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Editor's Choice

ORIGINAL ARTICLE

Phage biocontrol of enteropathogenic and Shiga toxin-producing *Escherichia coli* during milk fermentation

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Significance and Impact of the Study: Coliphages DT1 and DT6, isolated from faeces and selected on the basis of their host range, showed to be valuable tools for the control of pathogenic *Escherichia coli* during milk fermentation, without compromising the starter culture performance. Both phages, either individually or as a cocktail, may function as an extra safety barrier beyond traditional pasteurization, effectively reducing O157:H7 Shiga toxin-producing *Escherichia coli* (STEC) counts during early growth, thus avoiding Shiga toxin production and accumulation.

Keywords
bacteriophage, *Escherichia coli*, milk fermentation, O157:H7 STEC, phage biocontrol.

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Abstract

Two bacteriophages, isolated from faeces, were assayed as biocontrol agents of pathogenic *Escherichia coli* during milk fermentation. Phage DT1 was tested on the strain *E. coli* DH5α, one enteropathogenic *E. coli* (EPEC) strain and one Shiga toxigenic *E. coli* O157:H7 (STEC) strain. Phage DT6 was tested on two STEC strains (O157:H7 and non-O157). One additional assay was performed by using a cocktail of both phages against the O157:H7 STEC strain. *Streptococcus thermophilus* 10-C, the strain used as lactic starter, reached 10^9 CFU ml^{-1} after 4 h, while pH values fell to 4·5 after 8 h, regardless of the presence of *E. coli* strains and/or phages. In absence of phages, *E. coli* strains reached 4–6 log CFU ml^{-1} at 5–6 h. *Escherichia coli* DH5α and O157:H7 STEC strains were rapidly and completely inactivated by phage DT1 and phage cocktail, respectively, while O157:H7 STEC was completely inactivated either by DT1 or by DT6, after 8 h. The EPEC strain was not detected at 1 h (<10 CFU ml^{-1}) but grew afterwards, though at lower rates than without phage. For non-O157:H7 STEC, reductions lower than 1 log CFU ml^{-1} were observed for all sampling times. Phages DT1 and DT6, either individually or as a cocktail, effectively reduce O157:H7 STEC counts during milk fermentation, without compromising the starter culture performance.

Introduction

The incidence of foodborne pathogens such as *Salmonella* spp., *Shigella* spp. and *Escherichia coli* continues to increase considerably in many countries (Rivas *et al.* 2008; Farrokh *et al.* 2012). Particularly, Shiga toxin-producing *Escherichia coli* (STEC) are human pathogens that can cause diarrhoea, as well as severe clinical diseases including haemorrhagic enterocolitis, haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (Su and Brandt 1995; Griffin *et al.* 2002). Moreover, the *e*nterocyte *a*ttaching-and-*e*ffacing lesion gene (*eaeA*) present in enteropathogenic strains (EPEC) could contribute to the virulence of STEC (Frankel *et al.* 1998). Recent epidemiological studies showed that there is a sustained global increase in the isolation of emerging non-O157 STEC serogroups responsible of infection: *E. coli* O26, O103, O111, O121, O45 and O145 (Mathusa *et al.* 2010). In Argentina, HUS is endemic, with an annual rate incidence of 13·9 cases per 100 000 children under 5 years of age, as informed by Hospital Nephrology Units (NCASP 1995; Roldán *et al.* 2007; Governmental Agency of Control 2012).

