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Evaluation of a novel cocktail of six lytic bacteriophages against Shiga toxin-producing *Escherichia coli* in broth, milk and meat

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ABSTRACT

Phages are potentially useful as antimicrobial agents in food, especially cocktails of different phages which may prevent the development of bacterial resistance. Biocontrol assays with a six-phage cocktail, which is lytic against DH5α, an enteropathogenic (EPEC) and two Shiga-toxigenic (STEC) *Escherichia coli* strains, were performed in Hershey-Mg broth, milk and meat at refrigerated (4 °C), room (24 °C) and abusive (37 °C) temperatures. At 4 °C, cell counts were significantly lower (2.2–2.8 $\log_{10}$ CFU/mL) when *E. coli* strains (~10^9 CFU/mL) were challenged against the phage cocktail (~10^9 PFU/mL) in Hershey-Mg broth after 24 h. However, reductions were higher (3.2–3.4 $\log_{10}$ CFU/mL) after a 48 h exposure for all the strains tested. In addition, reduction values reached up to 3.4 $\log_{10}$ CFU/mL (24 °C) and 3.6 $\log_{10}$ CFU/mL (37 °C) in challenge tests after 24 h, though the reductions achieved were slightly lower after 48 h for the four *E. coli* strains tested. In milk, the cocktail was highly effective since bacterial counts were below the detection limit (< 10^1 CFU/mL) at 4 °C, while the reductions ranged from 2 to 4 $\log_{10}$ CFU/mL at 24 °C after a 24 h exposure. At 37 °C, DH5α was eliminated within 2 h, and an average cell decrease of 4 $\log_{10}$ CFU/mL was observed for the three pathogenic strains tested. When the assays were performed in meat, biocontrol values ranged from 0.5 to 1.0 $\log_{10}$ CFU/mL after 48 h at 4 °C, while a higher cell inactivation was achieved at 24 °C (2.6–4.0 $\log_{10}$ CFU/mL) and 37 °C (3.0–3.8 $\log_{10}$ CFU/mL). Furthermore, higher inactivation values for O157:H7 STEC (1.55 ± 0.35 $\log_{10}$ CFU/mL) at 4 °C were obtained in meat when incubation was extended up to 6 days. As a conclusion, our six-phage cocktail was highly effective at 24 °C and 37 °C, though less effective at 4 °C in both food matrices evaluated. Thus, it might be applied against pathogenic EPEC and STEC strains to prevent foodborne diseases especially when the cold chain is lost.

1. Introduction

Foodborne pathogens such as enteropathogenic *Escherichia coli* (EPEC) and Shiga-toxigenic *Escherichia coli* (STEC) strains are the main causes of diarrhea and hemolytic uremic syndrome (HUS) in our country (Rivas et al., 2008). STEC infections are transmitted to humans through contaminated foods such as meat (Rivas et al., 2003), milk (Farrokh et al., 2013) and water (Swerdlow et al., 1992), while infection by EPEC is related to fecal contamination due to unhygienic handling of food (Hernandes et al., 2009).

The use of bacteriophages as biocontrol agents seems to be a promising alternative against several foodborne pathogens (O'Flynn et al., 2004; O'Flaherty et al., 2005; Bigwood et al., 2008; Mukhopadhyay and Ramaswamy, 2012). Phages are highly active and specific and have been extensively and safely used in clinical applications in Europe (Garcia et al., 2008). In addition, phages have a highly versatile use along the food chain since they have been employed for therapy, biosanitation and biopreservation (Modi et al., 2001; Gill et al., 2006; Raya et al., 2006; Kim et al., 2007).

Bacterial resistance may be a potential problem when using phages for the control of unwanted bacteria in food (Madera et al., 2004; O'Flynn et al., 2004; Endersen et al., 2013). Bacteriophage insensitive mutants (BIMs) mainly arise from the loss or mutation of phage receptors on targeted bacteria (Tanji et al., 2004). Further studies found that BIMs emerged when *E. coli* cells were challenged against coliphages (O'Flynn et al., 2004) as well as for other pathogens (Garcia et al., 2007) and bacteria, such as lactic acid ones (Chirico et al., 2014). On the other hand, the limitation of phages as pathogen control agents, *i.e.* limited host range and limited diffusion in solid food, may be bypassed by using a phage cocktail composed by many different phages

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and by adding large amounts of phage (O'Flynn et al., 2004), respectively.

The use of phage cocktails to control foodborne pathogens has been explored in milk (Garcia et al., 2007; Zuber et al., 2008), meat (O'Flynn et al., 2004), fruits and vegetables (Leverentz et al., 2003; Viazis et al., 2011). Preparations composed by several phages which use different receptors in the targeted bacteria may have the advantage of infecting a mutant resistant to another phage present in the cocktail. Thus, the use of several different phages which are lytic for the same pathogen will reduce the probability of selecting mutants (Sulakvelidze and Barrow, 2005), enhancing the effectiveness of biological control against foodborne pathogens.

In addition to resistance to phages, bacteria may show resistance to several antibiotics (Yilmaz and Özcengiz, 2017). Furthermore, the use of antibiotics as additives in animal feeds, also known as growth-promoting antibiotics (GPAs), could spread resistance among bacteria (Jia et al., 2017). Unlike phages, antibiotics may select many resistant bacterial species because of their broad spectrum of activity. Also, bacterial resistance mechanisms against phages and antibiotics differ (Sulakvelidze and Barrow, 2005). Therefore, phages could be used as hurdle technology, contributing to reduce the incidence of bacterial resistance to several antibiotics currently employed.

These cocktails have proved effective against several pathogens as *Staphylococcus aureus* (Garcia et al., 2007) and *Enterobacter sakazakii* (Zuber et al., 2008) in dairy products. Yet, their efficacy against *E. coli* in milk (McLean et al., 2013) as in meat products at refrigerated (Abuladze et al., 2008), room and abusive (O'Flynn et al., 2004) temperature have been scarcely explored. In our study, phages are proposed as promising tools to be used in combination and/or alternation with other current technologies. Thus, the aim of the present work was to evaluate the potential utility of six coliphages mixed in a cocktail to control *E. coli* strains by testing their efficacy under different conditions in milk and meat products.

