Evaluation of *Enterococcus*-infecting phages as indices of fecal pollution

Tasha M. Santiago-Rodriguez, Patricia Marcos, Silvia Monteiro, Miguel Urdaneta, Ricardo Santos and Gary A. Toranzos

ABSTRACT

No microbial source tracking tool satisfies all the characteristics of an ideal indicator of human fecal pollution. For this reason, the potential of *Enterococcus faecalis* phages (enterophages) as markers of this type of contamination was tested by using eight *Enterococcus* type strains as the possible hosts. The prevalence of enterophages in animal feces and domestic sewage were determined, as were the inactivation rates in raw sewage at 4 °C and surface and tap waters at 22 °C. Enterophages were exclusively detected in raw sewage (up to 66.0 plaque forming units (PFU)/100 mL), suggesting a strictly human origin; and exhibited inactivation rates of approximately 0.002 to 0.05, 0.3 to 0.5 and 0.4 to 1.4 log day^{-1} in raw sewage and surface and tap waters, respectively, similar to those of previous reports on human enteric viruses under similar conditions. Interestingly, phages infecting other *Enterococcus* type strains were detected in both animal feces and domestic sewage in concentrations of up to 335.8 PFU/g and 96.0 PFU/100 mL, and certain phage isolates infected several of the strains tested. This clearly indicates the possible promiscuous nature of some *Enterococcus* phages and thus opens up the opportunity to further characterize these as indices of specific fecal sources.

Key words | enterococci, enterophages, fecal pollution, microbial indicators, microbial source tracking

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INTRODUCTION

Human pathogens can be introduced into water sources as a result of fecal pollution. Among these, enteric viruses are of great concern since they: (i) can cause disease with infectious doses as low as 1 to 10 particles, (ii) affect 5 to 18 million people every year, and (iii) can be resuspended from sediments to surface waters after disturbances (Rao *et al.* 1984; Bosch 1998; Lipp *et al.* 2001; Rose *et al.* 2001). Enteric viruses are often detected by culture and molecular techniques (Jaykus *et al.* 1996; Schwab *et al.* 1996; Greening *et al.* 1999; Fout *et al.* 2003). However, culture methods are notoriously expensive and laborious and non-infectious viral particles or naked viral nucleic acids can be detected by molecular techniques (Schwab *et al.* 1998; Leclerc *et al.* 2000). Since not all laboratories have the facilities for enteric virus detection, microbial indicators have been used to infer the presence of these pathogens in waters and sewage; however, many microbial indicators fail to fulfill the characteristics of a reliable marker of fecal pollution (Havelaar *et al.* 1993). For instance, relatively large numbers of both enterococci and thermotolerant coliforms have been detected in pristine waters and are able to replicate in these environments (Rivera *et al.* 1988; Toranzos *et al.* 1996; Byappanahalli *et al.* 2003). Algae and sediments could also be sources of indicator bacteria and although certain type strains have been linked to fecal pollution, their presence in the feces of different warm-blooded animals makes them unreliable for microbial source tracking (MST) purposes (Baele 2002). In addition, enteric viruses can be present in waters that meet bacteriological

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standards and absent from waters failing to meet this criteria (Bosch 1998).

Bacteriophages have also been proposed as markers of fecal contamination and as models of human enteric viruses. Such is the case of phages infecting *Bacteroides fragilis* and *Escherichia coli* (coliphages). However, *B. fragilis* phages have been detected only in certain geographical regions (Allsop & Stickler 1985; Puig *et al.* 1997; Gantzer *et al.* 2002; Scott *et al.* 2002) and the techniques used for their detection are relatively difficult due to the strict anaerobic nature of the bacterial host (Weisberg *et al.* 1996; Gantzer *et al.* 1998). Coliphages may not discriminate the source of the fecal contamination since these have been detected in the feces of different animals (Brion *et al.* 2002; Cole *et al.* 2003). In addition, survival experiments have been performed with these bacteriophages (Grabow & Coubrough 1986; Hernandez-Delgado & Toranzos 1995; Gantzer *et al.* 1998; Long & Sobsey 2004). Coliphages can survive for long periods in pristine waters and may not necessarily indicate recent fecal pollution (Hurst & Gerba 1980; Hernandez-Delgado & Toranzos 1995; Long & Sobsey 2004).

There is a lack of microbial indicators of human fecal pollution. For this reason, we have proposed phages that infect a specific type strain of *Enterococcus faecalis* (enterophages) as markers of human fecal contamination. Most enterophage isolates characterized in our laboratory are tailed phages, with icosahedral capsids measuring between 13 and 80 nm and DNA genomes >23 kbp (Bonilla *et al.* 2010; Santiago-Rodriguez *et al.* 2010). In terms of their reliability as indicators of human fecal pollution, enterophages: (i) have been detected in raw and treated domestic sewage (Bonilla *et al.* 2010; Santiago-Rodriguez *et al.* 2010), (ii) possess a survival similar to that of many enteric viruses in fresh waters (Rao *et al.* 1984; Ward *et al.* 1986), and (iii) have not been detected in cattle feces. Even though previous results have been promising, more data are needed to fully determine the potential of enterophages as indicators of human fecal contamination. The present study focused on the use of various *Enterococcus* species (spp.) as the bacterial hosts, and fecal material from different animals and domestic sewage were tested for the presence and prevalence of enterophages. We also determined the inactivation rates and survival times of enterophage isolates under various conditions, and compared results to those studies using human enteric viruses. Therefore, the aims of the present study were to: determine the presence of enterophages in animal feces, their prevalence in raw and treated domestic sewage, inactivation rates and survival times under various conditions, including raw sewage at 4 °C, fresh and tap waters at 22 °C and various sterile water types at 37 °C.