Ground beef is still the most frequent source of *E. coli* outbreaks (Vugia *et al.* 2009), but dairy products can be directly contaminated by cattle faeces during milking process as well (Fremaux *et al.* 2008). Epidemiological studies on this matter led to the isolation of *E. coli* O157:H7 from milk handling pipes and bottling machines in dairy plants, indicating that inadequate pasteurization or postpasteurization contamination may have caused the outbreak. As a consequence, many studies have emphasized the design and application of several complementary strategies to reduce the incidence of these foodborne diseases related to dairy products (Viazis and Diez-Gonzalez 2011). Due to the high heat sensitivity of these pathogens, thermal treatments are the most widely applied in order to inactivate them when present in raw milk. However, the problem can not be solved if postpasteurization contamination occurs, since the survival of *E. coli* O157:H7 after 28 days in milk at 5°C (Wang *et al.* 1997) and after 21 days in cheese whey at 4, 10 or 15°C (Marek *et al.* 2004), was demonstrated. Consequences might be even worse if we consider that pasteurization temperatures were not validated for free Shiga toxin (Rasooly and Do 2010), which requires 5 min at 100°C for inactivation. Thus, the possible persistence of the heat-resistant toxin reinforces the need for a method to control the pathogen during early growth in order to block its production. Antibiotics are the most common alternative, but are restricted for application in foods. Taking into consideration the increasing antibiotic resistance of some pathogens, natural strategies such as phage control seem promising. Although reported effectiveness was variable, many studies have reported the isolation and use of *E. coli* bacteriophages as biocontrol tools in several food matrices, including meat and vegetables (O'Flynn *et al.* 2004; Abuladze *et al.* 2008; Viazis *et al.* 2011). Furthermore, the use of phage cocktails was highlighted as an enhanced tool when *E. coli* isolates exhibited resistance to some, but not all studied phages (Kudva *et al.* 1999; Viazis and Diez-Gonzalez 2011). Beyond the advantages of natural origin and high specificity, the ability of phages to act constantly, even during the manufacture process and food storage, make them a very interesting tool to be considered.

In this study, two bacteriophages isolated from faeces and selected on the basis of their host range were studied for their capability as biocontrol agents of pathogenic *Escherichia coli* during milk fermentation.

Results and discussion

Isolation, specificity and characterization of bacteriophages

Two bacteriophages (DT1 and DT6) obtained from diarrhoeic stool samples, which formed clearly defined plaques and exhibited different host ranges (Table 1), were selected for the experiments. Concentration of working stocks was $1{\cdot}2 \times 10^{10}$ PFU ml^{-1} and $4{\cdot}8 \times 10^{10}$ PFU ml^{-1} for phages DT1 and DT6, respectively.

Table 1 Host range of phages DT1 and DT6

Phage	*Escherichia coli* sensitive strains	Total sensitive strains
DT1	Two EPEC; one O157:H7 STEC; one non-O157 STEC; two UDEC	6
DT6	Four EPEC; six O157:H7 STEC; three non-O157 STEC; three UDEC	16

EPEC, enteropathogenic *E. coli*; non-O157 STEC, shiga toxigenic non-O157 *E. coli*; O157:H7 STEC, O157:H7 shiga toxigenic *E. coli*; UDEC, uncharacterized diarrheaogenic *E. coli*.

Awareness of the presence of virulence factors and phage-encoded genes is a prerequisite for phages to be included in food products for biocontrol purposes, since bacterial phenotype may be modified by phage gene expression (Miller and Day 2008). PCR amplification of major virulence factors (Stx1; Stx2; ST1; LT1 and Intimin) was negative for bacteriophages DT1 and DT6 (data not shown) making them suitable candidates for biocontrol applications. However, further sequencing and bioinformatic analysis are required to ensure that phages are free from other harmful factors and, in consequence, completely safe for use as biocontrol of pathogens in food. According to electron micrographs, phages DT1 and DT6 could be classified as T-even type of the *Myoviridae* family. Phages DT1 and DT6 had icosahedral heads and contractile tails. DT1 dimensions were of $89{\cdot}3 \pm 2{\cdot}2$ nm (head diameter), $127{\cdot}8 \pm 2{\cdot}3$ nm (tail length), $20{\cdot}8 \pm 1{\cdot}0$ nm (tail thickness) and $217{\cdot}1 \pm 4{\cdot}3$ nm (total length). DT6 measures were of $82{\cdot}1 \pm 1{\cdot}5$ nm (head diameter), $125{\cdot}7 \pm 2{\cdot}0$ nm (tail length), $17{\cdot}7 \pm 2{\cdot}1$ nm (tail thickness) and $207{\cdot}8 \pm 3{\cdot}1$ nm (total length) (Fig. 1).

