. Among ARGs, those genes coding for proteins involved in the modulation of antibiotic efflux pumps were the most abundant. Interestingly, the taxonomy of the bacterial ORFs identified into the viral metagenome did not reflect the microbial taxonomy as deduced by *16S rRNA* gene profiling and shotgun metagenomic analysis. A limited number of ARGs appeared to be mobilized, together with other bacterial genes coding for more general metabolic functions, from bacteria to phages or *vice versa* even in the absence of antibiotic treatment in the aquaculture plan studied, thus confirming the presence of a complex phage-bacterial network in the aquaculture environment.

INTRODUCTION

According to the recently released data (FAO -Fisheries and Aquaculture Department), world aquaculture production of food fish has exceeded 60 million tons in 2011, with an increase of 6.2% over the previous year (FAO 2011). This large growth of aquaculture has been accompanied by an increased use of a wide range of antibiotics (Armstrong *et al.* 2005). About half of the world's industrial production of antimicrobials is consumed in terrestrial animal agriculture with both therapeutic and prophylactic use (Levy and Marshall 2004; Sarmah *et al.*, 2006; Davies and Davies 2010). In aquaculture, the main use for antibiotics is to prevent and treat bacterial infection in fish; the prophylactic use of antibiotics is a common practice as well. The necessity of antibiotic use in aquaculture is a consequence of lowered host defenses associated with high-density culture with suboptimal hygiene (Grave *et al.*, 1999; Defoirdt *et al.*, 2007; Cabello *et al.*, 2013). Horizontal gene transfer among bacteria occurs by one of the three following mechanisms: conjugation, free DNA transformation and transduction through bacteriophages. Bacteriophages can move any sort of bacterial DNA, including linear chromosome fragments and all sorts of mobile elements such as plasmids, islands, transposons and insertion elements (Mann and Slauch 1997; Schmieger and Schicklmaier 1999; Muniesa *et al.*, 2013). Despite the potential importance of bacteriophages in transferring resistance genes from the environment to human and animal body microbiomes, the studies focusing on this topic are limited (Modi *et al.*, 2013). Concerning the virome associated to fresh water environments, the literature reports only studies that describe the taxonomic diversity and composition (López *et al.*, 2009; Rosario *et al.*, 2009; Roux *et al.*, 2011; Fancello *et al.*, 2012; Tseng *et al.*, 2013) or the viral and microbial community dynamics (Rodriguez-Brito *et al.*, 2010). Only few studies reported the metabolic profile deduced by viral metagenomes (Fancello *et al.*, 2012; Modi *et al.*, 2013).

In this work, we report a comprehensive characterization of viral and microbial communities in an experimental aquaculture sample through a metagenomics approach. The contemporary study of

both communities allowed the identification of different genes mobilized in the virome, with particular attention to ARGs.

Viral taxonomic characterization

Water sample (20 liters) was collected on March 2014 at Edmund Mach Foundation – Technology Transfer Centre (San Michele all'Adige – TN – Italy; 46°11'42.9"N 11°08'10.1"E). The sample was collected from the breeding tank wastewater. Antibiotics treatments have not been applied. The tank was used for the breeding of different salmonids as *Salmo carpio*, *Salmo trutta marmoratus* and *Salvelinus alpinus*. Water sample was filtered at 0.45 μm on a Sartorious filtering system (Sartorius AG., Goettingen, Germany). The filter was recovered and used to collect microbial cells as described later. The permeate containing all the viral like particles (VLPs) was later filtered at 100 KDa (Pall, Life Sciences, Milan, Italy) using a tangential flow filtration systems (TFF) (Quattro systems, Parma, Italy). VLPs were precipitated with PEG 8000, and than concentrated through a step of CsCl gradient centrifugation and DNA extraction according to the protocol proposed by Thurber et al. (2009). In order to increase the amount of DNA available, whole-genome amplification was performed using reagents and protocols in the Illustra GenomiPhi V2 kit (GE Healthcare) to generate sufficient material for metagenomic analysis. A qPCR quality control assay was carried out in order to verify the absence of detectable, contaminating, non-viral DNA according to Modi *et al.* (2013). Metagenomic shotgun of viral DNA was determined by GenProbio srl (Parma, Italy) using an Ion Torrent PGM platform (Life Technologies, Carlsbad, CA) and the MIRA program version 4.0.2 was used for *de novo* assembly of contigs. According with other studies (Fancello *et al.*, 2012; Roux *et al.*, 2012*;* Zablocki *et al.*, 2014), largest part of the identified ORFs (accession number SAMEA3333506) was from Bacteria kingdom (86%). Viruses were the second most represented kingdom (13%). Eukariota and Archea were identified both under 0.5%. Contigs obtained from shotgun sequencing were analyzed via MetaVir pipeline performing a BLAST comparison with the Refseq complete viral genomes protein sequences database from.

