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Resistance of foodborne pathogen coliphages to thermal and physicochemical treatments applied in food manufacture

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ABSTRACT

In the present work, six bacteriophages (DT1 to DT6) with lytic activity against one enteropathogenic (EPEC) and two Shiga-toxigenic (STEC) *Escherichia coli* strains were tested for their resistance to physicochemical conditions/treatments applied on food industry, either under conditions found in the food matrix such as different pH, cation concentrations, and water activity (A_w), and/or found during the manufacture process, namely thermal treatments at 63, 72 and 90 °C. Furthermore, phage viability was assessed at refrigeration and abusive temperature, different salt concentrations, and relevant pH values found in meat and dairy products. Phages were completely inactivated at 90 °C, though DT2 and DT6 showed higher thermal resistance since phage particles were detected after 2 min. In addition, Tris-magnesium gelatin buffer seems to be the most protective suspension medium with increasing temperature. Phage viability was slightly or moderately affected at 63 °C and 72 °C, respectively. All the cations evaluated showed no influence on phage viability, and the same was true for the low A_w values assayed, namely 9.5 and 9.0. The six phages tolerated pH treatments well, being more resistant to alkaline conditions (up to pH 11). Results showed that the activity of the phages evaluated was only partially affected – at the lowest temperature (regarding control temperature; 37 °C), with increasing Na^+ concentration, and at the lowest pH value (regarding control pH; 7.5) – and most tested conditions allow phages to multiply in the three pathogenic *Escherichia coli* strains evaluated. These results help to improve both selection of phages and time point, *e.g.* on a HACCP system, where phages may be applied on food during their manufacture in order to maximize phage effectiveness against pathogenic STEC and EPEC strains in the food chain. Therefore, the phages evaluated in this study could be used on several food matrices since they are viable and active in a wide range of environmental food conditions.

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1. Introduction

Shiga-toxigenic *Escherichia coli* (STEC) is the virotype responsible for most cases of hemolytic uremic syndrome (HUS) (Johnson & Taylor, 2008). Also, enteropathogenic *E. coli* (EPEC) strains are responsible for human outbreaks worldwide (Varela et al., 2007; Viljanen et al., 1990). Argentina is the country with the highest incidence of HUS in the world (Rivas et al., 2008), representing 51% of the annual cases worldwide. In addition, health care costs to treat HUS are about US $12.1 million per year (Caletti, Petetta, Jaitt, Casaliba, & Gimenez, 2006).

A large diversity of physicochemical conditions are encountered in different food matrices. For instance, meat possesses a wide range of cation (*e.g.* Na^+) concentration depending on whether it is raw beef or cold cuts. Likewise, dairy products have typical parameters (*e.g.* high concentration of Ca^{2+}), depending on the lactic acid starters and ingredients/additives used in the manufacturing processes. In addition, foodstuffs are subjected to different processes during manufacturing and storage. Due to the high heat sensitivity of STEC and EPEC strains, heat treatments such as *low-temperature long-time* (LTLT) and *high-temperature short-time* (HTST) are commonly applied (D'Aoust et al., 1988). However, the problem may persist if post-pasteurization contamination occurs, since survival of *E. coli* O157:H7 was demonstrated after 28 days in milk at 5 °C (Wang, Zhao, & Doyle, 1997) and after 21 days in whey at 4, 10 or 15 °C (Marek, Nair, Hoagland, & Venkitanarayanan, 2004). Furthermore, considering that pasteurization temperatures were not validated for Shiga toxin (Rasooly & Do, 2010), which requires 5 min at 100 °C for inactivation, the possible persistence of this heat-resistant toxin reinforces the need for a method to control the pathogen during early growth in order to block toxin production.

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Bacteriophages are the natural enemies of bacteria and have proved to be useful tools against pathogenic *E. coli* strains (Abuladze et al., 2008; Moradpour et al., 2009; O'Flynn, Ross, Fitzgerald, & Coffey, 2004) and several other foodborne pathogens, such as *Listeria monocytogenes* (Carlton, Noordman, Biswas, de Meester, & Loessner, 2005; Dykes & Moorhead, 2002; Guenther, Huwyler, Richard, & Loessner, 2009; Holck & Berg, 2009), *Campylobacter jejuni* (Bigwood, Hudson, Billington, Carey-Smith, & Heinemann, 2008), *Salmonella enterica* (Bigwood et al., 2008) and *Staphylococcus aureus* (Bueno, Garcia, Martínez, & Rodríguez, 2012; Garcia, Madera, Martinez, & Rodriguez, 2007; Obeso et al., 2010). However, viability and activity of these phages must be assessed under typical physicochemical conditions found in each food matrix in order to evaluate real effectiveness of phages on these complex environments.