The 35 non-*E. coli* and nonpathogenic *E. coli* strains tested for assessing the host range were resistant to the lytic action of both phages, while all O157:H7 strains, most non-O157 STEC and some EPEC isolated from diarrhoeic faeces or food, were sensitive to one or both bacteriophages (Table 1). Although these findings encourage the use of the phages for food conservation without altering the gastrointestinal tract normal flora, a broader host range needs to be assessed to ensure safety for commensal bacteria, since no correlation between nonpathogenic *E. coli* and phage sensitivity has been reported by other authors. Although prophage induction is usually related to the expression of virulence factors and many phage-encoded virulence genes exist as well, no correlation between *E. coli* strains lacking virulence genes and phage sensitivity has been reported. Despite the reported

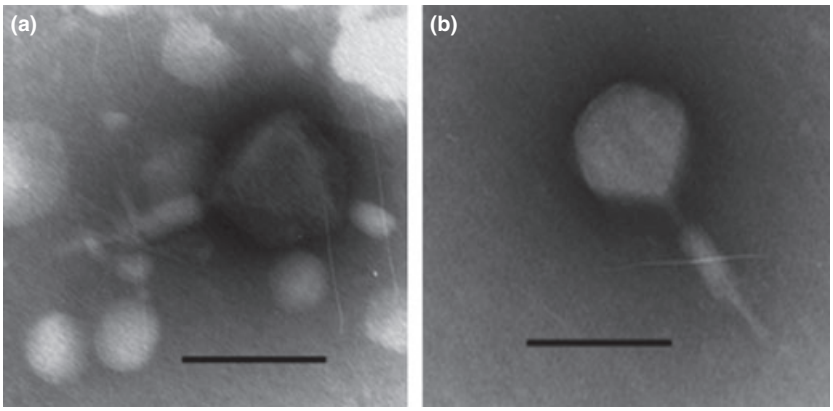

Figure 1 Electron micrographs of phages DT1 (a) and DT6 (b) negatively stained with 2% phosphotungstic acid. Bars represent 100 nm.

occurrence of O157-specific bacteriophages able to lyse all of the *E. coli* O157 (and none of the non-O157 *E. coli* or non-*E. coli*) strains tested (Kudva *et al.* 1999), no correlation was found between serogroups (O157 and non-O157) and phage sensitivity/resistance phenotype in our study. Likewise, other authors found phages to be species-specific, but usually capable of infecting more than one single serogroup (e.g. the virulent phage P100 can infect most *Listeria monocytogenes* strains; Carlton *et al.* 2005). On the other hand, one of the main drawbacks on the use of bacteriophages as biocontrol agents is their ability to lysogenize bacteria. In this study, phages DT1 and DT6 produced clear plaques, suggesting a lytic phenotype. In addition, BIMs (bacteriophage insensitive mutants) were isolated for both phages at a frequency similar to the normal occurrence of point mutations (ranging from $6{\cdot}5 \times 10^{-7}$ to $1{\cdot}3 \times 10^{-6}$) (Tomat, D., Mercanti, D., Balague, C., Quiberoni, A., unpublished data), whereas frequencies reported for temperate phages are much higher (about 10^{-4}, Garcia *et al.*, 2007). Besides, all of our BIMs reverted to phage sensitivity and no lysis plaques could be recovered after exposition to UV light, additional evidence of the incapability of the phages to lysogenize bacteria (Tomat, D., Mercanti, D., Balague, C., Quiberoni, A., unpublished results).

Biocontrol tests during milk fermentation

Phages DT1 and DT6, either alone or mixed in a cocktail, were evaluated for their efficiency to inhibit the growth of *E. coli* during milk fermentation. Phage DT1, with a narrower host range, was tested on the strains DH5α, EPEC920 and O157:H7 STEC464, whereas phage DT6 was tested on the two STEC strains (O157:H7 and non-O157). One additional assay was conducted by using a cocktail of DT1 + DT6 phages against the strain O157:H7 STEC464. The growth of *Streptococcus thermophilus* 10-C

