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ORIGINAL ARTICLE

Treatment of *in vitro* enterohemorrhagic *Escherichia coli* infection using phage and probiotics

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Abstract

Aims: To assay the combination of phage and probiotics against EHEC *in vitro* on infected Hep-2 cells.
Methods and Results: Phage and probiotics treatments on EHEC O157:H7-infected Hep-2 cells were assayed individually or combined. The effect of freeze-drying on phage and probiotic antimicrobial activity was also studied. While treatment with phage alone increased cell detachment caused by EHEC infection, the treatments with MM alone or in combination with phage proved to effectively diminish cell damage caused by EHEC infection. Combined treatment showed a decrease in apoptotic cell count of 57·3% and a reduction in EHEC adhesion to cell monolayer of 1·2 log CFU. The simultaneous use of phage and probiotics showed no antagonistic effect, and freeze-drying did not affect their antipathogenic activity.
Conclusions: The combination of phage and probiotics has great potential for reducing the number of pathogens adhered to epithelial cells during EHEC O157:H7 infection and attenuating the cytotoxic effect derived from it. Further *in vivo* assays are needed for assessing the actual effectiveness of the treatment.
Significance and Impact of the Study: This study presents a freeze-dried formulation of phage and probiotics capable of controlling EHEC infections and reducing epithelial cell damage *in vitro*.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) are bacterial pathogens, included in a larger group of pathogens known as Shiga toxin-producing *E. coli* (STEC), which are also capable of producing an attachment and effacing (A/E) lesion on epithelial cells.

Shiga toxins (Stx) are considered the main virulence factor of EHEC, exerting their cytotoxic effect by the inhibition of protein synthesis and induction of cell apoptosis on renal endothelium and intestinal epithelial cells (Doyle *et al.* 2001). A/E lesions are characterized by the erasure of microvilli and formation of pedestal like structures on intestinal epithelial cells where the pathogen is located and can inject effector proteins directly into the host cell.

EHEC are usually associated with haemorrhagic colitis outbreaks and represent the main trigger of haemolytic uremic syndrome (HUS) worldwide (Johnson and Taylor 2008). In particular, O157:H7 is the main serotype associated with haemorrhagic colitis and HUS outbreaks (Karmali *et al.* 2010; Masana *et al.* 2010). HUS affects mostly children under age 10 (Gamage *et al.* 2003) and is characterized by the production of acute renal failure, thrombocytopenia and microangiopathic haemolytic anaemia, with a mortality rate of 1–4% (Spinale *et al.* 2013).

Antibiotic treatment is contraindicated for *E. coli* O157:H7-infected children since there is evidence that it may enhance bacterial production of Stx (Kimmitt *et al.* 2000) and increase the risk of the development of HUS (Wong *et al.* 2000). Besides, there is a current trend of reducing the usage of antibiotics in order to control the

proliferation of resistant pathogens. Following this trend, alternative treatments including vaccines, antibodies to endotoxins, and micro-organisms with antimicrobial activity are being researched (Gu *et al.* 2009; Fernebro 2011; Rahal *et al.* 2012).

Phage therapy has proven to be efficient in the biocontrol of pathogenic *E. coli* (Chibani-Chennoufi *et al.* 2004; Tanji *et al.* 2005; Alam *et al.* 2011), and presents the advantage of using a self-replicating therapeutic agent which, unlike antibiotics, has high specificity and increased activity when the number of pathogens rises. Nonetheless, the use of lytic phage to kill bacteria with intracellular toxins has the disadvantage of producing a burst release of toxins during bacterial lysis (Paul *et al.* 2011).

Probiotics have also been successfully assayed for the control of *E. coli* O157:H7 infections (Ogawa *et al.* 2001; Shu and Gill 2002; Medellin-Pena and Griffiths 2009). Probiotic micro-organisms can execute their antipathogenic activity by several mechanisms such as maintaining intestinal epithelial integrity (Madsen *et al.* 2001), increasing mucin production and secretion from goblet cells (Mack *et al.* 2003; Ohland and MacNaughton 2010), production of bacteriocins (Corr *et al.* 2007; Oelschlaeger 2010; Arqués *et al.* 2015) and enhancing immunomodulatory effects (Tien *et al.* 2006; Boirivant and Strober 2007; Romanin *et al.* 2010). Some probiotics can also exhibit colonization resistance, which is the avoidance of pathogen binding through the formation of a protective barrier between the infecting organism and the epithelial cells (Fedorak and Madsen 2004; Eutamene and Bueno 2007; Jandu *et al.* 2009). The action mechanisms of probiotics against pathogens are completely different from those of phage, the latter showing an exponential reduction in the number of pathogens during the lytic cycle. Therefore, it is expected that late-stage infections are more likely to be controlled by phage than by probiotics.

