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Hard surfaces decontamination of enteropathogenic and Shiga toxin-producing *Escherichia coli* using bacteriophages

David Tomat [a,*], Andrea Quiberoni [b], Diego Mercanti [b], Claudia Balagué [a]

[a] *Área de Bacteriología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, S2002LRK Rosario, Argentina*
[b] *Instituto de Lactología Industrial (UNL — CONICET), Facultad de Ingeniería Química, Santiago del Estero 2829, 3000 Santa Fe, Argentina*

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ABSTRACT

Three *Myoviridae* phages (DT1, DT5 and DT6) specific for pathogenic *Escherichia coli* were studied, either individually or as cocktails, for their lytic activity on *in vitro* challenge tests. Also, cocktail ability to reduce artificial contamination on hard surfaces (glass coverslips and stainless steel coupons) by three pathogenic *Escherichia coli* strains (EPEC920, non-O157 STEC ARG4827 and O157:H7 STEC464) was tested. Assays of phage stability during refrigerated storage showed that the three phages evaluated retained a high viability after two months at 4 °C. Challenge tests showed high reductions in viable cells, of up to 6.4 log CFU ml^{-1}, for all tested strains at 37 °C. Efficiency was somewhat lower at 4 °C, though biocontrol levels were still good, reaching values of up to 3.8 log CFU ml^{-1}. Considering only results obtained at 37 °C, phage cocktails produced the highest reduction in most cases. Treatments with phage cocktails produced complete inactivation (ca. 5–6 log CFU ml^{-1}) of EPEC920 and O157:H7 STEC464 on glass coverslips, and of EPEC920, non-O157 STEC ARG4827 and O157:H7 STEC464 on stainless steel coupons, at both temperatures (4 °C and 37 °C) and multiplicity of infection (ca. 10^3 and 10^7) tested. However, some strains not detected at 3 h were sometimes detected at 24 h, and inactivation of non-O157 STEC ARG4827 on glass coverslips was never accomplished; viable cell reductions in all these cases ranged from 1.2 to 5.4 log CFU ml^{-1}. Our results suggest that lytic phages, either individually or as a cocktail, may be useful for reducing contamination on hard materials used in food processing surfaces. To our knowledge, this is the first study focused on the use of bacteriophages to reduce contamination of food processing surfaces by EPEC and non-O157 STEC strains.

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1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are human pathogens responsible for foodborne diseases worldwide (Farrokh et al., 2012). Infections by STEC often lead to diarrhea as well as severe clinical manifestations such as hemolytic uremic syndrome (HUS) (Griffin, Mead, & Sivapalasingam, 2002). In Argentina, where HUS is endemic, the estimated annual incidence rate is 17 cases/100,000 children under five years of age (Rivas, 2012). Moreover, enteropathogenic *E. coli* (EPEC) possess the enterocyte attaching-and-effacing lesion gene (*eaeA*) which could contribute to the virulence of STEC (Frankel et al., 1998).

Contamination of industrial equipment and hard surfaces such as utensils and food processing tables by pathogenic *E. coli* strains represents a major concern since cross contamination can occur. Contaminated fruits and vegetables are increasingly implicated as a source of *E. coli* infections (WHO, 2011). In addition, it has been reported that attachment of *E. coli* O157:H7 biofilms is stronger for bacteria grown in nutritionally limited environments occasionally found in inanimate surfaces at food processing facilities (Dewanti & Wong, 1995; Sharma, Ryu, & Beuchat, 2005). Therefore, decontamination may not be enough to assure the destruction of *E. coli* O157:H7 biofilms on hard surfaces (Kusumaningrum, Riboldi, Hazeleger, & Beumer, 2003). Moreover, the extensive use of chemical sanitizers increases bacterial resistance, resulting in a reduced efficacy, can kill commensal bacteria and alter organoleptic properties of treated foods as well (Abuladze et al., 2008).

Bacteriophages are i) everlasting natural entities, easy to isolate and propagate at very low cost; ii) ubiquitous, since they are distributed among all environments such as soil, water and foods; iii) the most abundant forms of life on the planet (ca. 10^{31} virions) (Rohwer & Edwards, 2002); and iiv) highly specific, self-perpetuating and self-limited entities, thus replicating only in the presence of susceptible host. As natural bacterial predators, phages represent a powerful biocontrol tool of foodborne pathogens, consequently improving food safety. On the other hand, phages can be also useful for the decontamination of hard surfaces used in food processing, as an environmentally friendly alternative to toxic chemical compounds. Regarding bacterial resistance, phages constantly evolve and can adapt *in situ* to regain the ability of infecting resistant bacteria. In spite of this natural ability, one strategy used to prevent resistance development is the application of

* Corresponding author at: Área de Bacteriología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario (U.N.R.), Suipacha 531, S2002LRK Rosario, Santa Fe, Argentina. Tel.: +549 0341 4804592.
E-mail address: dtomat@fbioyf.unr.edu.ar (D. Tomat).