MATERIALS AND METHODS

Detection of *Enterococcus* phages in feces and sewage

Poultry, cattle, pigs, and humans are among the most common sources of fecal pollution (USEPA 2005; Ahmed *et al.* 2008). For this reason, the presence of enterophages in animal feces and domestic sewage was tested by using *Enterococcus* type strains from the American Type Culture Collection (ATCC) and included: *E. faecalis* (ATCC 19433), *E. faecium*, *E. gallinarum*, *E. durans* (ATCC 19432), *E. dispar* (ATCC 51266), *E. hirae* (ATCC 8043), *E. casseliflavus* (ATCC 25788), and *E. pseudoavium* (ATCC 49372) as the possible bacterial hosts. In addition, the presence of *Enterococcus* phages in dog feces was tested since close contact with humans and lateral transmission of microorganisms can occur (Guardabassi *et al.* 2004). Samples were also processed for coliphages using *E. coli* C3000 (ATCC 15597) as the bacterial host as a means of comparison. Composites of the fecal samples were processed by using the single layer method: Briefly, 1 gram of chicken ($n = 30$), cattle ($n = 30$), pig ($n = 10$), and dog ($n = 12$) feces were eluted in 50 mL of a saline solution (0.85% w/v) per gram of feces and mixed with an equal amount of liquefied media as described previously (Santiago-Rodriguez *et al.* 2010). In order to detect the possible presence of different bacteriophage thermal groups, the mixture was poured into four Petri dishes and incubated at 22, 37, and 41 °C and viral plaques were enumerated at 24 h. The presence of *Enterococcus* phages was tested in raw sewage and primary effluent from three wastewater treatment plants (WTP) in Puerto Rico. Samples were collected and processed monthly from August 2010 to August 2011 using the single layer method with a modification (Bonilla *et al.* 2010). Sixty-milliliter aliquots were added to

equal amounts of molten media and processed individually by using the *Enterococcus* type strains described above as the bacterial hosts. Petri dishes were incubated at 22, 37, and 41 °C, viral plaques were enumerated at 24 h and the percent removal of each phage group was calculated.

Isolation and characterization of *Enterococcus* phages

Individual viral plaques were isolated as described previously (Bonilla *et al.* 2010). Several of the isolates were tested against different *Enterococcus* type strains using a spot test to determine the host specificity and replication temperature. Briefly, 1 μL of the bacteriophage isolate was placed on top of a Petri dish containing Trypticase Soy Broth (TSB) (Difco), agar (1.5% w/v), $CaCl_2{\cdot}2H_2O$ (Fisher Scientific Co., NJ, USA), and NaN_3 (MCB, OH, USA) (final concentration of 2.6 and 0.4 mg/mL, respectively) and a lawn of the bacteria tested. A total of three dishes was incubated at 22, 37, or 41 °C for 24 h. In addition, the morphology of two *E. faecalis* phages was determined by using transmission electron microscopy, as described previously (Bonilla *et al.* 2010).

Inactivation rates and survival studies

Current regulations require that water and sewage samples be processed within 6 h and stored at 4–7 °C prior to analysis. To test if storage time could affect the detection of *Enterococcus* phages, we determined the inactivation rates of phages infecting *E. faecalis*, *E. casseliflavus*, *E. faecium*, and *E. coli* in raw sewage. One liter of raw domestic sewage was seeded with a concentration of 10^4 plaque forming units (PFU)/100 mL from prepared laboratory stocks and kept at 4–7 °C. Aliquots were obtained daily and processed using the single layer method as indicated above. To simulate the survival of enterophages and coliphages under environmental conditions, raw domestic sewage was added to three fresh water samples collected from the Rio Grande de Arecibo watershed in Puerto Rico (Duran *et al.* 2001; Noble *et al.* 2003). Experiments were performed during a period of low and higher rainfall events and included: (i) the highest and thus one of the less polluted sites of the watershed (site 1), (ii) immediately after a WTP (site 2), since it represents a point-source of fecal contamination, and (iii) the estuary (site 3). The survival experiments were conducted as described above and seeded samples were kept at 22 °C, the average temperature of waters in Puerto Rico (Santiago-Rodriguez *et al.* 2010). All precipitation data were obtained from the US Geological Survey (USGS) Caribbean Water Science Center, stations 50020100, 50024950, and 50021700 (http://pr.water.usgs.gov/). Similarly, for the survival of enterophages and coliphages in tap water, 2 L were collected in a sterile container and seeded with enterophages and coliphages and aliquots were processed as described (Bonilla *et al.* 2010). In order to determine the possible effect of chlorine in the survival of enterophages and coliphages, one of the samples was dechlorinated using sodium thiosulfate (final concentration of 10.0 mg/L) and kept at 22 °C. Similarly, survival experiments with an enterophage isolate were performed at 37 °C (the optimal growth temperature of the host and the isolation temperature of the enterophage tested) in sterile tap, distilled, and wastewater, as described previously.

Results for all inactivation rates and survival experiments were plotted as semi-log plots of time (days) versus bacteriophage PFU/100 mL. Decay constants (*kd*) were calculated by using the slopes of the linear regressions of the semi-log plots ($-\log_{10}$ PFU day^{-1}) (Yates *et al.* 1985; Santiago-Rodriguez *et al.* 2010). T_{90} values, or the time to reach a 90% reduction in PFU densities, were calculated as ln (0.1)/*kd* (Sinton *et al.* 1994; Noble & Fuhrman 1996; Noble *et al.* 2003).