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 strains were used in the biocontrol experiments. Two of them, an enteropathogenic *E. coli* (*eae*+) (EPEC) and a Shiga toxigenic *E. coli* O157:H7 (*stx2* + and *eae*+) (O157 STEC), were previously isolated from stool samples, identified using API-20E system (Biomerieux, Buenos Aires, Argentina), and further characterized by PCR. The third strain was *E. coli* Shiga toxigenic non-O157:H7 (ARG4827; serogroup O18; *stx1* + and *stx2*+) (non-O157 STEC) (Balague et al., 2006). All the strains were maintained as frozen (−70 °C) stock cultures in Hershey broth (8 g/L Bacto nutrient broth, 5 g/L Bacto peptone, 5 g/L NaCl and 1 g/L glucose) (Difco, Detroit, Michigan, USA) supplemented with 15% (v/v) glycerol and routinely reactivated overnight at 37 °C in Hershey broth supplemented with $MgSO_4$ (5 mmol/L) (Cicarelli, San Lorenzo, Santa Fe, Argentina) (Hershey-Mg).

Myoviridae bacteriophages (T-even type) (Tomat et al., 2013a), DT1 to DT6, were previously isolated from stool samples of patients with diarrhea treated at the Hospital Centenario, Rosario (Tomat et al., 2013b) and characterized by electron microscopy, host range and PCR assays (Tomat et al., 2013a). Phages were grown to high titers as previously described (Tomat et al., 2013b). Phage stocks were enumerated (plaque-forming units per millilitre; PFU/mL) by the double-layer plate titration method (Jamalludeen et al., 2007) by mixing 0.1 mL of decimal dilutions with 0.1 mL of a log-phase culture of *E. coli* DH5α and 3 mL of warm (45 °C) Hershey soft agar (0.7% w/v) and pouring the mixture onto Hershey-Mg agar (1.5% w/v). Stocks were stored in Tris-magnesium-gelatin (0.05 mol/L Tris, 0.008 mol/L $MgSO_4$, 0.01% w/v gelatin, pH = 7.5) (TMG) buffer at 4 °C.

2.2. Biocontrol studies

2.2.1. Biocontrol in broth

Overnight cultures of the *E. coli* strains DH5α, EPEC, non-O157 STEC and O157 STEC were used to inoculate (1% v/v) fresh Hershey-Mg broth (pH 7,2), separately. When the appropriate cell density was reached (OD_{600} = 0.5), the phage cocktail (the six phages in equal proportions) was added (ca. 10^9 PFU/mL). Cultures were incubated at 4 °C, 24 °C or 37 °C with constant shaking (150 rpm) and samples (100 µL) were removed after 24 h and 48 h for viable cell count on Hershey-Mg agar plates (Tomat et al., 2013a). Phage enumeration (PFU/mL) was also carried out at the beginning and at the end of each experiment by the double-layer plate titration method. Cultures containing only bacteria and Hershey-Mg broth containing only phages were used as a control of viable cell reduction and to verify the absence of contamination, respectively. Three independent experiments and two replicates per assay were carried out.

In addition, qualitative assays were conducted using the phage cocktail and the four *E. coli* strains described above. Cultures in Hershey-Mg broth containing ~10^3 CFU either alone (strain; controls) or together with the phage cocktail (~10^9 PFU) (strain and cocktail; treatments) were incubated in 1.5 mL micro centrifuge tubes at 4 °C, 24 °C or 37 °C. Evidence of bacterial development to the naked eye was recorded every 24 h for a period of seven (7) days. After this incubation, cultures at 4 °C and 24 °C were shifted to 37 °C. Finally, cultures where no development was observed were plated in Hershey-Mg agar plates.

2.2.2. Biocontrol in milk

Biocontrol experiments were carried out at 4 °C, 24 °C and 37 °C in parallel batches in sterile, commercial, reconstituted (10%, w/v) powder skim milk (RSM) (pH 6.7), added with $CaCl_2$ (0.28 g/L) to replace the lost during the sterilization of milk. All the batches were inoculated (except one; contamination control) with overnight cultures of the four tested *E. coli* strains (one strain by batch; final concentration ~ 10^3–10^4 CFU/mL; control assays) described above. Next, batches are split into two aliquots, one aliquot of every batch infected with each *E. coli* strain was inoculated with the phage cocktail (~10^8 - 10^9 PFU/mL; experimental assays) in order to evaluate their potential as biocontrol agents, getting a multiplicity of infection (MOI) ranging from ~10^4 to 10^6. The other aliquot was used as control. The incubation proceeded for 24 h at 24 °C and 37 °C and for six (6) days at 4 °C. During incubation, bacterial cell counts were performed in MacConkey agar (37 °C, 18 h) and phage enumerations were carried out by the double-layer plaque titration method described above, at the beginning and at the end of each experiment. After milk assays, ten randomly selected colonies isolated from the experiments, *i.e.* bacteria exposed to phages, were checked for phage resistance against the cocktail. Assays were carried out in triplicate. Three independent experiments and two replicates per assay were carried out.

2.2.3. Biocontrol in meat

Beef was aseptically cut into pieces (1 cm^2 of surface and 0.4 cm thick; pH 5.6), placed in petri dishes and pre-equilibrated to 4 °C, 24 °C or 37 °C. Host strains employed in this study, namely DH5α, EPEC, non-O157 STEC and O157 STEC, were grown in Hershey-Mg broth for 18 h at 37 °C. Twenty µL of each diluted bacterial suspension were pipetted onto the surface of the meat sample (one strain by sample; final concentration ~ 10^3–10^4 CFU; control assays) and allowed to attach for 10 min at room temperature. Next, 20 µL of the phage cocktail (composed by six phages: DT1 to DT6 in equal proportions) were pipetted on the meat (final concentration ~ 10^8 - 10^9 PFU; experimental assays) at high MOI (~10^5 PFU/CFU). Control assays were also inoculated with 20 µL of TMG buffer instead of the phage cocktail. Controls and treatments were incubated at 4 °C, 24 °C or 37 °C. After each incubation time, meat pieces were transferred to a sterile bag, 5 mL of TMG buffer were added and samples processed for 2 min in a Stomacher (Seward,

London, UK). A portion (1 mL) of the stomacher fluid was transferred to a sterile tube and cells were pelleted by centrifugation at 3000 X g for 10 min. The supernatant was removed and cells were resuspended in 1 mL of TMG buffer. Finally, a sample (0.1 mL) was removed, serially diluted (if necessary) in TMG buffer and 0.1 mL of each dilution were plated on MacConkey agar for viable cell enumeration (Bigwood et al., 2008). Phages were enumerated by the double-layer plaque titration method (Jamalludeen et al., 2007) at the beginning and at the end of each experiment. Uninoculated controls were tested to determine the presence of naturally occurring bacteriophages. After meat assays, ten randomly selected colonies isolated from the experiments were checked for phage resistance against the cocktail. Three independent experiments and two replicates per assay were carried out.