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lytic phage mixtures (phage cocktails), which would achieve this goal by making less likely the survival of resistant cells arising for different phages. Despite cocktail advantages, increasing their complexity could hinder their application, partly due to regulatory issues (Viazis, Akhtar, Feirtag, & Diez-Gonzalez, 2010). However, consumer acceptance is probably the main hurdle to overcome in order to use phages to improve food safety.

Some authors have reported the effectiveness of specific phages to biocontrol *E. coli* O157:H7 on glass coverslips, on gypsum board surfaces (Abuladze et al., 2008) and on stainless steel, ceramic tile and high density polyethylene coupons (Sharma et al., 2005; Viazis et al., 2010). In addition to O157:H7 STEC, foodborne pathogens such as EPEC and non-O157 STEC can be found also in food processing environments (FDA, 2012; Kaspar, Doyle, & Archer, 2010). Considering that some bacteria attach to surfaces as their predominant form of survival in nature (Lindsay & Von Holy, 1999) and the increasing resistance to disinfectants such as hypochlorous acid and benzalkonium chloride (Davidson & Harrison, 2002) as well as the emergence of antibiotic-resistant strains, new non-antibiotics and natural biocontrol methods are needed. These methods should be effective, safe, inexpensive and usable in the presence of natural flora for food biocontrol purposes. In this study, three previously characterized bacteriophages were investigated, either individually or as cocktails, to determine their biocontrol activity against pathogenic *E. coli* strains during *in vitro* challenge tests, as well as on experimentally contaminated hard surfaces. *E. coli* strains tested belong to different serogroups (O157 and non-O157) and virotypes (STEC and EPEC) thus covering a wide range of pathogens on hard surfaces decontamination assays.

2. Materials and methods

2.1. Bacterial strains and phages

E. coli DH5α was used as sensitive 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, identified using API-20E system (Biomerieux, Buenos Aires, Argentina) and further characterized by PCR. 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 routinely reactivated overnight (37 °C) 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) supplemented with $MgSO_4$ (5 mmol l^{-1}) (Cicarelli, San Lorenzo, Santa Fe, Argentina) (Hershey-Mg).

E. coli DH5α has been previously used to isolate phages DT1, DT5 and DT6 from 50 stool samples of patients with diarrhea treated at the Centenary Hospital, Rosario (Tomat, Migliore, Aquili, Quiberoni, & Balagué, 2013). Phage isolation and purification were performed by the double-layer plaque technique according to Tomat et al. (2013).

2.2. Phage stability on storage

Phage stability at refrigeration temperature was determined at regular intervals over a two month period. Aliquots (100 μl) of phage stocks stored at 4 °C were serially diluted and phage titre was determined by the double layer plaque technique (Tomat et al., 2013). Results were expressed as the percentage of viable phages with respect to the initial phage concentration.

2.3. Challenge test

Overnight (18 h) cultures of the *E. coli* strains DH5α, EPEC920, non-O157 STEC ARG4827 and O157:H7 STEC464 were used to inoculate (1% v/v), separately, fresh Hershey-Mg broth. When the appropriate cell density was reached ($OD_{600} = 0.5$), bacteriophage DT1, DT6 or a mixture thereof in equal proportions (phage cocktail) were added (ca. 10^9 PFU ml^{-1}). Owing to resistance of non-O157 STEC ARG4827 strain to phage DT1, phage DT5 was used instead. Cultures were incubated at 4 °C or at 37 °C with constant shaking (150 rpm) and samples (100 μl) were removed after 2, 6 and 24 h for viable cell count on Hershey-Mg agar plates. Phage titres were also determined by the double layer plaque technique (Tomat et al., 2013). Cultures without phage (containing only bacteria) and without cells (containing only phage) were used as a control of viable cell reduction at each sampling time and to verify absence of contamination, respectively. Sampling was performed in duplicate in three independent experiments.

2.4. Efficiency of plating

Efficiency of plating (EOP) was calculated for phages DT1, DT5 and DT6 as the ratio: Phage $titre_{E.\ coli\ strain}$ (PFU ml^{-1})/phage $titre_{DH5\alpha}$ (PFU ml^{-1}), where *E. coli* strain refers to either EPEC920, STEC464 O157:H7 or non-O157 STEC ARG4827, and DH5α is the host strain for all the phages.