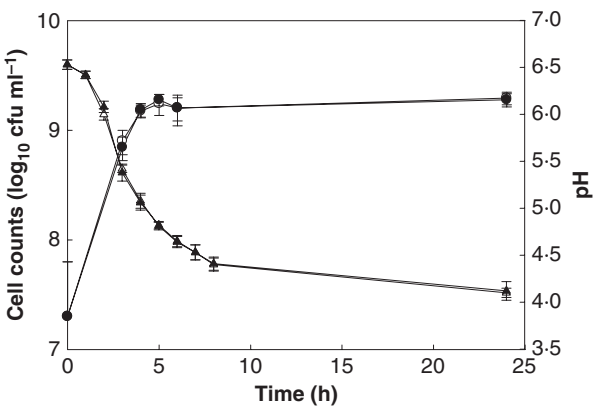

Figure 2 Evolution of *Streptococcus thermophilus* with O157:H7 STEC (464) in absence (○) and presence (●) of phage cocktail, and pH evolution with O157:H7 STEC (464) in absence (Δ) and presence (▲) of phage cocktail. Error bars represent the standard deviation of three determinations.

was similar in all the experiments, as well as the decrease of pH, regardless the presence of *E. coli* strains and phages tested (Fig. 2). *Strep. thermophilus* reached 10^9 PFU ml^{-1} after 4 h and maintained this level all throughout the experiment (24 h) (Fig. 2). The pH values evolved accordingly, falling to 4·5 at 8 h and 4·0 at the end of the fermentation process (24 h) (Fig. 2).

Excluding the phage cocktail (DT1 + DT6), which showed a slight but constant reduction (1·6 log PFU ml^{-1} after 24 h) (Fig. 3d), all phage titres remained constant (Fig. 3a,c,e) or increased slightly (0·5 log PFU ml^{-1}) (Fig. 3b,f) throughout the first 8 h, with a subsequent decrease between 8 and 24 h (1–2 log PFU ml^{-1}). The low pH and the accumulated lactic acid might be related to the partial phage inactivation observed at the final of the fermentation process. Regarding *E. coli* strains, phage DT1 and phage cocktail rapidly (<1 h) and completely inactivated DH5α [multiplicity of infection (MOI) = $2{\cdot}3 \times 10^5$]