Although many authors have studied phage viability and activity at various physicochemical conditions such as thermal treatments (Lee, Kim, & Park, 2013; Li et al., 2010), varying pH (Dini & De Urraza, 2010; Kerby et al., 1949; Sharp, Hock, Taylor, Beard, & Beard, 1946) and cation (Adams, 1949) concentrations, only one report has exhaustively evaluated lytic coliphages in several physicochemical conditions found in food matrices (Coffey et al., 2011). Furthermore, suspension media such as reconstituted skim milk (RSM) and Buffer Tris-magnesium-gelatin (TMG) have never been evaluated in order to assess their effect as potential phage protectants during thermal treatments. Moreover, even though many phage-host studies were previously performed, those focusing on EPEC and STEC strains have been scarcely documented.

In previous studies, phages of *E. coli* have been demonstrated to be efficient biocontrol agents of EPEC and STEC strains in meat (Tomat, Migliore, Aquili, Quiberoni, & Balagué, 2013) and milk matrices (Tomat, Mercanti, Balague, & Quiberoni, 2013), as well as surface decontaminants (Tomat, Quiberoni, Mercanti, & Balague, 2014). Thus, the aim of the present work was to evaluate the influence of physicochemical parameters on phage viability and to evaluate the interaction of six coliphages with three pathogenic host strains representing STEC and EPEC in order to maximize lytic efficiency on food environments.

2. Materials and methods

2.1. Bacterial strains and phages

E. coli DH5α was used as the sensitive host strain to propagate all the bacteriophages used in this study. Three additional *E. coli* strains were used in the experiments. Two of them, an enteropathogenic (EPEC920) (*eae*) and a Shiga-toxigenic O157:H7 (STEC464) (*stx2* and *eae*) strains, were isolated from stool samples (Tomat, Quiberoni, Casabonne, & Balague, 2014). The third strain was an *E. coli* Shiga-toxigenic non-O157:H7 (STEC) (ARG4827; serogroup O18; *stx1* and *stx2*) (Balagué et al., 2006). All the strains were maintained as frozen (−80 °C) stocks in Hershey broth (8 g l^{-1} Bacto nutrient broth, 5 g l^{-1} Bacto peptone, 5 g l^{-1} NaCl and 1 g l^{-1} glucose) (Difco, Detroit, Michigan, USA) (Cicarelli, San Lorenzo, Santa Fe, Argentina) supplemented with 15% (v/v) glycerol and routinely reactivated overnight at 37 °C in Hershey broth.

Bacteriophages DT1, DT2, DT3, DT4, DT5 and DT6 were isolated from stool samples of patients with diarrhea treated at the Centenary Hospital, Rosario (Tomat, Mercanti, et al., 2013). High-titre phage suspensions were prepared as previously described (Tomat, Mercanti, et al., 2013). Namely, Hershey–Mg broth was inoculated (1%, v/v) with an overnight culture of DH5α, aliquots (100 μl) of phage stocks were added, incubated (37 °C) with shaking until complete lysis. Next, chloroform was added (0.1 ml) and cultures centrifuged at 4000 *g* for 10 min. Phage stocks were stored at 4 °C and enumerated (plaque-forming units per milliliter; PFU ml^{-1}) by the double-layer plaque technique. Briefly, aliquots of 100 μl of phage stocks were mixed with 100 μl of recipient strain culture (OD_{600} = 1.0), then added with 3 ml of Hershey–Mg soft agar (Hershey–Mg with 0.7% agar, w/v) at 45 °C. The mixture was poured into plates with Hershey–Mg agar (1.4%, w/v) and incubated overnight at 37 °C (Jamalludeen et al., 2007).

2.2. Viability studies

2.2.1. Influence of thermal treatments

Phage suspensions, containing between 10^7 and 10^8 PFU ml^{-1}, were prepared in three different suspension media and then subjected to thermal treatments. The media assayed were Hershey broth, reconstituted (10% w/v) commercial skim milk (RSM) and Tris-magnesium gelatin (TMG) buffer (10 mM Tris–Cl, 10 mM $MgSO_4$ and 0.1% (w/v) gelatin). Temperatures of 63 °C (30 and 60 min) and 72 °C (5, 10 and 20 min), corresponding to traditional *low-temperature long-time* (63 °C–30 min) —LTLT- and *high-temperature short-time* (72 °C–20 s) —HTST-pasteurization, respectively, and stronger heating applied during manufacture of fermented milks (90 °C; 2 min), were tested. Phage suspensions were immediately cooled after each incubation time assayed and enumerated by the double-layer plate titration method (Jamalludeen et al., 2007). Assays were carried out in triplicate.

2.2.2. Influence of cations

The influence of Na^+, Mg^{2+} and Ca^{2+} on phage (10^7–10^8 PFU ml^{-1}) viability was investigated by incubation at 25 °C in TG buffer (10 mmol l^{-1} Tris–Cl and 0.1% p/v gelatin) with and without NaCl (1, 2, 4 and 6%), $MgSO_4$ (1, 5 and 10 mmol l^{-1}) or $CaCl_2$ (1, 10 and 20 mmol l^{-1}). After 2 and 24 h, phage viability was determined as described above (Jamalludeen et al., 2007). Assays were carried out in triplicate.