2.5. Hard surfaces decontamination

Glass coverslips (GC) (18 mm × 18 mm) and stainless steel coupons (SSC) (25 mm × 15 mm) were used to represent two different food processing materials. Matrices were cleaned with 70% ethanol, autoclaved, placed in Petri dishes, and stored at room temperature until use. Each bacterial culture, namely EPEC920, non-O157 STEC ARG4827, and O157:H7 STEC464, was independently inoculated as spots (10 μl) onto hard surfaces (GC and SSC) at two concentrations (ca. 10^2–10^3 and 10^4–10^5 CFU ml^{-1} for GC, and ca. 10^3–10^4 and 10^5–10^6 CFU ml^{-1} for SSC) and allowed to dry for 30 min at room temperature (ca. 25 °C) in a laminar flow biosafety cabinet (Telstar Bio II A, Teslar, Inc., Buenos Aires, Argentina). Hard surfaces with dried *E. coli* cultures were then treated with aliquots (100 μl) of phage cocktails (DT1 + DT6 for EPEC920 and O157:H7 STEC464; DT5 + DT6 for non-O157 STEC ARG4827) (1:1 ratio, ca. 10^9 PFU ml^{-1}) or Hershey-Mg without phages (control hard surfaces); some pieces were not treated at all (dry controls). GC and SSC were then incubated inside Petri dishes at 4 °C and 37 °C for 1 h, 3 h, and 24 h; incubation at 37 °C was carried out in a thermostatic bath to prevent evaporation. At the indicated incubation times, GC and SSC were placed in 50 ml centrifuge tubes containing 1 ml of phosphate buffer saline (PBS) (pH 7.2), vortexed for 2 min, serially diluted in PBS, plated on agar Hershey-Mg and incubated 24 h at 37 °C; viable cells (CFU ml^{-1}) were subsequently enumerated. Viable cell reductions (CFU ml^{-1}) were calculated by subtracting cell counts in treated hard surfaces from cell counts in control hard surfaces (Hershey-Mg without phages). Duplicate samples at each sampling time were tested and three independent experiments were carried out.

2.6. Statistical analyses

For statistical analysis, data in CFU ml^{-1} were transformed to logarithm (log_{10}). Two-way ANOVA was applied and, when significant differences existed, Scheffé method was selected for multiple comparisons among treatments ($p < 0.05$).

3. Results

3.1. Phage stability on storage

Bacteriophages studied showed high stability at refrigeration temperature, with only small viability decreases observed after 60 d of storage at 4 °C (data not shown). After 30 d, reductions in PFU ml^{-1} were very similar, ranging from 22 to 37% for all phages evaluated whereas

Table 1
Reduction of *Escherichia coli* viable cells after *in vitro* treatment at 37 °C with either individual phages or a cocktail thereof (1:1 ratio).

E. coli strain	Phage	MOI	*E. coli* inactivation (log_{10} CFU ml^{-1})[a,b] at different times of phage treatment		
			2 h	6 h	24 h
DH5α	DT1	4.6×10^2	[1]3.4 ± 0.4	[2]5.2 ± 0.7	[1]2.0 ± 0.3
	DT6	4.2×10^2	[2]3.8 ± 0.5	[1]4.4 ± 0.7	[1]2.1 ± 0.4
	Cocktail	4.4×10^2	[3]5.5 ± 0.3	[1]4.4 ± 0.5	[1]2.3 ± 0.2
EPEC920	DT1	8.6×10^1	[1]3.3 ± 0.2	[1]4.7 ± 0.3	[2]1.5 ± 0.2
	DT6	7.8×10^1	[3]5.5 ± 0.4	[2]5.2 ± 0.2	[1]1.2 ± 0.2
	Cocktail	8.2×10^1	[2]3.9 ± 0.3	[2]5.0 ± 0.2	[1,2]1.4 ± 0.3
Non-O157 STEC (ARG4827)	DT5	2.9×10^2	[1]4.5 ± 0.3	[1]5.2 ± 0.4	[1]0.5 ± 0.2
	DT6	2.6×10^2	[2]6.3 ± 0.5	[1]5.1 ± 0.8	[2]1.1 ± 0.2
	Cocktail	3.0×10^2	[2]6.4 ± 0.4	[1]5.5 ± 0.3	[3]1.7 ± 0.4
O157:H7 STEC464	DT1	1.4×10^2	[1]3.3 ± 0.1	[2]4.4 ± 0.3	[0]0.1 ± 0.1
	DT6	1.3×10^2	[2]3.9 ± 0.4	[1]3.8 ± 0.2	[1]0.5 ± 0.4
	Cocktail	1.4×10^2	[2]3.6 ± 0.3	[3]5.2 ± 0.4	[2]2.0 ± 0.4

MOI: multiplicity of infection. Identical superscripts indicate phage treatments not significantly different at $p = 0.05$.
[a] With respect to phage free control.
[b] Mean value of three determinations ± standard deviation.

between 30 and 60 d none of the three phages showed noticeable reductions.

3.2. Challenge with individual phages and phage cocktail

Phages, individually or as cocktails, presented high lytic activity at 37 °C after short sampling times (2 and 6 h), though lower lytic activity was observed after 24 h of incubation in all systems evaluated, which led to obtain the lowest bacterial reduction values at long sampling times (Table 1). Also, reductions observed in the pathogenic strains were lower than those achieved on DH5α after 24 h.

Reductions for all the strains tested ranged from 3.3 to 6.4 log CFU ml^{-1}, 3.8 to 5.5 log CFU ml^{-1} and 0.5 to 2.3 log CFU ml^{-1} after 2, 6 and 24 h, respectively, the cocktail treatments in all cases corresponding to the upper limit of the ranges reported. Furthermore, cocktail treatments showed higher lytic activity in all phage/strain systems after 24 h, showing significant differences regarding individual phage treatments on both STEC strains, namely non-O157 STEC ARG4827 and O157:H7 STEC464.