Statistical analyses

Non-parametric one-way analyses of variances (Kruskal–Wallis) were used to determine differences in the prevalence of *Enterococcus* phages in raw sewage as influenced by the bacterial host tested and incubation temperature. The same analyses were used to determine differences in the inactivation rates of the *Enterococcus* phages (*E. faecalis, E. faecium,* and *E. casseliflavus*) and coliphages in raw sewage at 4 °C (Brion *et al.* 2002; Allwood *et al.* 2003). For the survival experiments in fresh waters, the analyses were used to determine differences in the inactivation rate of enterophages and coliphages as influenced by the incubation temperature and sampled site. Statistical analyses

were performed using the R statistical software (version 2.11.1) (Team 2010).

RESULTS

Enterococcus phages in animal feces and domestic sewage

Interestingly, phages infecting *E. faecium, E. casseliflavus,* and *E. pseudoavium* were detected in chicken feces at 37 °C (Table 1). Phages infecting *E. gallinarum, E. durans, E. dispar,* and *E. hirae* were not detected in chicken, cattle, or pig feces. Coliphages were detected in chicken (1.0 ± 0.0 PFU/g) and cattle feces at 22 and 37 °C (40.3 ± 28.0 and 335.81 ± 4.0 PFU/g, respectively) and in dog feces at 41 °C (120.79 ± 29.45 PFU/g). Neither coliphages nor *Enterococcus* phages were detected in pig feces. Enterophages were detected at all temperatures tested in raw sewage collected from all WTP and at 22 and 37 °C in the primary effluents from two WTP. Interestingly, *E. faecium*-infecting phages were also detected in raw and treated sewage from two WTP at all temperatures tested. Higher concentrations of both *E. faecalis* and *E. faecium* phages were detected in domestic sewage compared to other enterococci phages and exhibited removal percents of 37.0 to ≥99.0% and 47.0 to ≥99.0%, respectively. Other *Enterococcus* phages exhibited various removal percents depending on the WTP and incubation temperature (Table 2). Phages infecting *E. casseliflavus* were detected at 22 and 37 °C in raw sewage collected from all WTP and at 22 and 37 °C in the primary effluents. *Enterococcus pseudoavium*-infecting phages were also detected at 22 °C in raw sewage from all WTP and at 37 °C in two WTP, but were not detected in the primary effluents. Other phages detected in raw sewage were those infecting *E. hirae, E. durans,* and *E. dispar,* but these were not constantly detected in primary effluent (Figure 1).

Replication and morphology of *Enterococcus*-infecting phages

Most of the *Enterococcus* phage isolates in this study did not infect a specific bacterial host, with the exception of those infecting *E. faecalis*, which replicated at all three replication temperatures tested as shown previously (Santiago-Rodriguez *et al.* 2010). Table 3 shows the host range and replication temperature of the tested phages. In terms of the morphology of the *E. faecalis* phages in this study, these exhibited long non-contractile tails of approximately 180 nm and icosahedral capsids of approximately 80 nm (Figure 2). This morphology is similar to the enterophages characterized previously (Santiago-Rodriguez *et al.* 2010).

Inactivation rates and survival of *Enterococcus* phages

The initial titer of *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. coli* phages in raw sewage at 4 °C remained constant for 6 days. Calculated decays for *E. faecalis* phages were 0.050 ± 0.030, 0.030 ± 0.030, and 0.080 ± 0.030 log day^{-1}

Table 1 | *Enterococcus* and *E. coli*-infecting phages per gram of feces in chicken ($n = 30$), cattle ($n = 30$), dogs ($n = 12$), and pigs ($n = 10$). *Enterococcus* spp., included *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae*, *E. durans*, *E. dispar*, *E. casseliflavus*, and *E. pseudoavium*. Only hosts exhibiting positive results in at least one fecal source were included in the table

	Host				
Source	***E. coli***	***E. faecalis***	***E. faecium***	***E. casseliflavus***	***E. pseudoavium***
Chicken	1.0 ± 0.0[a]	ND	107.5 ± 10.6[b]	65.0 ± 0.0[b]	3.8 ± 1.8[b]
Cattle	40.3 ± 28.0[a]	ND	ND	ND	ND
Dogs	335.81 ± 4.0[b]	ND	ND	ND	ND
	120.79 ± 29.45[c]				
Pigs	ND	ND	ND	ND	ND

ND = not detected.
[a]22 °C.
[b]37 °C.
[c]41 °C.

Table 2 | Mean removal percentages of *Enterococcus* phages in primary effluent from three WTP (PR-A, PR-B, and PR-C) in Puerto Rico. Numbers were calculated for different incubation temperatures, namely 22, 37 and 41 °C. Phages infecting several *Enterococcus* were not detected (ND)

	PR-A			PR-B			PR-C		
Host	**22 °C**	**37 °C**	**41 °C**	**22 °C**	**37 °C**	**41 °C**	**22 °C**	**37 °C**	**41 °C**
E. faecalis	**37.0 ± 44.0**	**44.0 ± 33.0**	≥99.0 ± 0.9	83.0 ± 16.8	80.0 ± 15.9	≥99.0 ± 0.09	≥99.0 ± 0.09	≥99.0 ± 0.09	≥99.0 ± 0.09
E. faecium	**63.0 ± 16.0**	86.0 ± 5.8	ND	47.0 ± 42.0	60.0 ± 37.0	≥99.0 ± 0.09	≥99.0 ± 0.09	≥99.0 ± 0.09	≥99.0 ± 0.09
E. gallinarum	ND	ND	ND	ND	ND	ND	ND	ND	ND
E. hirae	ND	ND	ND	≥99.0 ± 0.09	≥99.0 ± 0.9	ND	≥99.0 ± 0.09	ND	ND
E. durans	ND	ND	ND	≥99.0 ± 0.09	≥99.0 ± 0.09	ND	ND	ND	ND
E. dispar	≥99.0 ± 0.09	ND	ND	**48.0 ± 25.2**	≥99.0 ± 0.45	≥99.0 ± 0.09	≥99.0 ± 0.09	ND	ND
E. casseliflavus	**27.0 ± 32.4**	≥99.0 ± 0.9	ND	81.0 ± 12.1	≥99.0 ± 0.09	≥99.0 ± 0.09	73.0 ± 20.4	≥99.0 ± 0.09	ND
E. pseudoavium	≥99.0 ± 0.09	≥99.0 ± 0.9	ND	87.0 ± 1.0	≥99.0 ± 0.09	≥99.0 ± 0.09	≥99.0 ± 0.09	ND	ND

