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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.

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

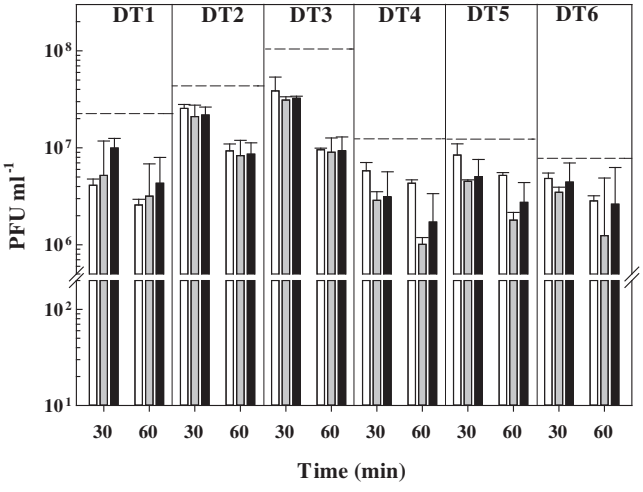

Fig. 1. Phage viability at 63 °C in reconstituted (10% w/v) commercial skim milk (RSM; □), Hershey broth (▒) and Tris-magnesium gelatin (TMG) buffer (■). Horizontal lines represent the titer for each phage at the beginning of the experiment. Error bars represent the standard deviation of three determinations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