In addition, *in vitro* biocontrol assays were also carried out at refrigeration temperature (4 °C) (Table 2). Biocontrol values obtained were evident but small. Average reductions for all the strains tested were 1.1 log CFU ml^{-1}, 2.8 log CFU ml^{-1} and 1.5 log CFU ml^{-1} after 2, 6 and 24 h, respectively, being the largest reduction (3.8 log CFU ml^{-1}) obtained by using the cocktail. Regarding to the influence of the temperature in challenge treatments, 37 °C led to higher reductions than 4 °C after 2 and 6 h, but values obtained after 24 h were similar at both temperatures, ranging from 0.5 to 2.3 log CFU ml^{-1}.

Phage particles were counted at different times throughout the challenge assays, in order to evidence phage replication and active adsorption at both temperatures evaluated. Evolution of phage counts was similar for phages DT1 and DT6. For both phages, free and total counts were significantly different (Fig. 1). Although bacteriophage counts using DH5α increased early (2 h) and decreased afterwards (6 h), final counts (24 h) fell within the same order of magnitude at both temperatures assayed. A similar behavior was observed for both phages in experiments with O157:H7 STEC464 at 4 °C, whereas at 37 °C phage count increased one order of magnitude, being maximum at 24 h.

3.3. Efficiency of plating (EOP)

EOP is closely related to the inactivation level achieved by individual phages on *in vitro* challenge assays. Thus, this parameter was measured to evidence correlation with the biocontrol values achieved. EOP values when phages were counted on pathogenic *E. coli* strains with respect to *E. coli* DH5α (reference strain) were always <1 (Table 3). Phage/strain systems DT1/EPEC920, DT1/O157:H7 STEC464, DT6/EPEC920 and DT5/non-O157 STEC ARG4827 showed the lowest EOP values, ranging from 5.70×10^{-2} to 2.43×10^{-1}. Regarding phage DT6, EOP values on both STEC strains evaluated were very close to 1.

Table 2
Reduction of *Escherichia coli* viable cells after *in vitro* treatment at 4 °C with either individual phages or a cocktail thereof (1:1 ratio).

E. coli strain	Phage	MOI	*E. coli* inactivation (log_{10} CFU ml^{-1})[a,b] at different times of phage treatment		
			2 h	6 h	24 h
DH5α	DT1	1.1×10^2	[1]1.3 ± 0.2	[1]3.3 ± 0.5	[1]1.6 ± 0.4
	DT6	1.2×10^2	[1]1.1 ± 0.3	[1]3.4 ± 0.5	[2]2.1 ± 0.3
	Cocktail	1.1×10^2	[1]1.3 ± 0.2	[1]3.4 ± 0.4	[2]2.1 ± 0.4
EPEC920	DT1	4.5×10^1	[1]1.1 ± 0.2	[2]3.0 ± 0.2	[1]1.4 ± 0.3
	DT6	5.2×10^1	[0]0.3 ± 0.3	[1]1.9 ± 0.4	[1]1.8 ± 0.4
	Cocktail	4.8×10^1	[1]1.0 ± 0.3	[3]3.8 ± 0.5	[1]1.6 ± 0.3
Non-O157 STEC (ARG4827)	DT5	3.1×10^2	[0]0.6 ± 0.4	[1]1.6 ± 0.3	[1]1.7 ± 0.3
	DT6	1.6×10^2	[1]1.2 ± 0.4	[2]3.5 ± 0.5	[1]2.3 ± 0.2
	Cocktail	2.3×10^2	[1]1.2 ± 0.3	[2]3.5 ± 0.4	[1]1.8 ± 0.4
O157:H7 STEC464	DT1	1.0×10^2	[1]1.6 ± 0.2	[1]1.9 ± 0.4	[1]1.3 ± 0.3
	DT6	1.1×10^2	[1]1.2 ± 0.3	[1]2.3 ± 0.3	[0]0.3 ± 0.3
	Cocktail	1.0×10^2	[1]1.3 ± 0.2	[1]2.1 ± 0.4	[0]0.2 ± 0.2

MOI: multiplicity of infection. Identical superscripts indicate phage treatments not significantly different at $p = 0.05$.
[a] With respect to phage free control.
[b] Mean value of three determinations ± standard deviation.