Lowest removal percentages are presented in bold.

at 22, 37, and 41 °C, respectively. *Enterococcus faecium* phages exhibited a decay of 0.0040 ± 0.0020 and 0.0020 ± 0.0020 log day^{-1} at 22 and 37 °C, respectively. For this specific *E. faecium* phage isolate, 41 °C was inhibitory. Similarly, the *E. casseliflavus* phage isolate in these experiments was able to replicate only at 22 °C and exhibited a decay of 0.020 ± 0.030 log day^{-1}. Coliphages exhibited decays of 0.010 ± 0.020, 0.040 ± 0.020, and 0.020 ± 0.050 log day^{-1} at 22, 37, and 41 °C, respectively.

Survival of enterophages and coliphages in fresh waters

During the period of low rainfall events, up to 1.01 mm of rain was reported at site 1 and no precipitation was reported at sites 2 or 3 24 h prior to collecting the samples. During this period, enterophages exhibited an average decay of 0.47 ± 0.03 log day^{-1} at all sites. Coliphages exhibited an average decay of 0.47 ± 0.03 log day^{-1} at sites 1 and 2 and 0.33 ± 0.07 log day^{-1} at site 3. During the period of higher rainfall events, up to 2.54 mm of rain was reported at sites 1 and 3 and 0.20 mm at site 2. During this period, enterophages exhibited an average decay of 0.40 ± 0.00 and 0.30 ± 0.00 log day^{-1} at sites 1 and 2 and 0.37 ± 0.03 log day^{-1} at site 3. Coliphages exhibited an average decay of 0.19 ± 0.12, 0.08 ± 0.03, and 0.20 ± 0.10 log day^{-1} at sites 1, 2, and 3, respectively. Calculated T_{90} values for enterophages and coliphages differed during the period of low and high rainfall and across the incubation temperatures as well (Table 4). The survival of enterophages and coliphages during both rainfall periods are shown in Figure 3.

Enterophages in chlorinated and dechlorinated tap water and sterile distilled, tap, and wastewater

The enterophage isolate in this study survived approximately 12 days in chlorinated tap water with an initial free chlorine concentration of 0.13 ± 0.02 ppm and more than 12 days in dechlorinated tap water (data not shown). These exhibited decays of 0.23 ± 0.16, 1.05 ± 0.72, and 1.42 ± 0.75 log day^{-1} in chlorinated tap water and 0.14 ± 0.045, 0.70 ± 0.38, and 1.02 ± 0.12 log day^{-1} in dechlorinated tap water at 22, 37, and 41 °C, respectively. Coliphages survived for more than 12 days in both chlorinated and dechlorinated tap water (data not shown) and