Thirteen families were identified (Table S1 Supporting Information) and prokaryotic viruses were observed as the most abundant in the sample. *Caudovirales* order accounted for more than fifty percent of the total taxonomic abundance. *Myoviridae* and *Siphoviridae* were the most represented families with respectively 28% and 18% of the total ORFs. The analysis revealed the presence of viral families that parasite *Eukaryotes* as *algae* and *amoebae*: *Mimiviridae* and *Phycodnaviridae* were the main component of this group representing 13-15% of the total respectively. Around 10% of sequences were assigned to unclassified viruses or phages. The largest variety of phages identified interacted with *Proteobacteria* coherently with the abundance of this phylum in the microbiome (Fig. S1 Supporting Information). Inside *Proteobacteria* the two main families represented in the water sample, *Sphingomonadaceae* and *Comamonadaceae,* and their related genera are typical constituents of freshwater environments, constituting the bacterioplankton or in some cases being recognized as opportunistic pathogens (Kallman *et al.*, 2006; Kilic *et al.*, 2007; Lin *et al.*, 2010; Vaz-Moreira *et al.*, 2011; Chen *et al.*, 2013). On the other hand, the most abundant ORFs identified in virome had best match score with phages that infect *Bacillus*, *Synechococcus* and *Mycobacterium*, bacterial genera that have not been identified in the microbiome, thus suggesting that these genera were under-represented in the water sample probably due to a phage infection resulting in a lytic cycle. In this context, the sharply reduction or local extinction of microbial taxa by viral predation is a phenomenon that would be expected according to the Kill-the-Winner dynamics hypothesis (Thingstad 2000). This dynamic model postulates a repetitive cycle in which an increase in prey population leads to an increase in the predator population that, in turn, decreases the prey population, thus causing its own subsequent decline.

Distribution and taxonomy of ARGs in virome and microbiome

Sartorious filter (0.45 μm) (Sartorius AG., Goettingen, Germany), used in the first step of VLPs recover, was washed in 20 ml of TE buffer pH 8.0 in order to release microbial cells. The cell suspension obtained was collected by centrifugation and subjected to total DNA extraction as

described by Arioli et al. (2007). Extracted DNA was used for *16S rRNA* gene profiling as described by Milani et al. (2013) (accession number SAMEA3334740), and for a metagenomics shotgun analysis [determined by GenProbio srl (Parma, Italy)] based on a genomic library generated from three μg of genomic DNA and using the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina inc, San Diego, CA, USA) and employing the MiSeq Reagent chemistry according to the user guide. The quality of the library was estimated and loaded onto a flow cell and subsequently sequenced using 500 sequencing cycles according **to** the Preparing Libraries for Sequencing on the MiSeq (Illumina inc, San Diego, CA, USA). 500 sequencing cycles resulted in an average reading length of approximately 250 nucleotides for both paired-end sequences (accession number SAMEA3334741. The ORFs obtained from shotgun metagenomic sequencing were analyzed using CARD database (2972 genes in CARD are tagged specifically for antibiotic resistance). The total amount of the ARGs identified were 950 (4.13% of the total ORFs identified with NCBI) in the microbiome and 214 (2.64%) in the virome. The distribution of the ARGs classes identified in the two metagenomes (Fig.S2 Supporting Information) showed that the most abundant genes were those coding for proteins involved in the modulation of antibiotic efflux pumps (CARD nomenclature), with values ranging from 16% to 19% of the total amount of identified ARGs. Among the different ARGs classes, antibiotic efflux pumps showed the most general function, acting on different target molecules thus having a cell detoxifying activity (Pao *et al.* 1998). "Macrolide-resistance genes" was the second most abundant group, including erythromycin, telithromycin and clarithromycin resistance genes. With few exceptions, the distribution of ARGs classes was similar between microbial and viral metagenomes. Glycopeptide efflux pump, and lincosamide ARGs classes showed higher values in virome than in microbiome (Fig.S2 Supporting Information), whereas gene modulating antibiotic efflux pumps and fluoroquinolone resistant genes were the most abundant in the microbiome. To each ARGs identified, the relative taxonomy of the respective ORF based on the results obtained from the BLAST comparison with the NCBI refseq database has been associated. The taxonomy of ARGs in microbiome (Fig. 1) showed the presence