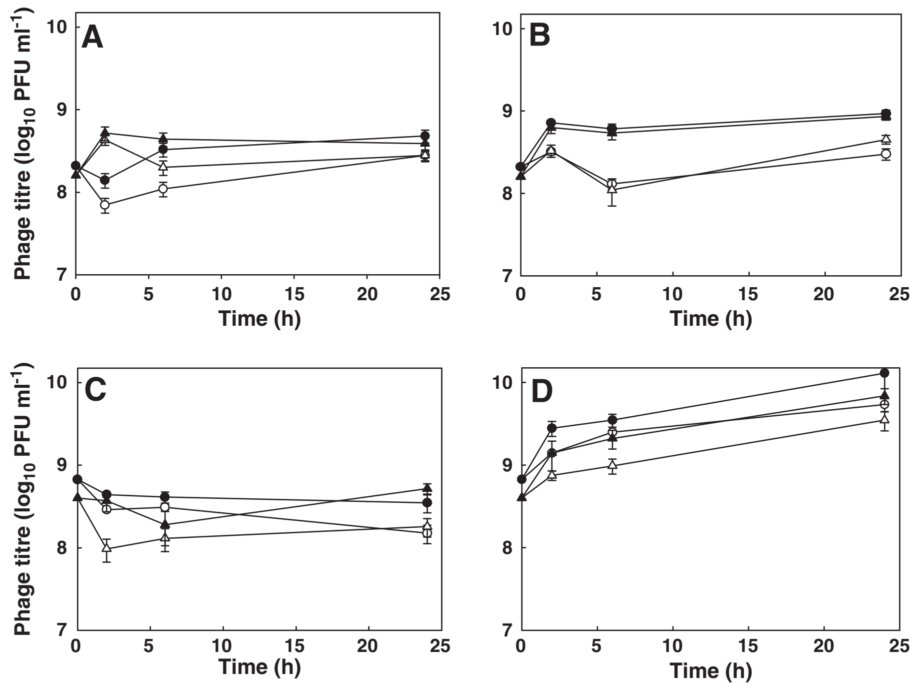

Fig. 1. Evolution of phage titre (PFU ml^{-1}) with *Escherichia coli* DH5α at 4 °C (A) and 37 °C (B) and *Escherichia coli* O157:H7 STEC464 at 4 °C (C) and 37 °C (D). (DT1: ● = total phage; ○ = free phage and DT6: ▲ = total phage; Δ = free phage). Error bars represent standard deviation of three determinations.

3.4. Hard surface decontamination using phage cocktails

3.4.1. Glass coverslips (GC) decontamination assays

Biocontrol experiments carried out on GC resulted in rapid and complete clearance, up to ca. 5 log CFU ml^{-1}, of *E. coli* EPEC920 (Fig. 2A and B) and *E. coli* O157:H7 STEC464 (Fig. 2A and B) at the different temperatures (4 °C and 37 °C) and MOI (1.8×10^4 and 1.8×10^6 for EPEC920; 1.2×10^5 and 1.2×10^7 for O157:H7 STEC464) tested. However, O157:H7 STEC464 cells were observed at 4 °C and low MOI after 24 h, though still at moderately lower concentrations than in control samples. *E. coli* non-O157 STEC ARG4827 was significantly reduced after 1 h and 3 h (Fig. 2A and B) at both 4 °C and 37 °C. Unexpectedly, this strain seemed to be better inactivated by lower MOI, except at 37 °C after 24 h where no difference was found (Fig. 2A and B). In addition, inactivation of *E. coli* remained constant after 24 h at 4 °C, but dropped at 37 °C, showing differences of up to 1.5 log CFU ml^{-1}. After 24 h of incubation, controls showed slightly lower values regarding initial bacterial inoculum at 4 °C, whereas at 37 °C there was active bacterial growth (Fig. 2). Dry controls were not significantly different from standard controls, recovering ca. 90% of viable cells.

3.4.2. Stainless steel coupons (SSC) decontamination assays

Treatment of experimentally contaminated SSC with phage cocktails significantly reduced ($p \leq 0.05$) the number of viable pathogenic *E. coli* cells recovered (Fig. 3); inactivation was complete (ca. 6 log CFU ml^{-1} reduction) for EPEC920 (Fig. 3A and B), non-O157 STEC ARG4827 (Fig. 3A and B) and O157:H7 STEC464 (Fig. 3A and B) using two different MOI levels (ca. 10^3 and 10^5) at 4 °C and 37 °C. Disregarding the MOI used, EPEC920 cells were detected at 37 °C after 24 h, nevertheless accomplishing similar (5.4 log CFU ml^{-1} at MOI = 1.8×10^3) and better (5.1 log CFU ml^{-1} at MOI = 1.8×10^5) inactivation than at shorter incubation times. Also, biocontrol at 37 °C of O157:H7 STEC464 at low MOI (9.3×10^3) was less effective after 24 h, although a reduction of 1.2 log CFU ml^{-1} was still observed. In addition, complete inactivation of *E. coli* non-O157 STEC ARG4827 was rapidly achieved (≤3 h) at 37 °C (at low and high MOI) and at 4 °C and high MOI, whereas at 4 °C and low MOI, 24 h was needed to attain a complete pathogen clearance (Fig. 3A). Standard and dry controls did not differ at either temperature tested.

4. Discussion

All the bacteriophages analyzed in this work were stable at 4 °C within 2 months, which is important if phages are intended to be incorporated in commercial phage cocktails. While the study was conducted for a relatively short period of time, results agree with previous studies carried out either in broth (Skurnik, Pajunen, & Kiljunen, 2007) or with phages inoculated on food (Guenther, Huwyler, Richard, & Loessner, 2009). Similar stability at 4 °C was also found for phages infecting other bacterial species (Merabishvili et al., 2009).