2.2.3. Influence of pH

Phages were diluted to a final concentration of 10^7–10^8 PFU ml^{-1} in Hershey broth with the pH adjusted to values ranging from 2 to 12. Hershey broth without adjustment (pH 6.4) was used as control. After 45 min and 3 h of incubation at 25 °C, phages were enumerated by the double-layer plate titration method as previously described (Jamalludeen et al., 2007). Assays were carried out in triplicate.

2.2.4. Influence of water activity (A_w)

Phages (10^7–10^8 PFU ml^{-1}) were suspended on TMG buffer adjusted with glycerol at different A_w, namely 1.0 (control), 0.95 and 0.90. After 8 and 24 h of incubation at 25 °C, phages were enumerated by the

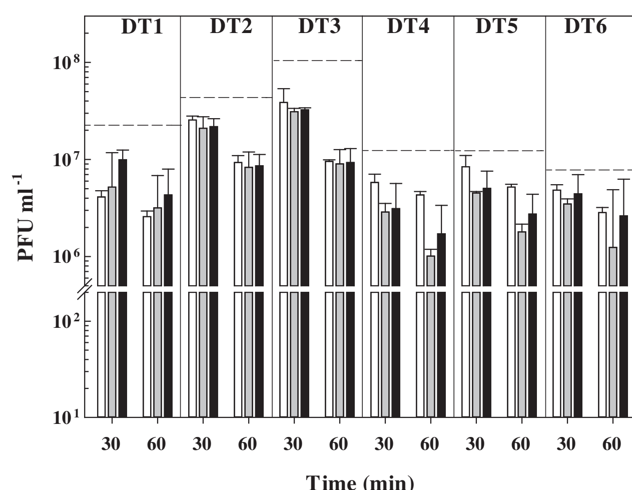

Fig. 1. Phage viability at 63 °C in reconstituted (10% w/v) commercial skim milk (RSM; □), Hershey broth (▒) and Tris-magnesium gelatin (TMG) buffer (■). Horizontal lines represent the titer for each phage at the beginning of the experiment. Error bars represent the standard deviation of three determinations ($p < 0.05$).

double-layer plate titration method as previously described (Jamalludeen et al., 2007). Assays were carried out in triplicate.

2.3. Activity studies

Activity studies were performed by mixing 100 μl of each phage, ca. 10^3 PFU, with 100 μl of the corresponding host bacteria (Optical density at $\lambda = 630$ nm – $OD_{630\ nm} - = 1$), at a multiplicity of infection (m.o.i) of ~0.001. The suspension was incubated at 37 °C and the phage titer following one lytic cycle (40 min) of each phage tested was determined by the double-layer plaque technique (Jamalludeen et al., 2007). Activity studies were carried out on phages representing narrow and broad host ranges. Phages DT1, DT2 and DT5 possess a narrow host range, while DT3, DT4 and DT6 possess a broader host range (Tomat, Migliore, et al., 2013). In addition, phage DT5 was used for studies in non-O157 STEC since this strain is resistant to phage DT1.

2.3.1. Influence of temperature

Activity of phages on *E. coli* cells was determined in Hershey broth at 4, 37 (control) and 50 °C. After 40 min of incubation, phage particles were enumerated as described above. Results were expressed as percentages of phages regarding *E. coli* optimal growth temperature (37 °C). Assays were carried out in triplicate.

2.3.2. Influence of Na^+

The influence of Na^+ on phage activity was investigated by incubation (37 °C) of infected *E. coli* cultures in Hershey broth, with and without added NaCl, namely 0.3 (control), 2 and 4%. After 40 min, phage particles