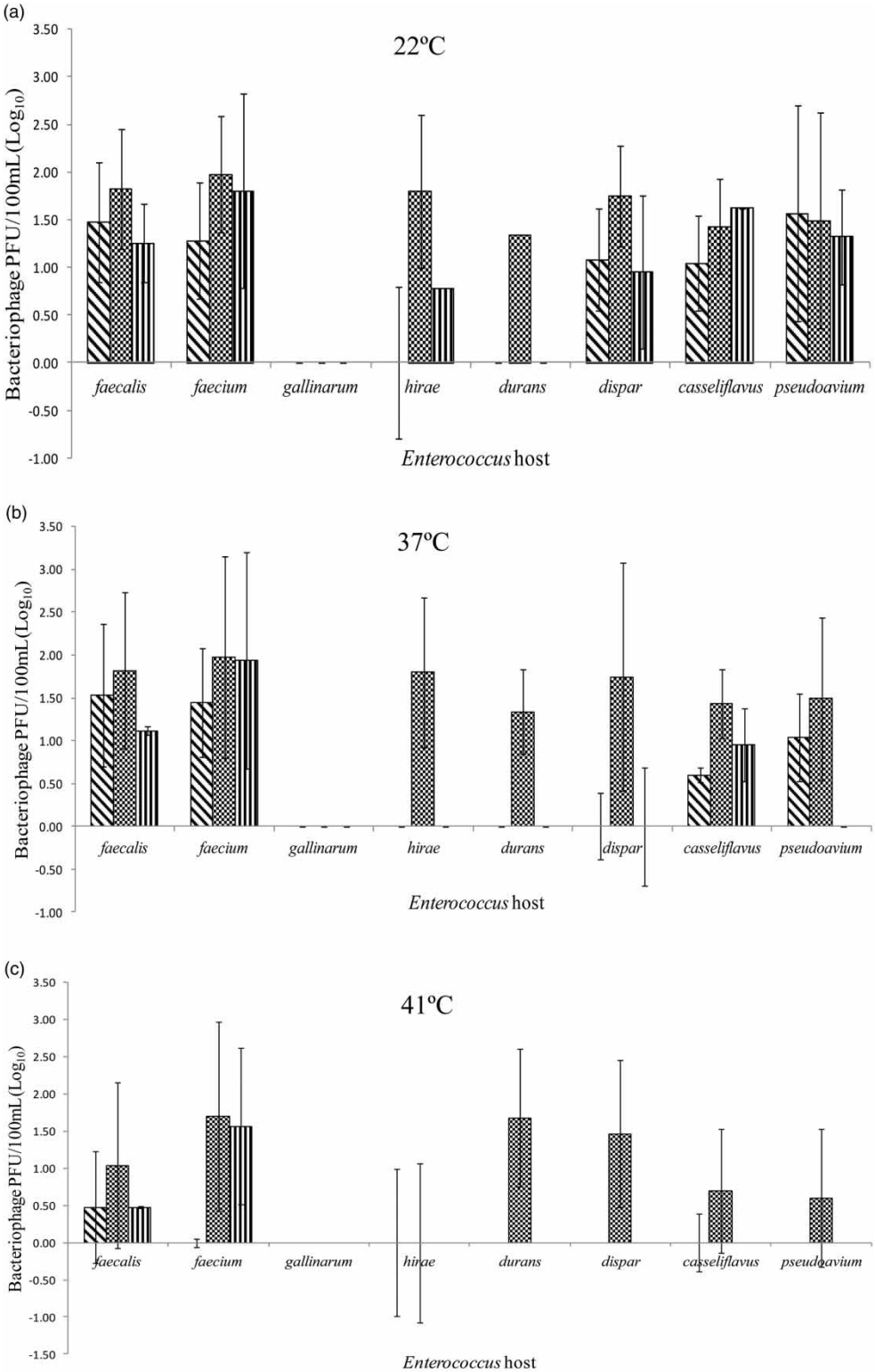

Figure 1 | Prevalence of *Enterococcus* phages in raw domestic sewage in three wastewater treatment plants (WTP) in Puerto Rico. Petri dishes were incubated at 22 (a), 37 (b) and 41 °C (c) and each column represents a WTP. *Enterococcus* spp. included *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae*, *E. durans*, *E. dispar*, *E. casseliflavus*, and *E. pseudoavium*. Results represent the log transformed geometric mean of $n = 12$ samples and standard deviations are represented by error bars.

Table 3 | Host range of *Enterococcus*-infecting phages at different incubation temperatures. Table shows the source of the isolated phages and the *Enterococcus* type strains used. Stocks tested contained only one phage type and were tested against other *Enterococcus* spp., as shown in the following columns

		Host range							
Source	**Host for isolation**	***E. faecalis***	***E. faecium***	***E. gallinarum***	***E. hirae***	***E. durans***	***E. dispar***	***E. casseliflavus***	***E. pdeudoavium***
Sewage	*E. faecalis*	+ [a,b,c]	–	–	–	–	–	–	–
	E. faecium	+ [a,b,c]	+ [a,b]	–	–	–	+ [a,b]	–	+ [a,b]
	E. hirae	–	–	–	+ [a,b,c]	–	+ [c]	–	+ [b,c]
	E. dispar	+ [a,b,c]	–	–	–	–	+ [b,c]	–	+ [a,b]
	E. casseliflavus	–	–	–	+ [b,c]	–	+ [b]	+ [a]	+ [a,b,c]
	E. pseudoavium	+ [b]	–	–	+ [c]	–	+ [b]	–	+ [a,b]
Fresh water	*E. faecalis*	+ [a,b,c]	–	–	–	–	–	–	–
	E. faecium	–	+ [b]	–	–	–	–	–	+ [a,b]
Poultry	*E. faecium*	–	+ [b]	–	–	–	+ [a,b]	–	+ [a,b]
	E. casseliflavus	–	+ [a,b]	+ [a,b,c]	+ [a,b,c]	+ [a,b,c]	+ [a,b,c]	+ [a,b,c]	+ [a,b,c]
	E. pseudoavium	–	+ [a,b,c]	+ [a,b,c]	+ [a,b,c]	+ [a]	+ [a,b,c]	–	+ [a,b,c]

[a]22 °C.
[b]37 °C.
[c]41 °C.

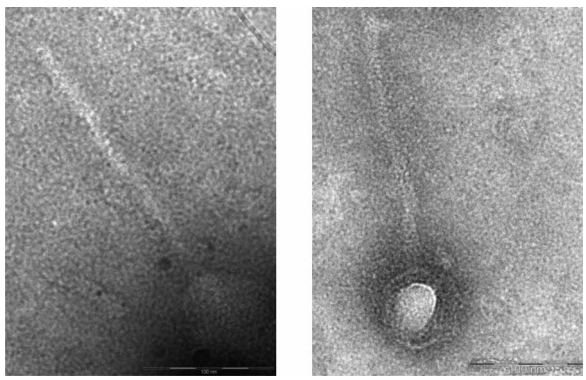

Figure 2 | *Enterococcus faecalis*-infecting phages isolated from domestic sewage. Isolates exhibited long non-contractile tails of approximately 200 nm and icosahedral capsids of 80 nm. Bar = 100 nm.

exhibited a decay of 0.60 ± 0.0062, 0.83 ± 0.26, and 1.10 ± 0.20 log day^{-1} in chlorinated tap water at 22, 37, and 41 °C, respectively. In dechlorinated tap water, coliphages showed a decay of 0.13 ± 0.045, 0.70 ± 0.38, and 1.02 ± 0.12 log day^{-1} at 22, 37, and 41 °C. Calculated T_{90} values for enterophages and coliphages in both chlorinated and dechlorinated tap water are described in Table 5. In terms of the survival experiments with the enterococci phage isolate at 37 °C, *kd* values for wastewater, tap, and distilled water were 0.038, 0.36, and 0.37 log day^{-1}, respectively. A decay of approximately 2 $\log_{10}$ was observed when testing sterile wastewater during 43 days (Figure 4(a)). On the other hand, when testing sterile tap and distilled water, enterophages exhibited a notable decay, surviving approximately 11 days (Figures 4(b) and 4(c)). T_{90} values were approximately 6 days for both tap and distilled water and 60 days for wastewater.