2.3. Statistical analysis

Means of two samples (treatment and control) were compared using the t student's test at $p < 0.05$ in each sampling time with n = 3 observations (three independent experiments) in each group.

3. Results and discussion

3.1. Biocontrol in broth

The activity of six coliphages together in a cocktail was evaluated under several conditions. The cocktail activity was first assessed against DH5α and pathogenic EPEC, non-O157 STEC and O157:H7 STEC strains in Hershey-Mg broth (Fig. 1).

At 4 °C, and after 24-h incubation, reduction in cell counts ranged from 2.2 to 2.8 $\log_{10}$ CFU, while after 48 h higher reduction values (3.2–3.4 $\log_{10}$ CFU) were observed (Fig. 1A). In contrast, O'Flynn and coworkers (2004) found that at low temperature (12 °C) bacterial cells were not significantly reduced by the phage cocktail at similar MOI values (~1–100) to those employed in this work. On the other hand, other previous studies proved the efficacy of phage cocktails at low temperature (4 °C), though at higher MOI values (~1000) (Kudva et al., 1999). Regarding other pathogens, challenge tests showed that *E. sakazakii* phages are effective as biocontrol tools at 12 °C (Kim et al., 2007). Viable cell reductions after 24 h of incubation at 24 °C (1.2–3.4 $\log_{10}$ CFU) (Fig. 1B) and 37 °C (1.0–3.6 $\log_{10}$ CFU) (Fig. 1C) were higher than those observed after 48 h (1.0–1.8 $\log_{10}$ CFU at 24 °C and 1.0 to 3.2 $\log_{10}$ CFU at 37 °C) at both temperatures, except for the non-O157 STEC strain at 37 °C, where reduction achieved after 24 h (1 $\log_{10}$ CFU) was lower than at 48 h (3 $\log_{10}$ CFU). As previously observed by other authors, phage cocktails are effective to reduce bacterial cell population at 4 °C and especially at 37 °C (Kudva et al., 1999) after a short exposure time; however, after longer treatments, a bacterial regrowth was reported (O'Flynn et al., 2004).

Moreover, phage titers at the end of each experiment were significantly affected at 4 °C and 24 °C, showing reductions ranging from 0.6 $\log_{10}$ PFU to 1.6 $\log_{10}$ PFU, while at 37 °C, a slight increase of ~1 $\log_{10}$ PFU in phage counts was observed for all the cocktail-strain systems evaluated (Fig. 1). Accordingly, phages present a self-amplification response at 37 °C (Stewart et al., 1998; Bourdin et al., 2013) and to a minor degree at non-optimal bacterial growth conditions (Bryan et al., 2016). In addition, phage count decreased at lower temperatures due to the mechanisms used to eliminate bacterial cells, namely abortive infection and/or lysis from without (LO), where phages kill bacteria preventing cell multiplication without phage production (Tarahovsky et al., 1994; Abedon, 2011).

Qualitative assays performed in Hershey-Mg broth revealed that, at 4 °C, development of the four strains tested was retarded for 7 days of incubation, while at 24 °C and 37 °C, only DH5α and O157:H7 STEC were inhibited for 7 and 2 days, respectively. After the 7-day period, cultures at 4 °C (DH5α, EPEC, non-O157 STEC and O157:H7 STEC) and 24 °C (DH5α) were shifted to 37 °C for 24 h, DH5α was the only strain where no development was observed. Finally, when cultures where no development was observed, namely cultures of DH5α at 4 °C shifted to 37 °C, DH5α at 24 °C shifted to 37 °C, and DH5α at 37 °C, were plated in Hershey-Mg agar plates, only the culture of DH5α at 37 °C was completely eliminated. However, the phage cocktail was able to inhibit DH5α (DH5α at 4 °C shifted to 37 °C, DH5α at 24 °C shifted to 37 °C) only in liquid medium after the shift to 37 °C, since ~ 10^3 CFU/mL were detected in Hershey agar plates (Table 1). This assay showed that cocktails are especially effective in liquid medium and can be the first approach to biocontrol in liquid foods where the use of phage cocktails may prevent or delay bacterial development. No further bibliography was found regarding qualitative studies with a coliphage cocktail such as those presented in this work.

3.2. Biocontrol in milk

The six-phage cocktail was also evaluated for its lytic activity against non-pathogenic (DH5α) and pathogenic (EPEC, non-O157 STEC and O157:H7 STEC) strains in milk at refrigeration (4 °C), room (24 °C) and abusive (37 °C) temperature.

The level of contamination of all the *E. coli* strains assayed in milk were to below the detection limit (< 10^1 CFU/mL) when the phage cocktail was applied at 4 °C (Fig. 2). Specifically, cell counts of DH5α (Fig. 2A) and O157:H7 STEC (Fig. 2D) dropped from ~10^2 CFU/mL to below the detection limit after a 1-d exposure, while cells of EPEC (Fig. 2B) and non-O157 STEC (Fig. 2C) were undetectable only after 13 d and 7 d of exposure to phages, respectively. At low temperatures, similar bacterial inactivation employing phages was also observed in different milk products against *E. coli* (McLean et al., 2013), *Enterobacter sakazakii* (Kim et al., 2007) and *Staphylococcus aureus* (Bueno et al., 2012), especially when a high MOI value is used (Carlton et al., 2005). The effective *E. coli* inactivation seen at low temperatures and high MOI values is possibly due to the mechanism LO, as suggested in previous studies (Abedon, 2011). However, at lower temperatures, the

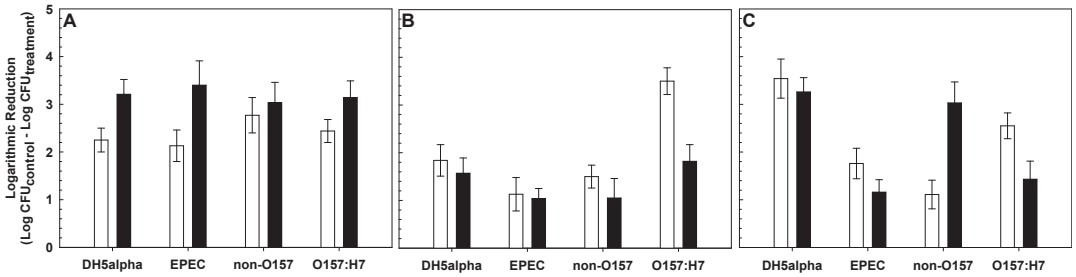

Fig. 1. Reduction of *Escherichia coli* viable cells treated with the six-phage cocktail in broth after 24 h (white bars) and 48 h (black bars) at 4 °C (A), 24 °C (B) and 37 °C (C). Phage titre at the beginning (0 h; 7.6 × 10^9 PFU/mL) and at the end (48 h) of each experiment were: DH5α (ranged from 1.0 × 10^8 to 4.0 × 10^{10} PFU/mL); EPEC (ranged from 6.0 × 10^7 to 3.8 × 10^{10} PFU/mL), non-O157 STEC (ranged from 1.0 × 10^8 to 4.6 × 10^{10} PFU/mL) and O157:H7 STEC (ranged from 1.7 × 10^9 to 4.1 × 10^{10} PFU/mL). Error bars represent the standard deviation of three determinations ($p < 0.05$).