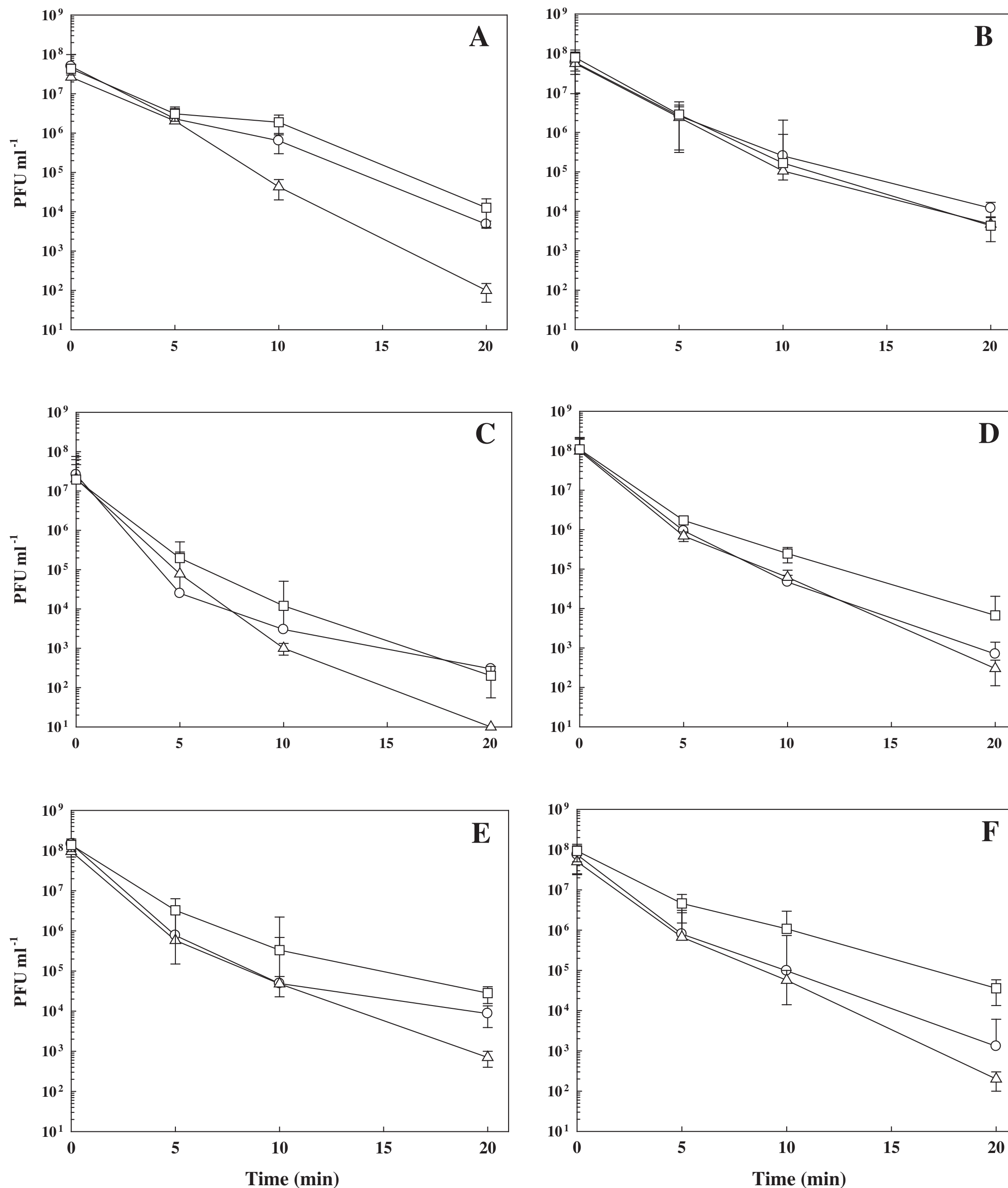

Fig. 2. Viability of phage DT1 (A), DT2 (B), DT3 (C), DT4 (D), DT5 (E) and DT6 (F) at 72 °C in reconstituted (10% p/v) commercial skim milk (RSM; ○), Hershey broth (△) and TMG buffer (□). Error bars represent the standard deviation of three determinations ($p < 0.05$).

Table 1
Lytic activity of phages DT1, DT5 and DT6 on *E. coli* cells subjected to different treatments.

Strain/phage system		Phage particles (%)[a,b]					
		Temperature		Na^+		pH	
		4 °C	50 °C	2%	4%	5.7	4.5
DH5α	DT1	20.2 ± 4.4	95.7 ± 2.7	86.3 ± 2.7	50.6 ± 3.4	22.3 ± 4.6	7.4 ± 5.8
	DT6	15.1 ± 4.5	96.5 ± 1.5	87.3 ± 4.6	42.4 ± 3.1	32.9 ± 2.8	6.3 ± 3.0
EPEC920	DT1	14.8 ± 5.9	87.3 ± 4.3	93.0 ± 4.5	58.8 ± 2.0	52.1 ± 5.3	5.2 ± 4.0
	DT6	15.2 ± 3.1	93.1 ± 2.3	78.5 ± 5.9	60.8 ± 1.5	65.8 ± 1.9	8.8 ± 1.2
Non-157 STEC	DT5	11.8 ± 2.2	70.8 ± 1.3	86.3 ± 1.4	56.5 ± 2.8	95.0 ± 4.1	7.1 ± 2.8
	DT6	5.0 ± 41	88.0 ± 2.2	96.6 ± 2.9	55.2 ± 3.2	42.6 ± 5.4	3.0 ± 1.4
O157:H7 STEC	DT1	16.2 ± 2.7	82.9 ± 2.0	84.7 ± 4.1	61.6 ± 1.3	29.5 ± 2.3	5.9 ± 4.2
	DT6	17.3 ± 4.1	78.9 ± 1.6	86.5 ± 3.2	72.0 ± 2.5	61.2 ± 1.2	7.1 ± 3.3

[a] Mean value of three determinations ± standard deviation.
[b] Percentage values regarding 100% of control conditions after 40 min of incubation.

were enumerated and the counts were compared with the standard conditions of the culture medium. Assays were carried out in triplicate.