On *in vitro* challenge tests carried out at 37 °C, viable cell reductions observed were similar to those obtained for biocontrol of *Enterobacter sakasakii* (Kim, Klumpp, & Loessner, 2007) and *Staphylococcus aureus* (Garcia, Madera, Martinez, & Rodriguez, 2007), and higher than those achieved by O'Flynn, Ross, Fitzgerald, and Coffey (2004) for *E. coli* O157:H7 at MOI of 100. In addition, all the systems evaluated showed great lytic activity at short sampling times and bacterial regrowth afterwards. One explanation for this behavior might be that bacterial growth at 37 °C may promote phenotypic variability in O-antigen expression

Table 3
Efficiency of plaquing (EOP) of bacteriophages on pathogenic *Escherichia coli* strains.

Phage	*E. coli* strain	EOP[a]
DT1	DH5α	$1 \pm 4.5 \times 10^{-2}$
	EPEC920	$2.4 \times 10^{-1} \pm 3.1 \times 10^{-2}$
	O157:H7 STEC464	$2.1 \times 10^{-1} \pm 5.8 \times 10^{-2}$
DT5	DH5α	$1 \pm 8.1 \times 10^{-2}$
	Non-O157 STEC ARG4827	$5.7 \times 10^{-2} \pm 3.8 \times 10^{-2}$
DT6	DH5α	$1 \pm 7.3 \times 10^{-2}$
	EPEC920	$1.1 \times 10^{-1} \pm 6.8 \times 10^{-2}$
	O157:H7 STEC464	$9.7 \times 10^{-1} \pm 1.1 \times 10^{-1}$
	Non-O157 STEC ARG4827	$7.9 \times 10^{-1} \pm 1.6 \times 10^{-1}$

[a] EOP values referred to *E. coli* DH5α reference strain. Mean value of three determinations ± standard deviation.

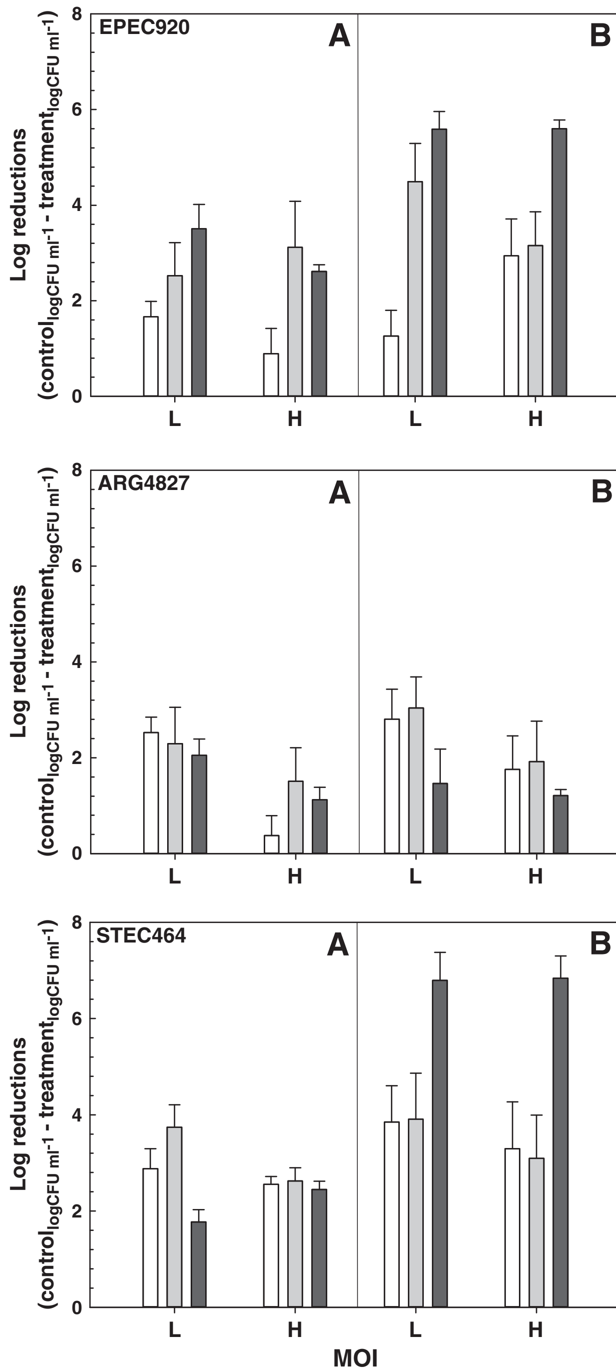

Fig. 2. Difference on *Escherichia coli* viable counts (log CFU ml^{-1}) after phage cocktail treatment (DT1 + DT6 for EPEC920 and STEC464; DT5 + DT6 for ARG4827; 1:1 ratio) using high (H; ca. 10^6) and low (L; ca. 10^4) MOI at 4 °C (A) and 37 °C (B) during 1 (□), 3 (■) and 24 (■) h of biocontrol on glass coverslips. Reported values indicate differences with respect to controls without phages and are expressed as the mean of three determinations ± standard deviation.