of three main families (*Comamonadaceae* 26-37%, *Sphingomonadaceae* 18-24%, and *Sinobacteraceae* 14-21%) equally distributed among the different drug classes, and a large part (15-26%) of the ARGs was shared among different microbial families accounting for a relative amount lower than 4%. The virome showed that ARGs were mainly distributed between *Flavobacteriaceae* (38% - 53%) and *Methylophilaceae* (19% - 33%) families, and other bacteria families (7-13%) with relative abundance below the 5% threshold (Fig. 1). None of the families that were present in both the virome and in the microbiome, accounted for a relative abundance higher than 5%. From a taxonomic point of view, it is interesting to note that microbial ORFs identified in virome did not reflect the microbiome taxonomy, as determined by *16S rRNA* gene profiling (Fig. S3 Supporting Information) or by shotgun metagenomics analysis (Fig. 1), thus suggesting that microbial genes mobilized in the genome of viruses could be considered as a reminiscence of the past recombination events rather than a picture of the current microbial diversity.

Virome and microbiome shared ARGs

In order to identify possible events of gene mobilization, ORFs located both in the microbiome and in the virome have been identified. To this aim, a BLAST comparison was performed using the ORFs identified in the virome as query and microbial ORFs as reference database; BLASTp with a threshold of 10^{-5} on the e-value were used. Only matches that showed an aminoacidic identity over 95% were considered. A total amount of 213 different ORFs have been identified to be shared between the two metagenomes, and mostly of them were related to microbial metabolism. With respect to the ARGs identified in the virome, 4% of them were shared with the microbiome with an aminoacidic sequence identity higher than 95%. Half of the identified ORFs encode for genes modulating the antibiotic efflux (i.e. transcriptional regulator); ORFs that encode for glycopeptide, tetracycline and beta-lactam resistance genes have also been identified. Among these genes, one was classified as a *Sphingopyxis alaskensis* β-lactamase coding gene, which was putatively mobilized together with other four genes including a pseudogene coding for a β-lactamase of *Sphingomonas* sp (Table 1). The other five genes shared between the virome and the microbiome,

were putatively involved in tetracycline and glycopeptide resistance, and antibiotic efflux mechanisms. In all cases, shared ARGs were not transferred alone but with other genes hosted in the same contig (Table 1). Our data indicated that ARGs are present in virome and some of these ARGs appeared to be mobilized from bacteria to phages or *vice versa*. The role of viruses in the mobilization of ARGs by transduction mechanisms have been recently described in a murine model (Modi *et al.*, 2013). The authors showed that the phageome became broadly enriched for functionally beneficial genes under stress-related conditions (*e.g.* an antibiotic treatment) and that antibiotic treatment expanded the interactions between phage and bacterial cells, leading to a more highly connected phage–bacterial network for gene exchange (Modi *et al.*, 2013). In our data, we showed that ARGs might be mobilized even in the absence of selective pressure, *i.e.* in the absence of antibiotic treatment in the aquaculture. Moreover, we showed that ARGs were mobilized together with other bacterial genes coding for more general metabolic functions, thus confirming the presence of a complex phage-bacterial network in the aquaculture environment.

ARGs in publically available viromes

In order to compare our data with those publically available, the relative amount of ARGs in 17 viromes obtained from freshwater samples was calculated. The sample analyzed in this work was basically groundwater pumped in aquaculture tanks containing approximately 7 kg/m^3 of salmonid (the fish density in a commercial aquaculture is close to 25 kg/m^3) with a water flow of approximately 83 m^3/h. Nevertheless, our sample showed a relative abundance of ARGs of 0.85%, the second highest value after the El Barbera lake (Fancello *et al.*, 2012). Analyzing the data reported (Table S2 Supporting Information), it is worth of mention that the relative amount of ARGs did not seems to correlate with the presence of anthropic activities geographically located near to the aquatic environments analyzed, being the highest in ARGs relative abundance found in the El Barbera sample, which was collected from the Mauritanian desert. The presence of ARGs in uncontaminated wild environment have been previously reported. Several studied, in fact, identified

ARGs in different ecosystems, from soil and permafrost (D'Costa *et al.*, 2006; Hallen *et al.*, 2010) to human gut (Hu *et al.*, 2013) as recently observed in the microbiome of members of an isolated Yanomami Amerindian village (Clemente *et al.*, 2015). Therefore, the diversity, the taxonomic distribution and the ecological role of antibiotic resistant genes in the environment is still far unknown. Moreover, it should be underlined that the presence of ARGs in metagenomes did not directly represent a risk for human health because a proper ranking of these risks should be carried out (Martinez *et al.*, 2015).