2.3.3. Influence of pH

Lytic activity of phages on *E. coli* cells was assayed in Hershey broth at pH values of 4.5, 5.7 and 7.5 (control). Results were expressed as percentages of phage particles after 40 min of phage-cell interaction with respect to controls. Assays were carried out in triplicate.

2.4. Statistical analysis

Means (three determinations) were compared using the one-way ANOVA procedure followed by Duncan's multiple range tests at $p < 0.05$.

3. Results and discussion

3.1. Influence of thermal treatments on viability and activity of phages

In order to determine the point of a Hazard Analysis and Critical Control Point (HACCP) system, *e.g.* in a dairy industry, at which phages could be used as biocontrol tools, the viability of phages at relevant pasteurization temperatures (*i.e.* LTLT and HTST) must be evaluated. When the six coliphages were tested for thermal resistance, they showed, at most, 1-log_{10} reduction after 60 min at 63 °C, indicating, although the significant reduction observed, a high resistance for all phages studied. Regarding the influence of the suspension media on phage viability, Hershey broth was the least protective medium at the three temperatures evaluated. Phage DT1 was protected by TMG, while RSM proved to protect DT4, DT5 and DT6 though this protection was variable (Fig. 1). At 72 °C, the six phages evaluated were more affected than at 63 °C, since an average of 4-log_{10} reductions were observed after a 20-min incubation. Again, DT1 was significantly more protected by TMG at this temperature, and unlike the results obtained at 63 °C, phages DT4, DT5 and DT6 were also significantly more protected by TMG, whereas DT2 showed the highest count in the RSM medium (Fig. 2). Although viability of *E. coli* phages had never been previously tested at these two particular temperatures on these three suspension media, Coffey et al. (2011) found that the number of two coliphages was significantly reduced in LB broth after exposure to 70 or 80 °C. In addition, Lee et al. (2013) evaluated viability in LB broth, and, although coliphage ECP4 showed heat resistance at temperatures below 65 °C, ECP4 was reduced by 6-log_{10} PFU ml^{-1} after 10 min, and was not detected after 20 min at 70 °C. Similar results were obtained by Li and coworkers at 63 and 72 °C (Li et al., 2010). Moreover, several authors have found high resistance at 63 °C and moderate resistance at 72 °C for lactic acid bacteria (LAB) phages (Mercanti, Guglielmotti, Patrignani, Reinheimer, & Quiberoni, 2012; Pujato et al., 2014). Next, when 90 °C was used, most phage populations were below the detection limit (<10 PFU ml^{-1}) after a 2-min incubation. However, phage particles of DT2 (namely 3.1×10^2 PFU ml^{-1} on RSM and 1.35×10^3 PFU ml^{-1} on TMG; resulting in 4.7- and 4.1-log_{10} reductions, respectively) and DT6 (1.25×10^3 PFU ml^{-1} on TMG; resulting in a 4.2-log_{10} reduction) were detected after the treatment, suggesting a high resistance at this extreme temperature. Likewise, heating at 90 °C was unable to complete coliphage EEP inactivation (Li et al., 2010). These results proved high thermal resistance of phages evaluated, remaining useful as biocontrol tools after physical treatments applied in food industry.

Although, from the aforementioned experiments, no clear influence of suspension media could be distinguished at each temperature assayed for all phages evaluated, TMG media showed a significant higher protective effect with increasing temperature. It might be hypothesized that gelatin forms a network providing protection to phages against the denaturing temperatures. On the other hand, RSM protection, the second protective medium, has been ascribed to the buffer capacity of milk, as well as to its higher protein, lipid and salt contents (Atamer et al., 2009; De Fabrizio, Ledford, & Parada, 1999).

Phage activity – with different host range (Tomat, Migliore, et al., 2013) – at refrigeration (4 °C), optimal growth for *E. coli* (37 °C), and abusive temperature (50 °C) – at which viability of phages remained unchanged (data not shown) – was evaluated. The effect of temperature on phage activity is shown in Table 1. The three phages evaluated

Table 2
Phage viability in TG buffer at 25 °C supplemented with mono- and divalent cations.