DISCUSSION

Enterococcus-infecting phages in feces and domestic sewage

The source (human) and host-specificity exhibited by enterophages suggest that the *E. faecalis* strain in this study may detect human-specific phages. The relative wide range of *Enterococcus* spp. infected by specific phages showed that these have a promiscuous nature, as shown with the coliphages elsewhere (Goodridge *et al.* 2003). This, in turn, suggests that future studies are needed in order to confirm whether *Enterococcus* phages may recognize receptors which are shared by the enterococci tested in the present study. Several of the *Enterococcus* strains tested also

Table 4 | T_{90} values for enterophages and coliphages in three fresh water samples. Samples were collected from the Rio Grande de Arecibo watershed in Puerto Rico during a period of low and higher rainfall events. Sites 1, 2, and 3 correspond to the unpolluted site, the point-source of fecal pollution, and the estuary, respectively

	Low rainfall			High rainfall		
	22 °C	37 °C	41 °C	22 °C	37 °C	41 °C
Enterophages						
Site 1	4.8 ± 0.8	4.8 ± 0.8	3.7 ± 1.0	4.0 ± 1.0	3.1 ± 0.7	2.9 ± 0.5
Site 2	4.6 ± 0.3	4.6 ± 0.2	5.2 ± 0.4	7.2 ± 2.3	7.2 ± 2.2	6.6 ± 1.7
Site 3	3.9 ± 1.4	3.2 ± 0.6	3.2 ± 0.9	5.3 ± 0.9	5.3 ± 1.0	4.8 ± 1.5
Coliphages						
Site 1	5.2 ± 0.5	4.6 ± 1.4	4.7 ± 0.9	32.2 ± 2.6	10.3 ± 1.3	6.9 ± 0.9
Site 2	5.1 ± 0.002	5.0 ± 1.4	4.6 ± 0.7	45.3 ± 9.2	22.2 ± 13.0	25.0 ± 5.0
Site 3	8.1 ± 4.1	7.0 ± 2.2	5.8 ± 3.3	24.0 ± 15.2	12.2 ± 5.0	6.5 ± 2.0

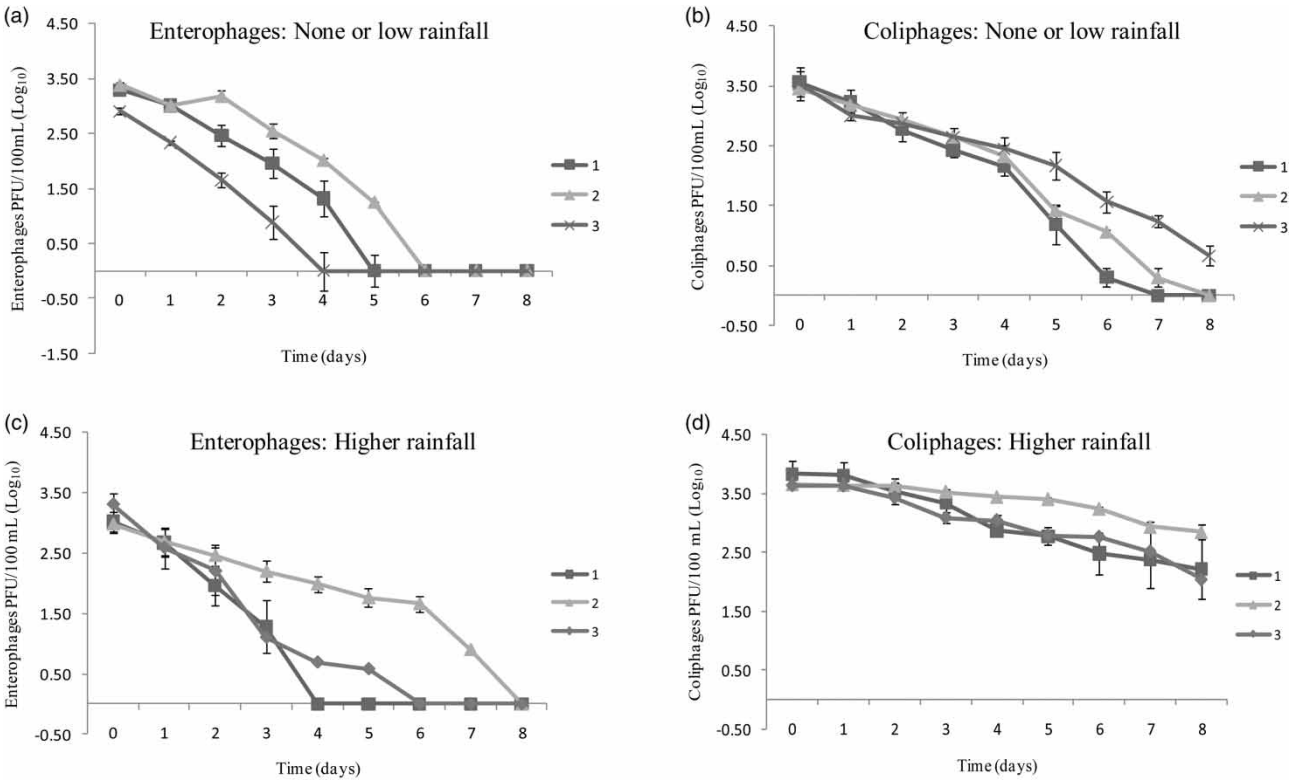

Figure 3 | Survival of enterophages and coliphages across a tropical watershed in Puerto Rico. Sampled sites included the highest site (1), after a waste treatment plant (2), and the estuary (3). Samples were collected during a period of low rainfall events and processed for the detection of enterophages (a) and coliphages (b). The survival of enterophages (c) and coliphages (d) during a period of higher rainfall events was also determined. Data represent those phages that replicated at 37 °C.