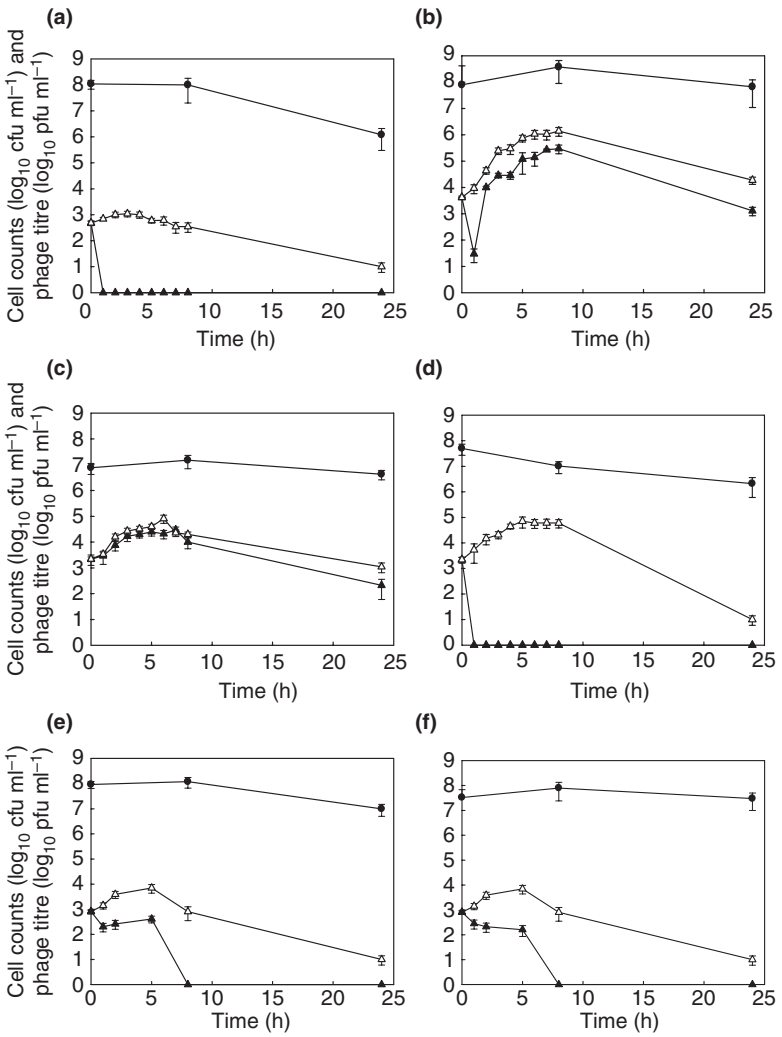

Figure 3 Evolution of phage titre (●) and *Escherichia coli* viable counts in absence (Δ) and presence (▲) of phage during production of fermented milk. DH5α/DT1 (a), EPEC920/DT1 (b), non-O157 STEC (ARG4827)/DT6 (c), O157:H7 STEC464/cocktail (d), O157:H7 STEC464/DT1 (e) and O157:H7 STEC464/DT6 (f) systems. Error bars represent the standard deviation of three determinations.

and O157:H7 STEC (MOI = $2{\cdot}2 \times 10^4$), respectively (Fig. 3a,d). *Escherichia coli* O157:H7 STEC464 was completely inactivated by either phage DT1 or phage DT6 at 8 h (Fig. 3e,f). *Escherichia coli* EPEC920 was not detected at 1 h (<10 CFU ml^{-1}) after infection with phage DT1 at a MOI of $2{\cdot}4 \times 10^4$ but showed a subsequent regrowth, although always lower than the control without phage, and yielding a reduction of 1·1 log CFU ml^{-1} after 24 h (Fig. 3b). For non-O157:H7 STEC ARG4827 (MOI = $3{\cdot}5 \times 10^3$), reductions lower than 1 log CFU ml^{-1} were obtained for all sampling times (Fig. 3c). In control assays without phages, all the *E. coli* strains reached 4–6 log CFU ml^{-1} after 5–6 h of incubation (Fig. 3).

Regardless the presence of phages, a decrease on the viability of *E. coli* strains was observed as a result of the low pH attained during the acidification, resulting DH5α (1·5 log CFU ml^{-1} at 24 h) and O157:H7 STEC464 (2·3 log CFU ml^{-1} at 24 h) the most affected ones. Even if acidity is known to affect growth and survival of STEC and EPEC strains, the pH values reached during milk fermentation are not enough to assure their inactivation (Farrokh *et al.* 2012). Moreover, acid adaptation to nonlethal pH has been suggested to enhance *E. coli* O157:H7 survival (Jordan *et al.* 1999). The antagonistic effect of organic acids, hydrogen peroxide and bacteriocins produced by dairy starters may also contribute to *E. coli* inhibition (Akpinar *et al.* 2011). Nevertheless, the inhibitory effect of low pH was only observed after a considerable *E. coli* multiplication occurred, when the risk of Shiga toxin accumulation is high. Consequently, and considering

our results, the use of phages DT1 and DT6, either individually or as a cocktail, could be a valuable tool to control *E. coli* early growth, especially O157:H7 STEC464, and therefore limiting toxin production and accumulation. It is worth noting that phages DT1 and DT6 were more effective as a cocktail than individually, as total inactivation of the pathogen O157:H7 STEC464 was achieved after 1 h of incubation in the first case, but required at least 8 h for individual phages. O'Flynn *et al.* (2004) also reported shorter inactivation times when using phage cocktails during *in vitro* challenge tests.