Phage (PFU ml^{-1})	Phage particles ($\times 10^7$)[a,b]												
	Na^+ (%)					Mg^{2+} (mM)				Ca^{2+} (mM)			
	C	1	2	4	6	C	1	5	10	C	1	10	20
DT1	1.81	1.51	1.95	1.74	1.68	1.75	2.12	1.96	1.72	1.49	1.38	1.64	1.90
DT2	3.68	3.70	3.88	3.07	3.20	1.51	1.39	2.01	2.33	2.53	2.61	2.86	3.26
DT3	6.43	7.84	6.07	6.96	5.76	3.60	3.66	3.89	3.62	6.32	5.68	6.97	6.04
DT4	4.59	4.48	4.56	5.35	5.16	2.06	2.58	2.18	2.98	5.36	5.08	5.24	5.68
DT5	4.72	5.12	4.88	4.80	4.94	3.19	3.31	3.39	3.60	6.20	6.44	6.76	5.69
DT6	3.36	3.23	3.41	3.32	3.12	2.63	2.16	2.39	2.22	3.17	3.31	3.02	2.81

C: Control (TG buffer) at 24 h of incubation. Similar behavior was found after 2 h of incubation.
[a] Mean value of three determinations after 24 h of incubation.
[b] Initial inoculum range ($\times 10^7$): DT1 = 1.4–1.8; DT2 = 1.6–3.0; DT3 = 2.8–8.3; DT4 = 2.1–5.0; DT5 = 3.1–6.7; DT6 = 2.0–3.0.

remained approximately 99% viable after exposure to low (4 °C) and high (50 °C) temperatures, which is consistent with the findings of Coffey et al. (2011). As expected, phage activity was maximum at 37 °C whereas it was affected at 4 °C while it was moderate at 50 °C for all phage/strain systems evaluated. Likewise, similar results were found for e11/2 and e4/1c, since these phages can withstand a wide range of conditions (*e.g.* refrigeration –4 °C– and abusive –50 °C– temperatures) and remain biologically active (Coffey et al., 2011) either at refrigeration temperatures or when the cold chain is lost. On the contrary, other authors found that all phage activity was destroyed after 10 min at 60 °C (Li et al., 2010). The slightly reduced activity observed in this work at 50 °C may be due to disorganization and/or partial denaturation of phage receptors on the bacterial cell surface, thus hindering phage multiplication. Although, Kudva, Jelacic, Tarr, Youderian, and Hovde (1999) proposed that low temperatures and the absence of bacterial growth may favor the first step on the lytic cycle, lower enzymatic activity and, thus, lower phage activity was observed at refrigeration temperature for all phage/strain systems evaluated.

3.2. Influence of cations on viability and activity of phages

Several cations, at a wide range of concentrations, are natural or added components of dairy and meat products. Thus, the viability of phages, as potential biocontrol agents for pathogenic *E. coli* in food applications, was assessed under several concentrations of different ions (Na^{+}, Mg^{2+} and Ca^{2+}). In addition, and in order to determine the effect