exhibited immunity to certain phages, suggesting the presence of prophages, prophage remnants or clustered regularly interspaced short palindromic repeats (CRISPR) (Canchaya *et al.* 2003; Palmer & Gilmore 2010). Host range and replication temperature may be used to group enterococci phages as those of an animal or human origin which, in turn, suggests their potential for MST purposes (Purnell *et al.* 2011). Results also suggest that humans are

Table 5 | T_{90} values (days) for enterophages and coliphages in chlorinated and dechlorinated drinking water

	Enterophages		Coliphages	
Temperature (°C)	Chlorinated	Dechlorinated	Chlorinated	Dechlorinated
22	9.4 ± 7.0	18.1 ± 7.7	18.1 ± 7.7	39.1 ± 4.3
37	8.9 ± 7.0	28.3 ± 6.0	32.1 ± 6.0	43.3 ± 8.3
41	6.9 ± 1.7	28.3 ± 3.7	28.3 ± 3.7	33.0 ± 3.9

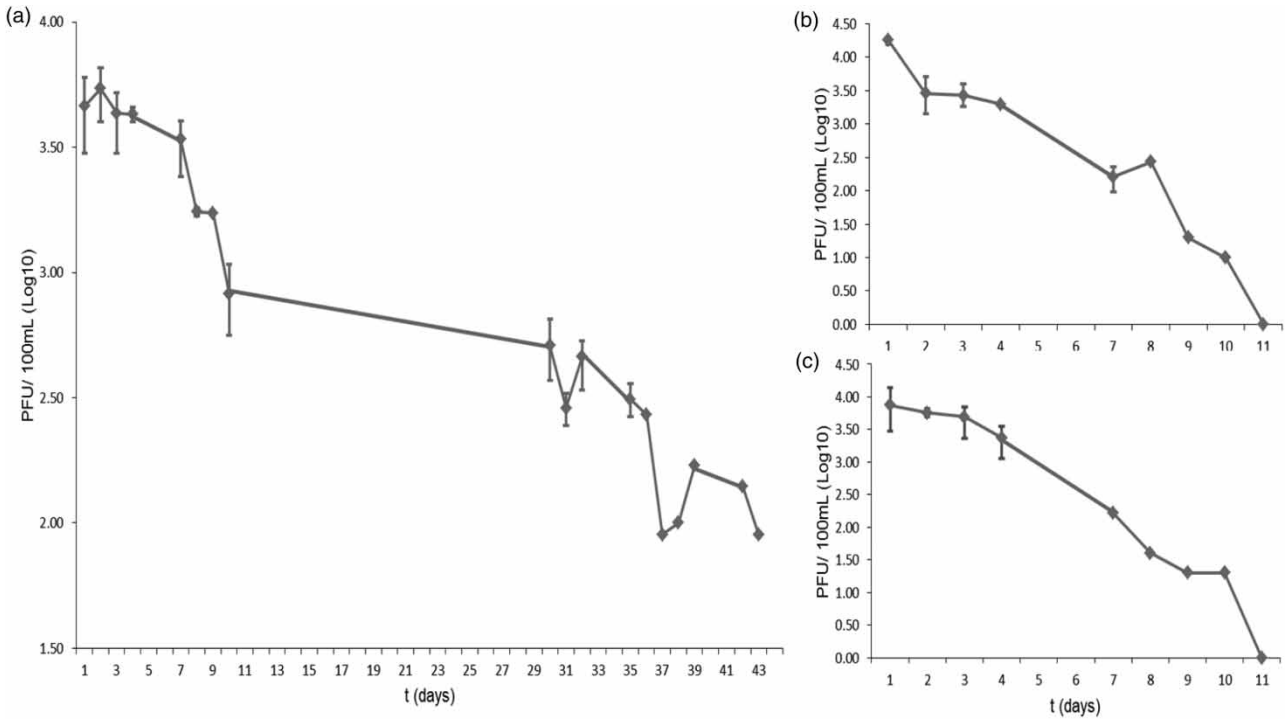

Figure 4 | Survival of enterophages in various sterile water types at 37 °C. Samples include (a) wastewater, (b) tap, and (c) distilled waters and standard deviation is represented by error bars.

reservoirs of various enterococci phage groups, as seen by the influence of the bacterial host used ($X^2 = 27.50$, df = 6, $p = 0.00012$) and temperature tested ($X^2 = 24.27$, df = 2, $p = 5.37^{-6}$. In addition, given that there is the need of characterizing markers of chicken fecal pollution, enterococci phages detected in chicken fecal matter could be further characterized and tested in various water types impacted by this source of fecal contamination. Future studies need to determine potential hosts for the detection of *Enterococcus* phages in other animal feces.

In terms of the coliphages, previous studies have shown that their concentrations vary according to each individual and can range from 10^0 to 10^7 PFU/g in cows, pigs, and humans and <10 PFU/g in dog feces (Dhillon *et al.* 1976; Calci *et al.* 1998). In the present study, coliphage concentrations in cows and humans are within the range reported previously, but higher concentrations were detected in dog feces. Although coliphages were not detected in pig feces in the present study, 0 to <10 PFU/g have been reported elsewhere (Calci *et al.* 1998).