On the other hand, the pathogens EPEC920 and non-O157 STEC ARG4827 were not eliminated by phages DT1 and DT6, respectively. Successful phage infection and host killing is strongly dependent on MOI (Cairns *et al.* 2009). The MOI used for non-O157:H7 STEC ($3{\cdot}5 \times 10^3$) and EPEC ($2{\cdot}4 \times 10^4$) may be not high enough to achieve a full biocontrol, though reduction of viable cells counts of those strains would reduce the possibility of toxin production. Low MOI were tested during milk fermentation assays, but effectiveness was low and a subsequent bacterial regrowth was observed (preliminary results, not shown). Other authors largely agree in using high MOI to obtain large bacterial reductions on food matrices (e.g. MOI = 10^6, O'Flynn *et al.* 2004; 6×10^7 PFU/cm^2, Carlton *et al.* 2005), as treatments with lower MOI values eventually led to bacterial regrowth. O'Flynn *et al.* (2004) assayed specific phages for *E. coli* O157:H7 on nine meat pieces, seven of which were completely host free after enrichment, but MOI used in that study were as high as 10^6. Kim *et al.* (2007) found that phage concentration between 10^7 and 10^9 PFU ml^{-1} (MOI = 10^5 to 10^7) was able to significantly inhibit the growth of *Enterobacter sakazakii* in reconstituted infant formula, although in a concentration-dependent way, as lower phage concentrations resulted in *Enterobacter sakazakii* regrowth. Taking into account the foregoing and evaluating the encouraging results obtained with DH5α and O157:H7 STEC strains, phages might be further concentrated up to about 10^{12} PFU ml^{-1} using PEG8000 and CsCl gradients, to test whether or not higher MOI values allow complete non-O157:H7 STEC and EPEC inactivation. Nowadays, it is possible to achieve such high concentrations at industrial scale. Indeed EcoShield™, a phage-based product commercially produced in large scale by Intralytix (www.intralytix.com/Intral_Food.htm), is composed by three lytic phages at a final concentration of $2{\cdot}7 \times 10^{11}$ PFU/ml.

Although many authors have reported the use of bacteriophages to control various foodborne bacterial pathogens, either in animals or in food matrices (Viazis and Diez-Gonzalez 2011), the present study is to our knowledge the first one focused on the biocontrol of EPEC and STEC during a milk fermentation process. Phages DT1 and DT6 were effective to reduce *E. coli* counts without compromising the performance of *Streptococcus thermophilus*, used as starter culture. In this sense, as *Lactococcus lactis* is even more extensively used than *Streptococcus thermophilus* worldwide (Mills *et al.* 2011), it is interesting to think about the usefulness of phages DT1 and DT6 as biocontrol tools in milk fermented by these mesophilic bacteria, either alone or as part of mixed cultures (Harnett *et al.* 2011). However, some parameters like temperature evolution and pH decrease during the fermentation process must be considered. Regarding temperature, and taking into account results previously obtained in meat during *in vitro* biocontrol assays, efficiency of both phages DT1 and DT6 at 30°C was not significantly different than at 37°C (unpublished data). With respect to acidification, final pH of milk is within the same range (4·0–4·5) regardless of the starter used (*Streptococcus thermophilus* or *Lactococcus lactis*). Therefore, a similar phage performance is expected in both cases.

Results suggest that novel phages such as those isolated in this study may indeed be applicable to the control of problematic pathogenic *E. coli* in dairy products. In summary, this research highlights that coliphages have significant potential as antimicrobial agents in milk and may function as an extra defence beyond traditional pasteurization. Their widespread occurrence and low cost of isolation and production may lead to their future exploitation in the dairy industry.

Materials and methods

Bacterial strains and culture conditions

Escherichia coli DH5α was used as sensitive strain to propagate all the bacteriophages used in this study. Three additional strains were used in the biocontrol experiments. Two of them, *E. coli* enteropathogenic (EPEC920) (*eae*) and *E. coli* Shiga toxigenic O157:H7 (STEC464) (*stx2* and *eae*), were 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 (STEC) (ARG4827; serogroup O18; *stx1* and *stx2*) (Balagué *et al.* 2006). The *E. coli* 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, MI, USA) supplemented with $MgSO_4$ (5 mmol l^{-1}) (Cicarelli, San Lorenzo, Santa Fe, Argentina) (Hershey-Mg). The strain *Streptococcus thermophilus* 10-C was used as starter culture for milk fermentation (Suárez *et al.* 2002); it was routinely reactivated and grown overnight in Elliker broth (Biokar, Beauvais, France). All the strains were

maintained as frozen (−80°C) stock cultures in Hershey (*E. coli*) or Elliker (*Strep. thermophilus*) broth supplemented with 15% (v/v) glycerol.