Table 1
Growth of *E. coli* strains on broth in the presence of the phage cocktail at 4, 24, and 37 °C.

Temperature/Strain		* Visible turbidity at time (d)										
		Controls					Cocktail treatment					Cell counts[a] (CFU/mL)[b]
		1 d	2 d	3 d	7 d	Shift to 37 °C	1 d	2 d	3 d	7 d	Shift to 37 °C	
4 °C	DH5α	–	–	–	–	+	–	–	–	–	–	8×10^3
	EPEC	–	–	–	–	+	–	–	–	–	+	nd
	non-O157 STEC	–	–	–	–	+	–	–	–	–	+	nd
	O157:H7 STEC	–	–	–	–	+	–	–	–	–	+	nd
24 °C	DH5α	+	+	+	+	nd	–	–	–	–	–	5×10^3
	EPEC	+	+	+	+	nd	+	+	+	+	nd	nd
	non-O157 STEC	+	+	+	+	nd	+	+	+	+	nd	nd
	O157:H7 STEC	+	+	+	+	nd	–	–	+	+	nd	nd
37 °C	DH5α	+	+	+	+	nd	–	–	–	–	–	< 10
	EPEC	+	+	+	+	nd	+	+	+	+	nd	nd
	non-O157 STEC	+	+	+	+	nd	+	+	+	+	nd	nd
	O157:H7 STEC	+	+	+	+	nd	–	–	+	+	nd	nd

* + (turbidity); - (no turbidity). nd: not done (cultures + were not shifted to 37 °C or plated).
[a] Cell counts were made when no turbidity (−) was observed.
[b] Detection limit: 10 CFU/mL.

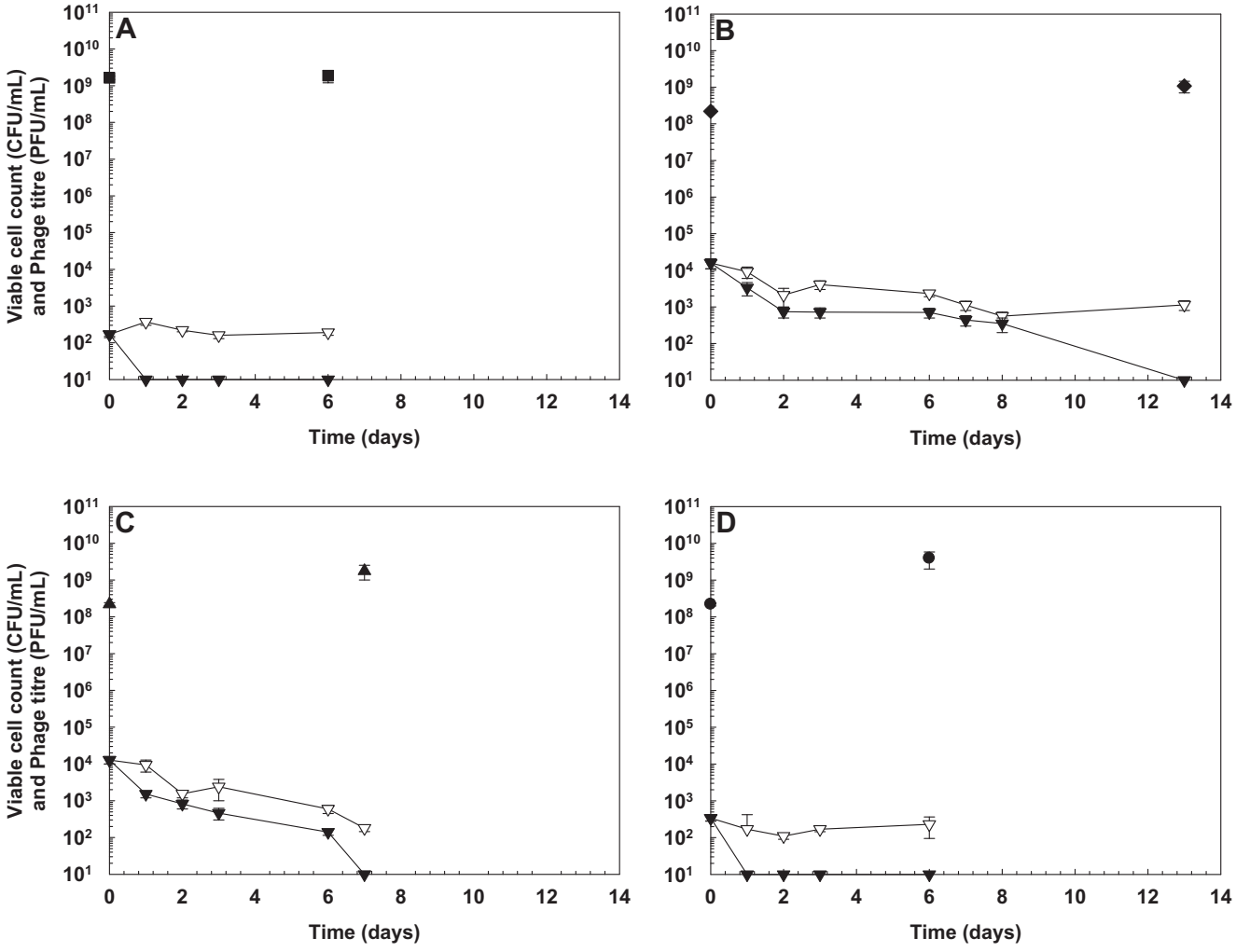

Fig. 2. Inactivation of *Escherichia coli* DH5α (A), EPEC (B), non-O157 STEC (C) and O157:H7 STEC (D) by the six-phage cocktail in milk at 4 °C. Filled symbols (▼) represent viable cell counts in the presence of phages and empty symbols ( ) are phage-free controls. Phage titre at the beginning (0 d) and at the end of each experiment with DH5α (■; 6 d), EPEC (♦; 13 d), non-O157 STEC (▲; 7 d) and O157:H7 STEC (●; 6 d). Error bars represent the standard deviation of three determinations ($p < 0.05$).