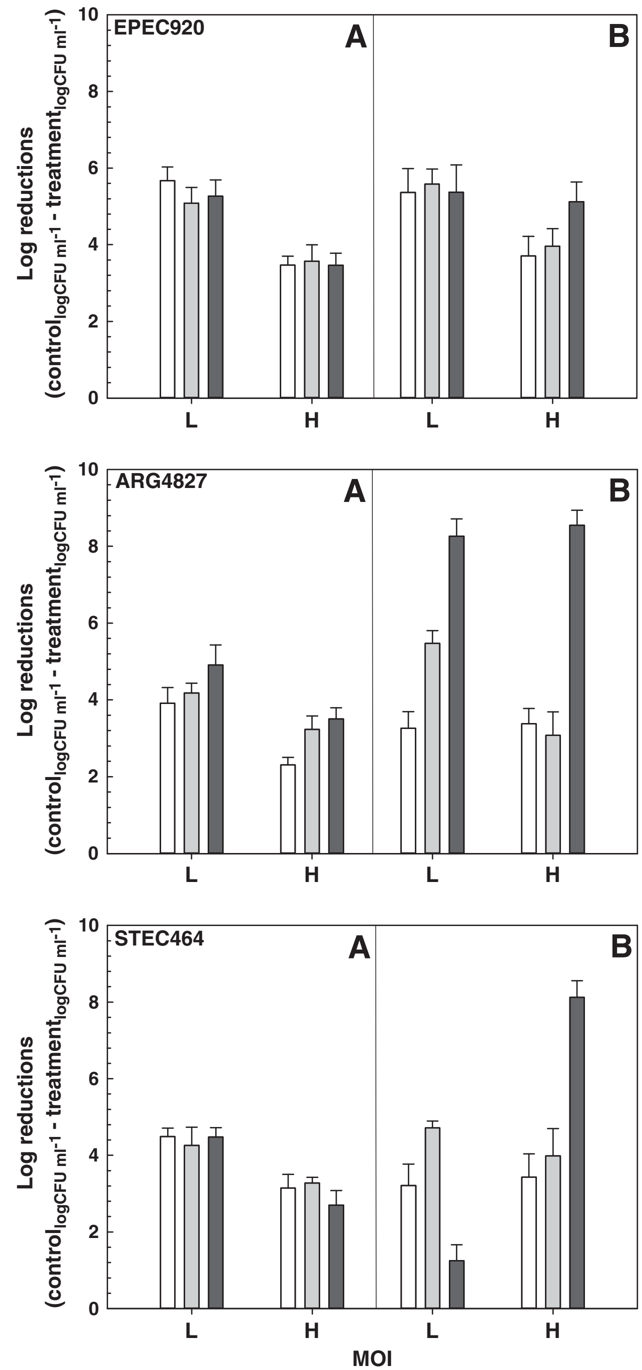

Fig. 3. Difference on *Escherichia coli* viable counts (log CFU ml^{-1}) after phage cocktail treatment (DT1 + DT6 for EPEC920 and STEC464; DT5 + DT6 for ARG4827; 1:1 ratio) using high (H; ca. 10^5) and low (L; ca. 10^3) MOI at 4 °C (A) and 37 °C (B) during 1 (□), 3 (■) and 24 (■) h of biocontrol on stainless steel coupons. Reported values indicate differences with respect to controls without phages and are expressed as the mean of three determinations ± standard deviation.

and consequently survival and secondary growth of cells resistant to phage infection (Dodds, Perry, & McDonald, 1987). Indeed, factors contributing to phage resistance include changes in the lipopolysaccharide fraction and point mutations causing complete loss or changes on proteinaceous receptors. However, these alterations provide bacteria with just a temporary advantage, since they could be detrimental in the absence of phages.

Both the frequency of the occurrence and the stability of bacteriophage insensitive mutants (BIM) were also analyzed (unpublished data). BIM occurrence was similar to the normal frequency of point mutations (from 6.5×10^{-7} to 1.3×10^{-6}; data not shown), whereas greater frequencies were reported for temperate phages (about 10^{-4}, Garcia et al., 2007). In addition, the stability of all BIMs was variable, in agreement with previous findings (O'Flynn et al., 2004). For all the phages studied, the frequency of BIM occurrence was low enough to recommend the use of phages as biocontrol tools. O'Flynn et al. (2004) also observed a subsequent bacterial regrowth between 1 h and 4 h after phage treatment at 37 °C at MOI similar to those used in our

study. Furthermore, while significant phage biocontrol of *E. coli* O157:H7 was achieved at short sampling times, regrowth after 24 h was also reported (Kudva, Jelacic, Tarr, Youderian, & Hovde, 1999). *E. sakasakii*, another enterobacteria, also exhibited secondary growth at different phage concentrations, though at earlier sampling times (Kim et al., 2007).

When treatments' effectiveness was compared, phage cocktails evidenced equal or higher efficacy in 10 out of 12 assays; in 4 of these cases, phage cocktails were significantly more effective than the second best treatment. Other authors also had better results by using phage mixtures instead of individual phages (Kudva et al., 1999; O'Flynn et al., 2004).