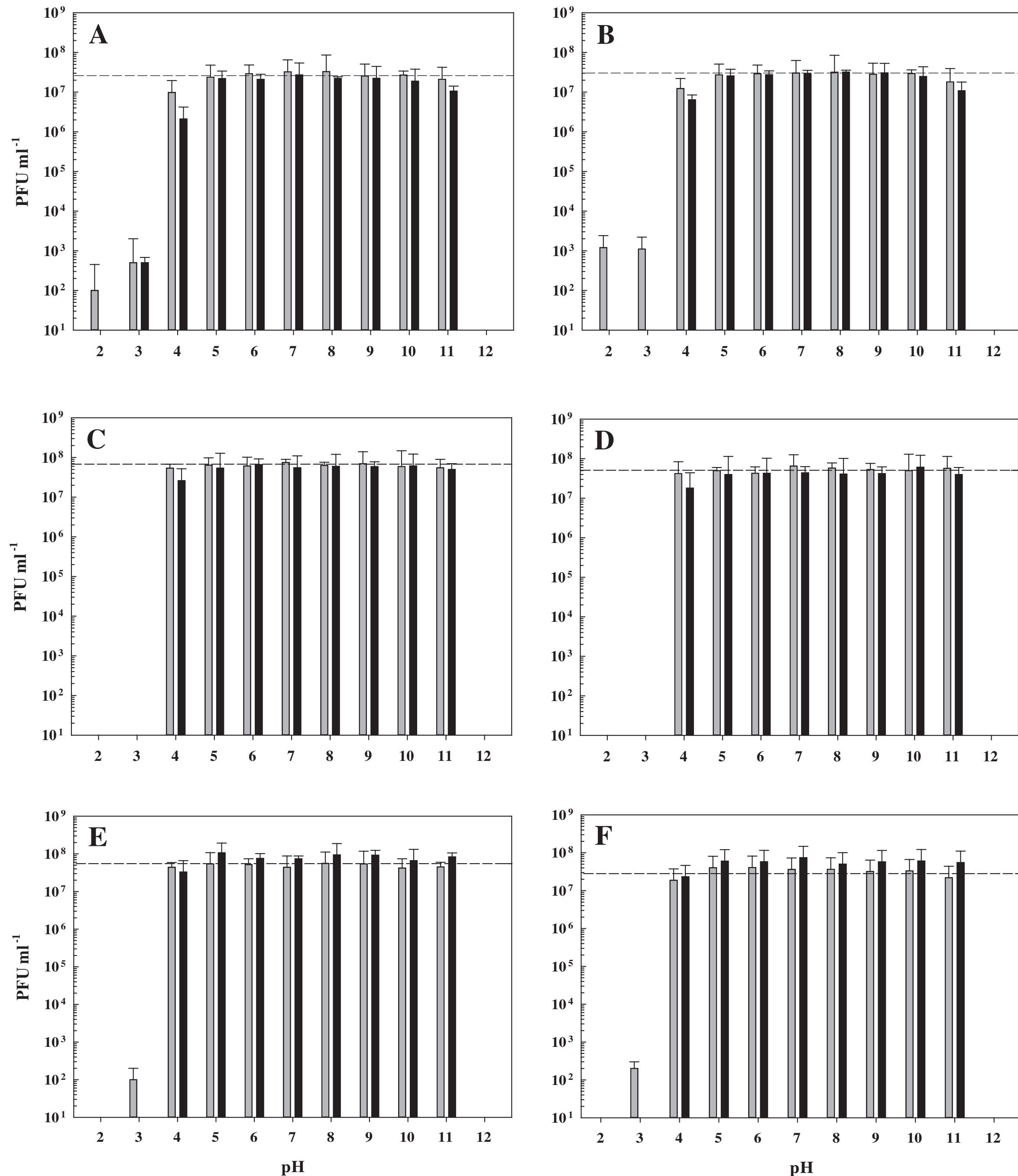

Fig. 3. Viability of phage DT1 (A), DT2 (B), DT3 (C), DT4 (D), DT5 (E) and DT6 (F) after 45 min (▒) and 3 h (■) in Hershey broth with pH adjusted to values ranging from 2 to 12. Horizontal lines represent the titer for each phage on Hershey broth without pH adjustment (control). Error bars represent the standard deviation of three determinations ($p < 0.05$).

of monovalent ions present in the food matrix, phage activity was determined following exposure to varying sodium concentration.

Monovalent ions such as Na^+ may cause a rapid change in osmotic pressure (Lark & Adams, 1953) and increase the aggregation of phages (Langlet, Gaboriaud, Duval, & Gantzer, 2008), reducing their viability. In addition, chelating agents are able to inhibit phage activity by binding divalent cations (Hicks & Surjawan, 2002; Suarez et al., 2008), interfering with phage adsorption (the first step on the lytic cycle) (Capra, Quiberoni, Ackermann, Moineau, & Reinheimer, 2006) or inactivating phage particles (Bassel, Shaw, & Leon Campbell, 1971; Issinger & Falk, 1976). However, results of the present work showed that phage viability in the presence of monovalent ions (Na^+) as well as divalent cations (Mg^{2+} and Ca^{2+}) was unaffected for all the phages evaluated (Table 2). In accordance with our findings, Coffey et al. (2011) found that NaCl (from 1.0 to 2.5%) did not significantly affect the number of phages evaluated as other authors found by monovalent (Mylon et al., 2010) and divalent (Adams, 1949) cations.

The three phages evaluated showed similar values of lytic activity at 2% and 4% of NaCl in all the phage/strain systems studied. Table 1 shows the percentage of viable phage particles after each treatment against the control condition (0.3%). At NaCl 2%, phages DT1 (DT5 for non-O157 STEC ARG4827, strain resistant to DT1) and DT6 showed an average phage count of 85.9% and 87.2%, respectively, after one lytic cycle. When NaCl concentration was 4%, the phage activity was reduced only in 57.6% at most. Results indicate that with increasing NaCl concentration, phage activity decreases, in accordance with our previous work where increasing NaCl concentration resulted in lower adsorption values in the phage/strain systems evaluated in this study (Tomat, Quiberoni, et al., 2014). Accordingly, Coffey et al. (2011) found that lytic capacity of phages was affected at all NaCl concentrations assayed. However, the three phages evaluated showed a relatively high activity at the highest salt concentration used, thus phages may be useful on food with these characteristics.

3.3. Influence of pH on viability and activity of phages

After cattle slaughtering, the meat pH reaches values as low as 5.5–6.0. In addition, during milk fermentation process the pH falls to 4.0–4.5. Therefore, it is important to evaluate the viability and activity of phages in order to determine if they can withstand these adverse conditions found in food environments. In addition, the range of pH from 2 to 12 was assessed in order to determine phage viability.