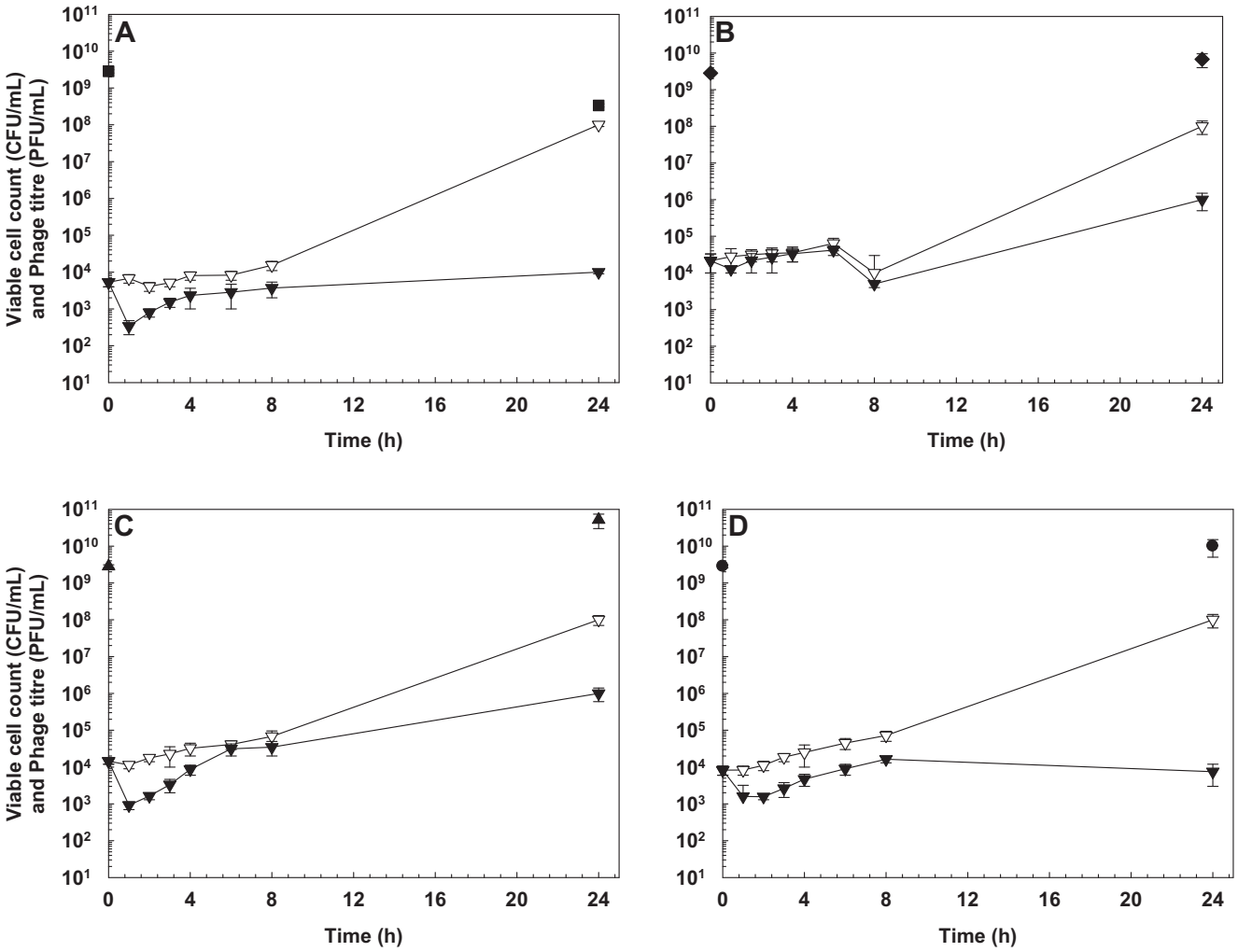

Fig. 3. Inactivation of *Escherichia coli* DH5α (A), EPEC (B), non-O157 STEC (C) and O157:H7 STEC (D) by the six-phage cocktail in milk at 24 °C. Filled symbols (▼) represent viable cell counts in the presence of phages and empty symbols ( ) are phage-free controls. Phage titre at the beginning (0 h) and at the end (24 h) of each experiment with DH5α (■), EPEC (♦), non-O157 STEC (▲) and O157:H7 STEC (●). Error bars represent the standard deviation of three determinations ($p < 0.05$).

abortive infection process becomes more relevant (Tarahovsky et al., 1994). In addition, it is likely that the low temperature (4 °C) also contributed to viable cell inactivation as observed in the control assays (Fig. 2B and C) since other authors also reported this behavior for two *E. coli* strains in milk (McLean et al., 2013) and for O157:H7 STEC at 4 °C (Reitsma and Henning, 1996) and non-O157 STEC at 11 °C (Montet et al., 2009) during cheese ripening.

To determine whether bacteriophages can eliminate or inhibit grown of *E. coli* (~10^4 CFU/mL) during an interruption in the cold chain, challenge tests with the cocktail were carried out on milk at room temperature (24 °C) (Fig. 3). Cell counts in treatment samples were always below control counts. After 24-h incubation, reductions achieved were ≥4 $\log_{10}$ CFU for DH5α (Fig. 3A) and O157:H7 STEC (Fig. 3D), while only a 2 log10 CFU reduction was observed for EPEC (Fig. 3B) and non-O157 STEC (Fig. 3C). Thus, the phage cocktail maintained bacterial contamination at an acceptable level, similar to the initial contamination, for DH5α and O157:H7 STEC after 24 h, while EPEC and non-O157 STEC strains were inhibited only within 8-h incubation. Phage cocktails used with biocontrol purposes in milk products are known to be highly effective against *E. coli* (McLean et al., 2013), *Staphylococcus aureus* (Bueno et al., 2012) and *Mycobacterium smegmatis* (Endersen et al., 2013). Namely, McLean et al. (2013) used a three-phage cocktail (EC6, EC9, and EC11) (10^9 PFU/mL) in raw milk artificially contaminated with *E. coli* (10^5 CFU/mL) at 25 °C and achieved a bacterial reduction to non-detectable levels. In addition, individual phages were able to eliminate *Enterobacter sakazakii* cells in reconstituted infant formula at 24 °C (Kim et al., 2007). Taking into account that the level of contamination by pathogenic STEC in milk products is low (Omiccioli et al., 2009; Amagliani et al., 2016), and considering that highly perishable foodstuffs such as milk (Likar and Jevsnik, 2006; Gunders, 2012) exposed to room temperature, *e.g.* milk with breaks in the cold chain for periods of time longer than a few hours should be discarded, the phage cocktail proved to be useful when milk is subjected to non-refrigerated temperatures. However, additional hurdles may be needed in order to significantly reduce or eliminate bacterial contamination by *E. coli* in this food matrix at room temperature.