Bacteriophages

Escherichia coli DH5α was used to isolate the bacteriophages from 50 stool samples of patients with diarrhoea treated at the Centenary Hospital, Rosario. A portion of faeces (5 g) was added to 10 ml of a DH5α culture (OD_{600} = 1·0) grown in Hershey broth and the culture was incubated at 37°C for 12 h. Next, 0·5 ml of chloroform (Cicarelli) was added and the preparation was mixed and centrifuged at 4000 ***g*** for 10 min. The supernatant was sterilized by filtration through a 0·45-μm pore filter (Gamafil S.A., Buenos. Aires, Argentina) (Kennedy and Bitton 1987). Bacteriophage isolations were performed 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 three 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). To isolate and purify phages, well-defined single plaques on the soft agar were picked and placed in 5 ml of Hershey-Mg broth (phage stock). High-titre phage suspensions were prepared as follows: Hershey-Mg broth was inoculated (1%, v/v) with an overnight culture of the recipient strain and aliquots of 100 μl of phage stocks were added. Incubation at 37°C with discontinuous shaking was performed until complete lysis. At that point, 0·1 ml of chloroform was added and cultures centrifuged at 4000 ***g*** for 10 min. Phage stocks were stored at 4°C and enumerated (plaque formation units per millilitre; PFU ml^{-1}) by the double-layer plaque technique (Jamalludeen *et al.* 2007).

Bacteriophage characterization

PCR amplification of virulence factors

Phages were tested for the presence of toxin-encoding genes (*stx1*, Shiga toxin 1; *stx2*, Shiga toxin 2; *eaeA*, attaching-and effacing; *LT1*, thermolabile toxin and *ST1*, thermostable toxin) of diarrheaogenic *E. coli* by the polymerase chain reaction (PCR) using primers detailed in Table 2. PCR conditions were as follows: initial denaturing step at 95°C for 2 min, followed by 25 cycles of 95°C for 30 s, annealing at 63°C for 30 s and elongation at 72°C for 30 s and a final elongation step at 72°C for 5 min. *Escherichia coli* ATCC43889 (*stx2* and *eaeA*), ATCC43890 (*stx1*) and ATCC43895 (*stx1*, *stx2* and *eaeA*, and also harbouring the *stx2* phage, 933W) were used as positive controls, while enterotoxigenic *E. coli* ATCC35401 was used for *LT1* and *ST1* genes. *Escherichia coli* HB101 and ATCC98222 were utilized as negative controls. Amplified products were resolved by electrophoresis using 3% agarose gels in TBE buffer (89 mmol l^{-1} Tris borate, 2 mmol l^{-1} EDTA, pH 8·0) (Promega, Madison, WI, USA) at 100 V for 3 h. Gels were stained with ethidium bromide (0·5 μg/ml) (Sigma, St. Louis, MO, USA) and PCR products were visualized under UV light.

Table 2 Sequences of primers used in this study

Gene	Primer* (Pass *et al.* 2000)	Product size (bp) expected
stx1	fp: 5′- ACGTTACAGCGTGTTGCRGGGATC-3′ bp:5′- TTGCCACAGACTGCGTCAGTRAGG-3′	121
stx2	fp: 5′- TGTGGCTGGGTTCGTTTATACGGC-3′ bp: 5′- TCCGTTGTCATGGAAACCGTTGTC-3′	102
eaeA	fp: 5′- TGAGCGGCTGGCATGATGCATAC-3′ bp: 5′- TCGATCCCCATCGTCACCAGAGG-3′	241
LT1	fp: 5′-TGGATTCATCATGCACCACAAGG-3′ bp: 5′-CCATTTCTCTTTTGCCTGCCATC-3′	360
ST1	fp: 5′-TTTCCCCTCTTTTAGTCAGTCAACTG-3′ bp: 5′-GGCAGGACTACAACAAAGTTCACAG-3′	160

*bp, backward primer; *eaeA*, intimin encoding gene; fp, forward primer; *LT1* and *ST1*, thermolabile and thermostable toxins encoding genes; S*tx1* and *stx2*, Shiga toxin1 and 2 encoding genes.