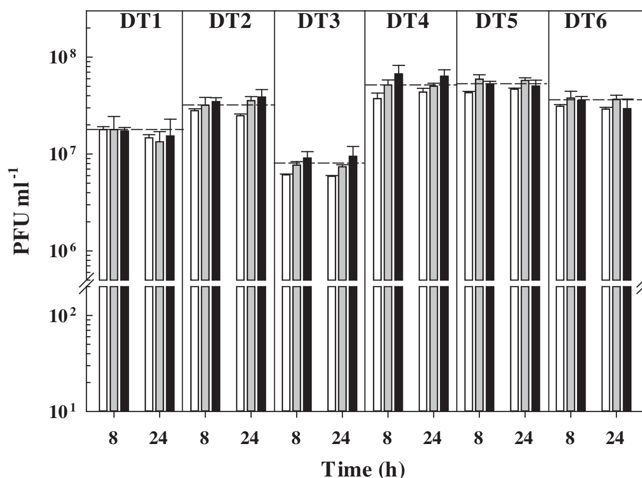

Fig. 4. Phage viability at water activity (A_w) of 1.0 (control; □), 0.95 (▒) and 0.90 (■). Horizontal lines represent the titer for each phage at the beginning of the experiment. Error bars represent the standard deviation of three determinations ($p < 0.05$).

Our results indicated that the six phages evaluated showed similar resistance within the range of pH values analyzed, and resulted highly stable within a wide range of pH, namely 4 to 11 (Fig. 3). The viability of the phages was significantly affected at low, namely 2 and 3, and at the highest pH values assayed. Phages DT1 and DT2 were still detected at pH 2 and 3, though a 5-$\log_{10}$ reduction was observed. Moreover, phages DT5 and DT6 showed high resistance and reductions similar to those of DT1 and DT2, but only at pH 3, since at pH 2 phage particles were completely inactivated. Accordingly, Dini and De Urraza (2010) found that no viable phage particles were detected below pH 3. On the other hand, the six phages evaluated exhibited high resistance to basic pH; however, no viable particles were found at pH 12. Several authors evaluated phage viability at pH range from 2 to 12 and found similar results in coliphages (Coffey et al., 2011; Li et al., 2010), LAB phages (Mercanti et al., 2012) and mycophages (Endersen et al., 2013). Overall, the six phages evaluated seemed to be more resistant in an alkaline than in an acidic environment, in agreement with Feng, Ong, Hu, Tan, and Ng (2003) who found similar results for one (Qβ) of the two phages evaluated.

Table 1 shows that lytic activity of the phages assayed was significantly lower at pH 5.7 as well as at 4.5 with respect to control conditions (pH 7.5). At pH 5.7, the highest lytic activity observed, namely 95.0%, was for the DT5/non-O157 STEC ARG4827, while the activity for the other systems oscillated from 22.3 to 65.8%. Moreover, at pH 4.5 the activity obtained for the phages tested was always lower than 10%. In agreement with the results obtained in this study, the activity of several phages was limited at pH lower than 4.5 (Ly-Chatain, 2014). This may be because irreversible virion coagulation (at pH 2) and precipitation (at pH 3 and 4) might be the factors limiting phage activity (Jonczyk, Kłak, & Górski, 2011). Jamalludeen et al. (2007) found high phage resistance up to pH 5, while both viability and lytic activity were affected at a lower pH value. In addition, other coliphages showed similar behavior at low and high pH values (Jepson & March, 2004; Kerby et al., 1949; Sharp et al., 1946), whereas phages such as PM2 proved sensitive to moderate pH, losing all activity at pH 5.0, and M13 that survived even at pH 2 (Jonczyk et al., 2011). Although most virions were inactivated at the extreme pH values assayed, namely 2, 3 and 12, phages evaluated endure the pH values found in food matrices such as meat and dairy.

3.4. Influence of A_w on phage viability

Although many foodborne pathogens require high water activity to grow or survive, others may remain viable and even grow in foods with low water activity. In order to determine whether phages could be useful as a biocontrol tool on these particular food environments, the viability of the six phages at different A_w was assessed.

After 8 and 24 h of incubation at A_w of 0.95 and 0.90, the six phages evaluated showed no significant reduction on their numbers indicating that all of them were stable at these A_w values (Fig. 4). These results are in accordance with a study carried out by Coffey et al. (2011), wherein one of the two coliphages evaluated was highly resistant to A_w as low as 0.87, while the other was resistant at A_w higher than 0.91 following 1 h exposure. However, phages evaluated in the present study seem to be more resistant, since they resisted A_w of 0.90 up to 24 h of incubation. As for other phages, no viability data at low A_w was found.

4. Conclusion

Although phages represent a useful tool against foodborne diseases, limited information is available concerning phage viability and activity in the physicochemical conditions found in food environments. In order to significantly improve phage treatments regarding biological control of foodborne pathogens, such as STEC and EPEC strains, applied on food environments, viability and lytic efficiency of phages in the food matrix and the timing of application of the phage treatment must be optimized.

The present study demonstrates that phages can withstand a wide range of conditions found in food matrices, or during their manufacturing process, and remain biologically active under a wide range of food environmental conditions. In addition, phages remained viable even after incubation at pasteurization temperatures, indicating that these thermal treatments are not an obstacle for their application on the food chain production, namely, prior to these sanitizing processes, yielding greater food protection. However, further experiments should be conducted in various foods in order to obtain more information since the food matrix may have a great impact in the stabilities and the activities of phages.

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