In biocontrol assays performed in milk at 37 °C (Fig. 4), the phage cocktail significantly reduced EPEC (Fig. 4B), non-O157 STEC (Fig. 4C) and O157:H7 STEC (Fig. 4D) strains by 4, 3 and 4 $\log_{10}$ CFU/mL respectively; though 10^3–10^4 bacterial cells remained viable after 24 h of incubation. These results are similar to those observed for other pathogens such as *Staphylococcus aureus* (Garcia et al., 2007) and *Mycobacterium smegmatis* (Endersen et al., 2013) in milk products treated with a phage cocktail at 37 °C. In addition, Kim et al. (2007) observed a reduction of 4 $\log_{10}$ CFU/mL in the bacterial population of *Enterobacter sakazakii* with phage ESP 1–3 and a complete cell inactivation using phage ESP 732–1 at high phage concentration (10^9 PFU/mL) and high exposure time (10 h). Likewise, the non-pathogenic strain was rapidly reduced when exposed to our cocktail since DH5α counts were below

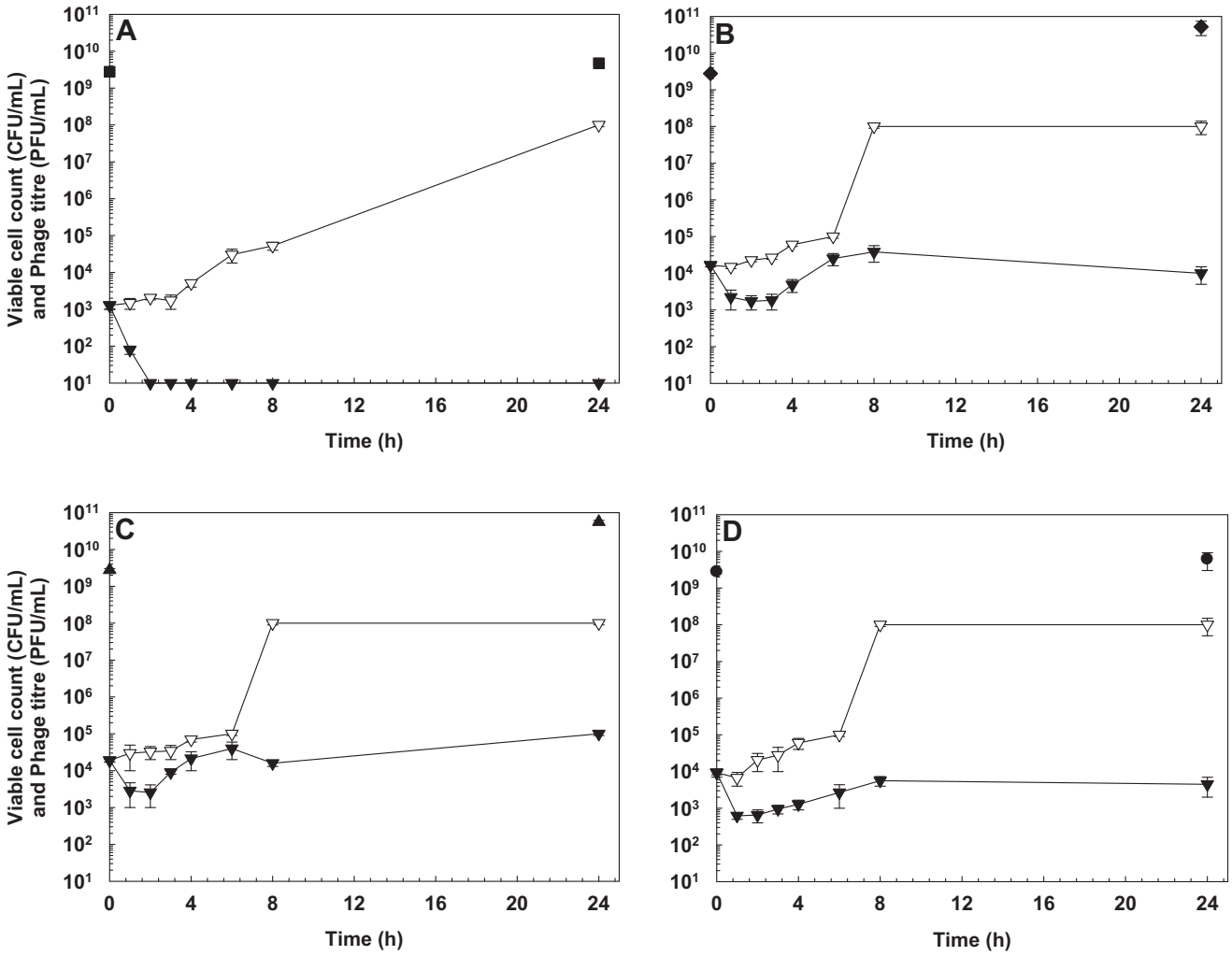

Fig. 4. Inactivation of *Escherichia coli* DH5α (A), EPEC (B), non-O157 STEC (C) and O157:H7 STEC (D) by the six-phage cocktail in milk at 37 °C. Filled symbols (▼) represent viable cell counts in the presence of phages and empty symbols ( ) are phage-free controls. Phage titre at the beginning (0 h) and at the end (24 h) of each experiment with DH5α (■), EPEC (♦), non-O157 STEC (▲) and O157:H7 STEC (●). Error bars represent the standard deviation of three determinations ($p < 0.05$).

the detection limit within 2 h (Fig. 4A). In accordance, Moradpour et al. (2009) reported complete inactivation of *E. coli* O157:H7 after a 2-h exposure when cells were challenged with a genetically engineered phage (10^9 PFU/mL) in milk at 37 °C.