In conclusion, this study addresses for the first time a complete description of microbiome and virome in an aquaculture sample, giving information on the presence of ARGs and their mobilization by transduction mechanisms. In this context, the monitoring of the ARGs in a microbial community could be a useful tool to follow environmental perturbations, particularly in aquaculture where prophylactic use of antibiotics is a common practice.

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Conflict of interest statement. None declared.

212 Table 1. ARGs genes shared between viral and microbial metagenomes

ARGs shared between viral and microbial metagenome	Coding function [a]	Identity [b] (%)	Mobilized ORFs hosted in the same contig	contig *n.*	
				virome	microbiome
β-lactam resistant gene (*Sphingopyxis alaskensis*)	beta-lactamase (class C family)	100%	β-lactam resistant gene partial (*Sphingomonas* sp.) hypothetical protein (*Sphingomonas* sp.) peptidase (*Sphingopyxis alaskensis*) membrane protein (*Sphingopyxis alaskensis*)	c4174	c208
Tetracycline resistant gene (*Caulobacter* sp.)	Transcriptional regulator, subgroup of AraC transcriptional regulators having an N-terminal Type 1 glutamine amidotransferase (GATase1)-like domain	100%	integrase (*Novosphingobium* sp.) sarcosine oxidase subunit beta (*Novosphingobium* sp.)	c4836	c92
Gene modulating antibiotic efflux (Oxalobacteraceae bacterium IMCC9480)	Transcriptional regulator (repressor function), TetR family	100%	cyclohexanone monooxygenase (*Brevundimonas subvibrioides* acyl-CoA ligase (*Brevundimonas subvibrioides*) TonB-denpendent receptor (*Brevundimonas subvibrioides*) L-carnitine dehydratase/bile acid-inducible protein F (*Caenispirillum salinarum*) alpha/beta hydrolase (*Deinococcus phoenicis*) putative flavin-containing monooxygenase (*Rhodococcus wratislaviensi*s NBRC 100605)	c4172	c321
Glycopeptide resistance gene (*Sphingopyxis baekryungensis*)	AAA+ (ATPases Associated with a wide variety of cellular Activities)	100%	GTPase HflX (*Sphingopyxis baekryungensis*) RNA-binding protein Hfq (*Sphingopyxis baekryungensis*)	c4235	c143
Glycopeptide resistance gene (*Sphingobium baderi*)	HATPase_C (Histidine kinase-like ATPases)	100%	ATPase AAA (*Sphingopyxis baekryungensis*)		
Gene modulating antibiotic efflux (*Limnohabitans* sp. Rim28)	REC (cheY-homologous signal receiver domain)	97%	/	c73	c713
	HATPase_C (Histidine kinase-like ATPases)	98%	/		

213 [a], function assigned by NCBI conserved domains database; [b], amino acidic identity between the ORFs in the virome and the ortholog in the microbiome
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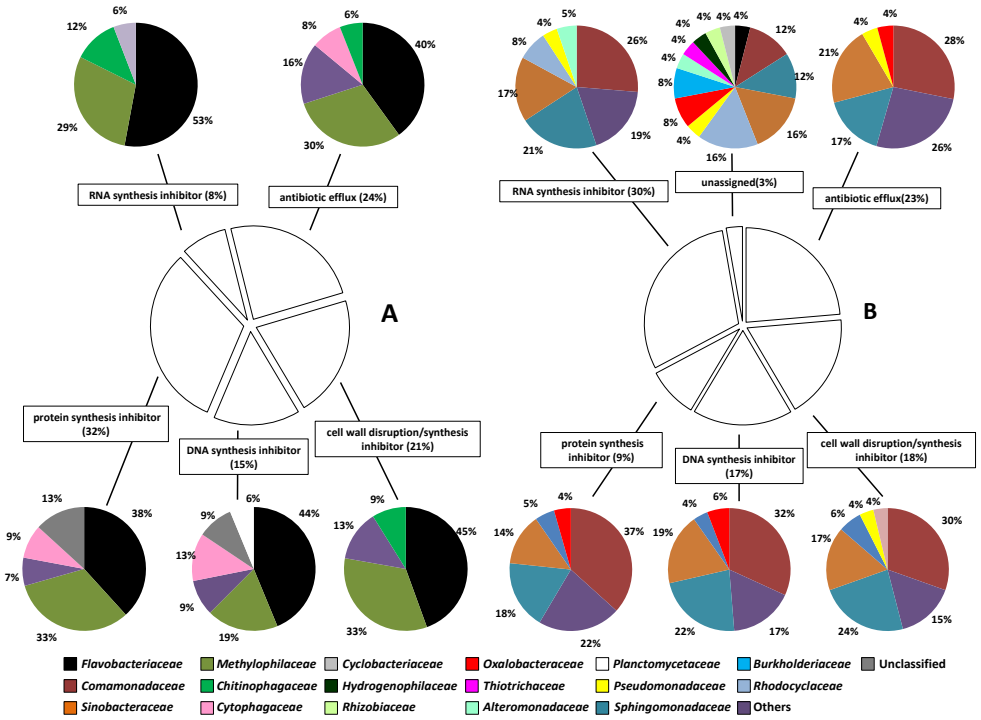