Temperature proved to influence phage lytic activity; inactivation attained for most systems at 4 °C was quite good, but lower than at 37 °C. O'Flynn et al. (2004) obtained poor biocontrol of *E. coli* O157:H7 using a cocktail of three bacteriophages at 12 °C, but inactivation was complete when cultures were infected with a cocktail of three phages at 4 °C (Kudva et al., 1999), similar to that observed in this study.

Moreover, phage replication during *in vitro* challenge experiments was evidenced with both a non-pathogenic and a pathogenic strain, at 4 °C and 37 °C. Significant differences found between total and free phage counts indicated active phage adsorption. On DH5α, a slow-growing strain if compared to pathogenic ones, phage replication was observed only at 37 °C, but the same behavior was observed in the pathogenic strains. Raya et al. (2006) found similar results for CEV1 type phages at 37 °C and low MOI; an initial phage decrease was followed by a subsequent increase of ca. 2 PFU ml^{-1}. Besides, Kudva et al. (1999) found a 10-fold increase in the number of phages at 37 °C, while it remained constant at 4 °C (MOI = 10^3), suggesting that phage replication occurs only at temperatures allowing bacterial growth. In addition, Chibani-Chennoufi et al. (2004) obtained 4 log PFU ml^{-1} increases for *E. coli* phages at 37 °C and a MOI low enough (0.1) to allow for more than one replication cycle.

Machinery surfaces and utensils such as meat grinders, cutting boards and knives, routinely used in food industry, represent potential routes of contamination with pathogenic bacteria such as *E. coli*, *Salmonella* spp. and *Listeria monocytogenes* (Abuladze et al., 2008), due to either poor hygiene or cross contamination. In this context, our results are promising, as phage cocktails significantly reduced the levels of pathogenic *E. coli* strains on two different inanimate surfaces (glass coverslips and stainless steel coupons) experimentally contaminated, at both temperatures assayed. On glass coverslips, a complete bacterial elimination was achieved in most systems, but non-O157 STEC ARG4827 and O157:H7 STEC464 were still detected at 4 °C and reduction of the former at 37 °C reached only 1.5 log UFC ml^{-1}. Similar results were found by Abuladze et al. (2008), who reported a 4 log orders (99.99%) inactivation of a mixture of *E. coli* O157:H7 strains by using a cocktail of three lytic bacteriophages (10^{10} PFU ml^{-1}) on glass coverslips.

On the other hand, all the *E. coli* strains tested were completely eliminated from stainless steel coupons at either 4 °C or 37 °C. However, EPEC920 and O157:H7 STEC464 cells were detected after 24 h of incubation at both high and low MOIs, respectively. Reduction of EPEC920 cells was very good at both MOIs evaluated, whereas for O157:H7 STEC464 it was of only 1.2 log CFU ml^{-1}. Viazis et al. (2010) obtained comparable results on stainless steel coupons at both 4 °C and 37 °C. While some studies reported an effective pathogen inactivation on food processing surfaces at a MOI of 10^3 (Abuladze et al., 2008; Hibma, Jassim, & Griffiths, 1997), Sharma et al. (2005) found only moderate biocontrol levels on stainless steel coupons at 4 °C using a MOI of 10^5. Regarding phage biocontrol of *L. monocytogenes*, another relevant food pathogen, encouraging results were obtained on contaminated stainless steel (Roy, Ackermann, Pandian, Picard, & Goulet, 1993; Soni & Nannapaneni, 2010).

EOP values were somewhat lower than 1 only for phage DT6, especially against the enteropathogenic strain. However, no correlation was found between EOP values and the inactivation level achieved by individual phages on *in vitro* challenge assays, though on glass coverslip trials, non-O157 STEC ARG4827 strain, which produced the lower EOP value with DT5, showed the lowest bacterial reductions at both MOIs evaluated. Low EOP values obtained may be due to host characteristics such as masking produced by the O-antigen of lipopolysaccharide (LPS), which affect phage binding as well as DNA injection. The foregoing, together with the presence of restriction endonucleases, may cause EOP values to oscillate considerably (Kutter, 2009). In addition, DH5α, used as reference strain in our experiments, possesses a truncated LPS, thus being more accessible to phage attack compared to STEC and EPEC strains evaluated. Therefore, the reduced EOP observed on pathogenic strains may be related to LPS, in addition to other bacterial characteristics mentioned above.

It is noteworthy that studies carried out to date in both food and solid surfaces are specifically focused on *E. coli* strains belonging to the O157:H7 serogroup. Foodborne pathogens such as EPEC and non-O157 STEC are also found, in addition to food, in food processing environments (FDA, 2012). The phages used in this study are lytic for *E. coli* strains belonging to different serogroups (non-O157 and O157) and virotypes (STEC and EPEC), thus being useful to control a wider range of pathogens potentially found on food processing surfaces. Accordingly, this novel phage-mediated strategy would be the first one focused on the use of bacteriophages to reduce high-level contamination of food processing surfaces with EPEC and non-O157 STEC strains.

5. Conclusions

This study demonstrated that phages achieved a high killing activity against pathogenic *E. coli* strains on *in vitro* challenge tests. In addition, it can be concluded that phage cocktails are an effective tool for controlling pathogenic *E. coli* strains on food processing surfaces.

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