Regarding the bacterial cells that survived in our milk assays after a 24-h exposure at 24 °C and 37 °C, other authors found that some proteins in whey may inhibit the adsorption of phage on bacteria (Gill et al., 2006). Also, in another study it was suggested that the access of phage K to *Staphylococcus aureus* cells was prevented by immune factors present in bovine milk (O'Flaherty et al., 2005). From the findings above, we hypothesize that there may be a factor in the dairy matrix which is inactive at low temperature but which interferes with the phage processes at room and abusive temperatures. As mentioned, Moradpour et al. (2009) found complete inactivation of *E. coli* O157:H7 in milk at abusive temperature; however, virus particles used in this work were genetically engineered to contain the lethal CAP gen that kills bacteria by an additional mechanism other than lysis from within induced by the regular lytic phage cycle.

The behavior of phage titers in milk challenges was similar at the three temperatures evaluated. Specifically, phage counts showed a slight increase of about 1 $\log_{10}$ PFU/mL at 4 °C for the three pathogenic strains evaluated. Yet, it remained constant for DH5α after 24 h incubation (Fig. 2). The absence of phage propagation is characteristic at this low temperature; however, similar results were found for phage A511 in chocolate milk by Guenther et al. (2009). On the contrary, the counts of a phage cocktail dropped from ~10^8 PFU/mL to 10^2 PFU/mL after a 14-h exposure at 4 °C, though the pH values also decreased throughout the experiment (Bueno et al., 2012). At 24 °C (Fig. 3) and 37 °C (Fig. 4), a low phage multiplication was observed since increases from 0.5 to 1.3 log10 PFU/mL at most were recorded in milk. High MOI values are mandatory to LO (Tarahovsky et al., 1994), and high temperatures such as 24 °C and 37 °C can increase both the rate and degree of LO (Abedon, 2011), thus reducing the propagation effect that the lytic cycle of phages has. Likely, the high number of phage particles adsorbed to bacteria contributed to reduce the phage titers observed. On the other hand, several authors have found a variable propagation response among phages infecting *Staphylococcus aureus* at 37 °C (Obeso et al., 2010) and *Enterobacter sakazakii* at 30 °C (Zuber et al., 2008), depending on the initial concentrations of phages and bacteria employed.

None of 10 *Escherichia coli* isolates recovered from the samples at the end of the milk experiments proved to be resistant to infection by the phage cocktail (data not shown). The development of BIMs was observed for other pathogens from phage-treated liquid foods (Endersen et al., 2013; Guenther et al., 2012); however, more than 30 *Listeria* isolates retained sensitivity to P100 infection (Carlton et al., 2005), though colonies were isolated from Ch-easy plates. BIMs could arise if a greater number of colonies isolated from our milk experiments were tested for resistance, especially with assays carried out at the optimal grown temperature (37 °C) for *E. coli* (O'Flynn et al., 2004).

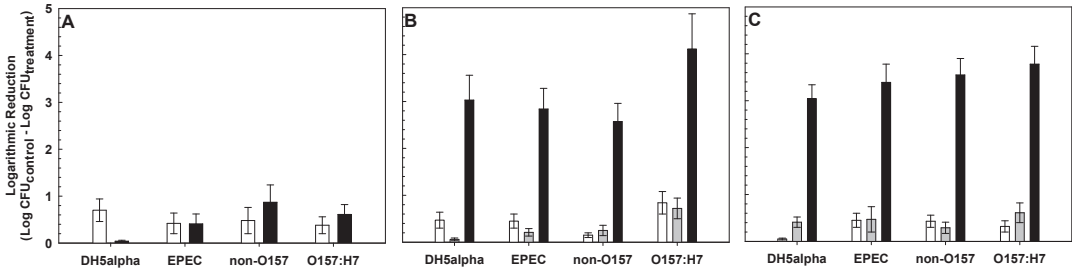

Fig. 5. Reduction of *Escherichia coli* viable cells treated with the six-phage cocktail in meat after 24 h (white bars) and 48 h (black bars) at 4 °C (A), and after 1 h (white bars), 3 h (gray bars) and 24 h (black bars) at 24 °C (B) and 37 °C (C). Phage titre at the beginning (0 h; ~1.2×10^9 PFU/mL) and at the end (24 h at 4 °C; 48 h at 24 °C and 37 °C) of each experiment were: DH5α (ranged from 3.2×10^6 to 1.5×10^8 PFU/mL), EPEC (ranged from 3.0×10^6 to 1.9×10^7 PFU/mL), non-O157 STEC (ranged from 3.1×10^6 to 2.8×10^7 PFU/mL) and O157:H7 STEC (ranged from 3.6×10^6 to 3.6×10^7 PFU/mL). Error bars represent the standard deviation of three determinations ($p < 0.05$).

3.3. Biocontrol in meat

Next, the potential of the cocktail to eliminate or significantly reduce bacterial contaminations on meat was evaluated. For these assays, meat pieces were inoculated with *E. coli* at ~10^3–10^4 CFU and with the cocktail (10^9 PFU/mL). After that, the meat samples were incubated at refrigeration, room and abusive temperatures (Fig. 5).

When the six-phage cocktail was applied to the meat surface, there was a small yet significant reduction (0.5–1.0 $\log_{10}$ CFU/mL) for all the cell counts after 24 h at 4 °C, though a bacterial regrowth was observed only for DH5α after a 48-h incubation (Fig. 5A). Several studies have demonstrated the usefulness of phage application on meat at low temperature (Carter et al., 2012; Hudson et al., 2013). However, variable results have been found for *E. coli* (Hudson et al., 2010; Hong et al., 2014) and for other pathogens (Dykes and Moorhead, 2002; Bigwood et al., 2008). Although high MOI values were used to increase the cells becoming infected at the beginning of our experiment, low inactivation levels were observed. This could be due to cell lysis during counting (Hudson et al., 2013), or the fact that most targeted bacteria may be embedded within the protein network on the meat matrix, becoming unreachable to phage particles (Tomat et al., 2013a). On the contrary, our phage cocktail showed better biocontrol values at higher temperatures. At 24 °C (Fig. 5B) and 37 °C (Fig. 5C), bacterial reduction increased over time in the presence of phages compared with the phage-free controls. Namely, reductions of *E. coli* in phage-treated meat ranged from 2.6 to 4.0 $\log_{10}$ CFU/mL when samples were incubated at room temperature (24 °C), and from 3.0 to 3.8 $\log_{10}$ CFU/mL when incubated under abusive temperature (37 °C), both after a 24-h exposure. In accordance, O'Flynn et al. (2004) obtained a complete inactivation of *E. coli* in seven out of nine meat samples within 1 h of contact at 37 °C by using a three-phage cocktail. Furthermore, Hudson et al. (2013) found that at high phage concentration (7.3 $\log_{10}$ PFU/piece) there was a ~2 $\log_{10}$ CFU/piece reduction in the concentration of *E. coli* after 1 h, though at lower concentrations there was no effect (3.5 $\log_{10}$ PFU/piece) or a slight decrease (5.6 $\log_{10}$ PFU/piece) compared to the phage-free controls.