Inactivation rates and survival of *Enterococcus* phages

Our results showed that titers of *E. faecalis, E. faecium, E. casseliflavus,* and *E. coli* phages do not decrease significantly at 4 °C (approximately 0.002–0.05 log day^{-1},

depending on the bacterial host), suggesting that samples may be stored longer at refrigeration temperatures prior to analyses. Similar outcomes have been reported for adenovirus 40 and 41 and poliovirus 1 in sewage at 4 °C, which have exhibited a decrease of 2.5, 2.0, and 2.2 log after 50 days (Enriquez *et al.* 1995). In fresh waters, the similar replication of enterophages at all temperatures tested suggests that a group predominated in the analyses. Unlike enterophages, coliphages exhibited differences in their replication at different temperatures ($X^2 = 11.46$, $df = 2$, $p = 0.0033$), suggesting that various groups were present, as previously suggested (Osawa *et al.* 1981). In terms of the inactivation rates, enterophages did not exhibit differences across the sampled sites during the period of low or high rainfall events. This is comparable with the inactivation rates of poliovirus, coxsackievirus and rotaviruses SA11, which exhibit similar inactivation rates in unpolluted and polluted sites (approximately 0.5–1.0 log day^{-1}) (Hurst & Gerba 1980). Similarly, coliphages did not show differences in their inactivation rates across the sampled sites during the period of low rainfall, but this was not the case for the period of higher rainfall events ($X^2 = 10.11$, $df = 2$, $p = 0.0064$). This is comparable with echovirus 7, which exhibits different inactivation rates in unpolluted (1.0 log day^{-1}) and polluted sites (0.5 log day^{-1}) (Hurst & Gerba 1980). Although there are no hard data that may explain this, several factors may be involved in these differences in survival times during both rainfall periods, including pH changes, dilution of proteolytic enzymes and antiviral chemicals, as described elsewhere (Sidwell *et al.* 1967; Sobsey & Cooper 1973; Ohgaki *et al.* 1986). Another possibility is that due to the relatively long survival times of coliphages in surface waters (20–160 days) and sediments (30 days) at 20 °C, it is reasonable to believe that the opportunity to attach to sediment particles is greater (Duran *et al.* 2001; Long & Sobsey 2004). Once attached, coliphages may be desorbed after rainfall events, and thus their numbers may be higher. In terms of the estuary, polioviruses, coxsackieviruses, rotaviruses SA11, and echoviruses can survive approximately 3 days in estuaries, similar to enterophages during the period of low rainfall events in the present study, but exhibit a more rapid decay compared to both enterophages and coliphages (approximately 0.5–2.5 log day^{-1}) (Hurst & Gerba 1980).

Survival of the tested bacteriophages may also be influenced by chlorine and the environmental microbiota. Few studies have determined the survival of enteric viruses in waters with chlorine levels similar to those found in tap water (Payment *et al.* 1984). Therefore, comparing the survival of enterophages and coliphages with that of enteric viruses in chlorinated tap water is relatively difficult. Certain enteric viruses can survive up to 30 minutes while others can survive 16 h and exhibit decays of approximately 1 log h^{-1} in waters with free chlorine levels of 0.10 ppm at 22 °C (Kelly & Sanderson 1960). This suggests that enteric viruses are more susceptible to chlorine than both enterophages and coliphages, which exhibited T_{90} values of approximately 7–9 and 18–32 days, and decays of approximately 0.20–1.40 and 0.60–1.10 log day^{-1}, respectively. This, in turn, suggests that chlorine may have a more visible effect on the survival of enterophages compared to coliphages. In dechlorinated tap water, T_{90} values for enterophages detected at 37 and 41 °C were similar to that of poliovirus 1 at 23 °C (approximately 30 days); however, neither the enterophages nor the coliphages possess a survival similar to that of adenoviruses 40 and 41 under similar conditions (Enriquez *et al.* 1995). These studies, however, did not consider the effect of the environmental microbiota. Results from using different sterile water types in the present study suggest that the absence of other microorganisms enhances the survival of enterophages; therefore, future studies should consider the environmental microbiota in the survival of enteric viruses and bacteriophages in various water types.

CONCLUSIONS

This is one of the few studies in which the potential of enterophages as markers of human fecal pollution has been further tested. The presence of enterophages in chicken, cattle, dog, and pig fecal materials and their prevalence in domestic sewage were determined by testing various *Enterococcus* spp. as the bacterial hosts. This, however, may represent one limitation to the present study as phages infecting other *Enterococcus* spp. may also be present in fecal material and fecally contaminated waters and thus future studies are needed in order to determine this.

Humans and animals are reservoirs of *Enterococcus* phages and those specifically infecting *E. faecalis* seem promising indices of human fecal pollution. The present study also determined the inactivation rates and survival times of enterophages and compared results with those of human enteric viruses reported elsewhere under similar conditions; however, the present studies must also be performed *in situ*. Enterophages could be considered models of certain human enteric viruses in various water types since their inactivation rates and survival times are similar to those reported previously. Phages infecting *E. faecium*, *E. casseliflavus,* and *E. pseudoavium* replicating at 37 °C may be used to infer the presence of chicken fecal matter in fresh water sources, but more studies need to determine whether these phages are present in waters impacted by chicken fecal matter. The present study opens the possibility to further characterize *Enterococcus* phages as indices of specific fecal sources, especially in various geographical regions, and to predict their behavior in different areas by using specific mathematical models, for example. Also, future studies need to develop the techniques for the detection of enterophages using molecular methods and compare results with culture techniques.

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