Electron micrographs

Phage electron micrographs were obtained by the procedure of Bolondi *et al.* (1995) using a JEOL 1200 EX II electron microscope (INTA Castelar, Buenos Aires, Argentina) operating at 85 kV. Phage morphologies and dimensions (head diameter, tail length and diameter) were recorded.

Host range of bacteriophages

The host range of each phage was determined by the double-layer agar technique using 70 strains isolated by streaking both stool samples (uncharacterized diarrheaogenic *E. coli*; UDEC) and urine cultures (uropathogenic *E. coli*; UPEC) in cystine lactose electrolyte–deficient agar plates. After incubation for 24 h at 35°C, lactose positive and citrate negative colonies were further identified using API-20E system. Sixteen *E. coli* strains from food (Balagué *et al.* 2006), one uropathogenic *E. coli* strain (*E. coli* T149) which expresses fimbriae P and α-haemolysin (Balagué *et al.* 2004) and five ATCC *E. coli* strains were also tested (ATCC 43890; 43889; 43895; 35401 and 98222). Previously characterized (API-20E system) isolates from stool samples were also tested: *Shigella flexneri*, *Shigella sonnei*, *Proteus mirabilis*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Salmonella enteritidis*, *Salmonella* Typhi and *Salmonella* Typhimurium. Strains tested against stock phages are listed in Table 3. Bacteriophage sensitivity was

Table 3 Strains tested against isolated phages

Source	Strains (No.)	Strain features/description
Food	*Escherichia coli* (16)	Eight non-O157 STEC (belonging to O8, O18, O44, O57 and O79 serogroups; Balagué *et al.* 2006); two O157:H7 STEC and six UDEC
	Escherichia coli (35)	Four O157:H7 STEC; five EPEC and 26 UDEC
	Escherichia coli (18)	Nonpathogenic (non-O157)
Stool samples	*Shigella* spp. (5) *Salmonella* spp. (5) *Proteus mirabilis* (4) *Citrobacter freundii* (1) *Klebsiella pneumoniae* (2)	Other enterobacteria
Urine culture	*Escherichia coli* (18)	UPEC (7 *fimH*+, *csgA*+, *papC*+; 5 *fimH*+, *csgA*+; 6 *fimH*+; all *bfp*-) All *papC*+ (fimbria type P) and one *papC*- (fimbria type 1) were mannose resistant
ATCC	*Escherichia coli* (5)	35401; 43889; 43890; 43895 and 98222

ATCC, American Type Culture Collection; EPEC, enteropathogenic *E. coli*; non-O157 STEC, shiga toxigenic non-O157 *E. coli*; O157:H7 STEC, O157:H7 shiga toxigenic *E. coli;* UDEC, uncharacterized diarrheaogenic *E. coli;* UPEC, urophathogenic *E. coli.*

assayed by placing 10 μl of phage suspension on the solidified soft-agar layer inoculated with 100 μl of each bacterial culture, incubated for 24 h at 37°C, and the presence of lysis zones or plaques was examined (Goodridge *et al.* 2003).

Biocontrol of Escherichia coli

During milk fermentation Biocontrol experiments were carried out at 37°C in parallel batches in sterile, commercial, reconstituted (10%, w/v) dry skim milk, added of $CaCl_2$ (0·28 g l^{-1}). All the batches were inoculated with overnight cultures of *Streptococcus thermophilus* 10-C at 1% (v/v), either alone (control assay) or together with overnight cultures of the tested *E. coli* strains (final concentration approx. 5×10^2–5×10^3 CFU ml^{-1}; experimental assays). One aliquot of each *E. coli*-infected batch was inoculated with the corresponding phage or phage cocktail (10^7–10^8 PFU ml^{-1}), so as to evaluate their potential as biocontrol agents, getting a MOI ranging from $3·5 \times 10^3$ to $2·5 \times 10^5$. The acidification proceeded during 24 h at 37°C in a thermostatic bath. During fermentation, changes in pH were assessed with a model SA 720 pH metre (Orion, Beverly, MA, USA), bacterial cell counts were performed in Elliker agar (37°C, 48 h) for streptococci or Hershey agar (37°C, 18 h) for *E. coli*, and phage enumeration (if applicable) was also carried out by the double-layer plaque titration method described above.

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