Taking into account that meat is commonly contaminated by *E. coli* O157 during slaughter and that this foodborne pathogen is responsible for the high incidence of HUS in our country (Rivas et al., 2006), further studies were carried out in meat with the O157:H7 STEC strain. At low temperature, experiments were conducted for an extended period of time (up to 6 days), while at 24 °C and 37 °C, incubations were accomplished only for 24 h due to meat spoilage, though more sampling times were added to the experiments.

Concentrations of O157:H7 STEC in phage-treated meat were 0.67 ± 017, 1.01 ± 0.21 and 1.55 ± 0.35 $\log_{10}$ CFU/mL less than those in untreated meat when samples were incubated at 4 °C for 1d, 3 d and 6 d, respectively (Fig. 6A). That is, O157:H7 STEC reductions increased as incubation was extended up to 6 days under refrigeration conditions, thus suggesting that this phage cocktail may be useful for cold-storage of meat products. When incubation was accomplished at 24 °C and 37 °C, the concentrations of O157:H7 STEC in phage-treated samples were 3.41 ± 0.51 (24 °C; Fig. 6B) and 3.78 ± 0.38 (37 °C; Fig. 6C) $\log_{10}$ CFU/mL less than those found in untreated ones after a 24-h exposure. Similar results were found in biocontrol assays with a three-phage cocktail against *E. coli* O157:H7 in meat at 37 °C (O'Flynn et al., 2004); while at the same abusive temperature, Hudson and coworkers achieved only a ~1 log10 CFU/mL reduction after a 4-h exposure, and a subsequent bacterial regrowth of *E. coli* O157:H7 cells (10^7 CFU) was observed after a 20-h incubation (Hudson et al., 2013). According to Hong et al. (2014), phages may possess different physiological properties, being more effective at low temperature than at high temperature or vice versa. According to our results, the phages used to design our six-cocktail may belong to the high-temperature or mid-temperature class of phages that lyses bacteria more effectively at or above 15 °C (Seeley and Primrose, 1980).

None of the 10 *Escherichia coli* isolates recovered from the samples at the end of the meat experiments were shown to be resistant to infection by the phage cocktail (data not shown), as also found by Abuladze et al. (2008). Besides, no resistance was found for other pathogens such as *Salmonella* (Bigwood et al., 2008) and *Listeria* (Guenther et al., 2009). These observations may indicate that, unlike in liquid foods, bacterial cells inoculated in meat escaped contact with phage particles.

Phage titers were significantly reduced at all temperatures assayed on meat (Figs. 5 and 6). At 4 °C, an average of ~1.8 $\log_{10}$ PFU/mL reduction in the titers was observed, while at 24 °C and 37 °C, reductions ranged from 1.2 to 1.8 log10 PFU/mL and from 0.6 to 1.4 $\log_{10}$ PFU/mL, respectively; while 10^6–10^8 phage particles remained viable. In accordance, some authors found that replication of phages does not occur in the food matrix at 5 °C (Hudson et al., 2013). However, when phages were added to growing *E. coli* O157:H7 cells (37 °C) on beef at a low concentration, an increase in the numbers of phages was detected after a 20-h incubation (Hudson et al., 2013). In our trials, decreases in phage titers may be due to phage particles that remain attached and/or trapped in the meat matrix, reducing the phage particles to be counted.

Our phage cocktail proved to be effective at reducing bacterial populations on food matrices at refrigerated, room and abusive temperatures, as also found with other coliphages (O'Flynn et al., 2004; Minh et al., 2016) and other pathogens as well (Bigwood et al., 2008; Guenther et al., 2009). However, in order to improve phage biocontrol, other authors have suggested that phages can be protected by microencapsulation (Ly-Chatain, 2014) or by improving delivery mechanisms (Inal, 2003); though in our experiments, high MOI values could be assayed in order to improve the reductions achieved.

4. Conclusion

The findings presented in this work showed that the use of

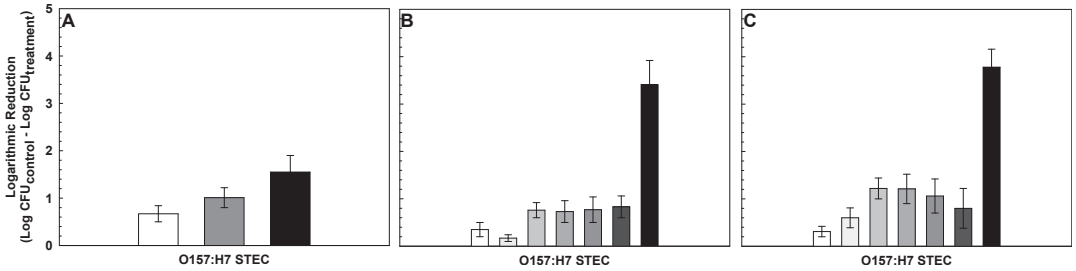

Fig. 6. Reduction of O157:H7 STEC viable cells treated with the six-phage cocktail in meat after 1 day (white bars), 3 days (gray bars) and 6 days (black bars) at 4 °C (A), and after 1 h (□), 2 h (■), 3 h (■), 4 h (■), 6 h (■), 8 h (■) and 24 h (■) at 24 °C (B) and 37 °C (C). Phage titre at the beginning (0 h; 1.6×10^8 PFU/mL at 24 and 37 °C and 3.1×10^9 PFU/mL at 4 °C) and at the end of each experiment were 1.4×10^7 PFU/mL (4 °C; 6 d), 2.0×10^7 PFU/mL (24 °C, 24 h) and 5.1×10^7 PFU/mL (37 °C, 24 h). Error bars represent the standard deviation of three determinations ($p < 0.05$).

coliphages mixed in a cocktail was effective against foodborne pathogens. Our phage cocktail may be a promising strategy to control foodborne pathogens such as *E. coli* EPEC and STEC at several temperatures in both milk and meat. In addition, the development of BIMs was also prevented. The phages survived at high levels at the end of all the experiments conducted, a desirable characteristic for the prevention of recontamination through the food chain production. However, further sequencing and bioinformatics analyses of our six phages are required prior its application to foods. Although several authors have found encouraging results with other cocktails composed by coliphages (O'Flynn et al., 2004; Abuladze et al., 2008), challenges in foods with a six-phage cocktail had never been previously carried out against pathogenic *E. coli* strains circulating in our region.

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