Figure 1. Comparison of the antibiotic resistance related ORFs distribution among the viral (A) and the microbial (B) metagenome. ARGs have been divided in six different drug classes: RNA synthesis inhibitor, Protein synthesis inhibitor, DNA synthesis inhibitor, Cell wall disruption and synthesis inhibitor, Antibiotic efflux and Unassigned; taxonomy distribution at family level has been associated to each drug class.

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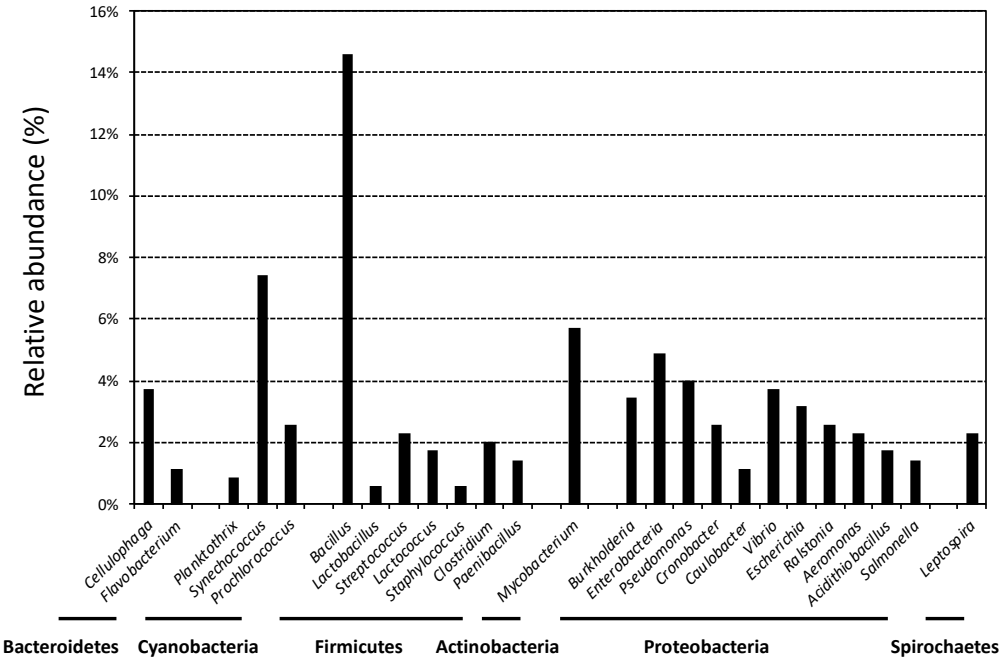

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320 Figure S1. Distribution of phage-hosts association. Percentages were respect to the total amount

321 of phage-related ORFs identified. Bacteria were grouped based on taxonomic level of phyla.

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Table S1. Taxonomic abundance of ORFs (BLASTp with a threshold of 10^5 for the E value) identified by MetaVir in the aquaculture virome

Virus order and family	Host(s)	Taxonomic abundance of viral ORFs (%)
Caudovirales		53,70%
Myoviridae	Bacteria, Archaea	27,78%
Podoviridae	Bacteria	6,67%
Siphoviridae	Bacteria, Archaea	17,59%
unclassified Caudovirales	Bacteria, Archaea	1,67%
Herpesvirales		0,37%
Alloherpesviridae	Vertebrates	0,19%
Herpesviridae	Vertebrates	0,19%
Virus not assigned into an order		
Ascoviridae	Invertebrates	1,11%
Baculoviridae	Invertebrates	0,37%
Inoviridae	Bacteria	1,85%
Marseilleviridae	Amoebae	0,19%
Mimiviridae	Amoebae	13,15%
Phycodnaviridae	Algae	15,37%
Poxviridae	Humans, arthropods, vertebrates	1,67%
Virus not assigned into families		
unclassified phages	N/A	3,70%
unclassified viruses	N/A	7,78%
ssDNA viruses	N/A	2,41%

N/A not assigned