In previous work, podophage CA933P isolated in our laboratory proved to be a promising candidate for the biocontrol of enterohemorrhagic *E. coli*. CA933P is capable of infecting certain strains of serotypes O145:H25, ONT:H12 and O13:H6 of Shiga toxin-producing *E. coli*, and can also infect enteroinvasive *E. coli*, *Shigella flexneri* 2, *Sh. flexneri* 3 and *Pseudomonas aeruginosa* (Dini and De Urraza 2010).

Probiotic *Lactobacillus plantarum* strain CIDCA 83114 isolated from kefir grains was also successfully assayed against EHEC pathogenesis *in vitro* (Hugo *et al.* 2008). A microbial mixture (MM) of five probiotic strains isolated from kefir grains (*Lact. plantarum, Lactococcus lactis, Lactobacillus kefir, Kluiveromices marxianus and Saccharomyces cerevisiae*), which includes strain CIDCA 83114, also exhibited antagonistic activity against pathogenesis of *Shigella sonnei* and *Clostridium difficile in vitro* and *in vivo* (Bolla *et al.* 2011, 2013a,b; Kakisu *et al.* 2013).

The aim of this work was to study the combined effect of phage CA933P and the probiotic microbial mixture (MM) *in vitro* on the attachment of EHEC O157:H7 to epithelial Hep-2 cells and its cytotoxic effect.

Materials and methods

Bacteriophage and bacterial strains

Bacterial strain EDL933 of EHEC O157:H7 (ATCC 700927), bacteriophage isolate CA933P (Dini and De Urraza 2010) and a probiotic microbial mixture (MM) (Bolla *et al.* 2011) were used for all the assays. MM is composed of bacterial and yeast strains isolated from kefir grains: *Lc. lactis* sub *lactis* CIDCA 8221, *Lact. plantarum* CIDCA 83114, *Lact. kefir* CIDCA 8348, *K. marxianus* CIDCA 8154 and *S. cerevisiae* CIDCA 8112. Probiotic strains were isolated, identified and characterized by Garrote *et al.* (2001) and Delfederico *et al.* (2006).

EHEC was cultured in LB medium (1% NaCl; 0·5% yeast extract; 1% tryptone) for 20 h at 37°C and 180 rev min^{-1} in an orbital shaker giving a final concentration of $4{\cdot}4 \times 10^8$ CFU ml^{-1} (OD600nm = 0·63).

Phage was propagated in LB medium using EHEC strain EDL933 as host as previously described (Dini and de Urraza 2013). The phage lysate was filtered through a 0·22-μm pore-size membrane, and phage titre was determined by the soft agar overlay method in LB medium (Kropinski *et al.* 2009). Phage lysate was diluted with LB medium to the concentration specified for each assay.

Probiotic strains (lactobacilli and yeasts) were grown in MRS-broth (Difco, Detroit, MI) for 48 h at 30°C. *Lactococcus lactis* was cultivated in 1 : 1 : 1 growth media (1% w/v of tryptone – Difco; 1% w/v of yeast extract – Biokar Diagnostic, Beauvais, France; and 1% w/v of lactose – Mann Research Laboratories, NY) for 24 h at 30°C. The same volume of each microbial suspension was centrifuged at 10 000 ***g*** for 15 min. Microbial mixture (MM) was obtained by resuspending both pellets together in 1 ml of the corresponding medium: Dulbecco's Modified Eagle's Medium (DMEM; GIBCO BRL Life Technologies, Rockville, MD) for fresh MM treatment, or UHT skim milk for freeze-drying, giving final bacteria and yeast concentrations of 10^9 and 10^6 CFU ml^{-1} respectively (Bolla *et al.* 2011).

Lyophilization of phage, probiotics and phage-probiotic mixture

The samples were prepared in sterile 1-ml glass vials as follows:

For phage freeze-drying, 20 μl of phage lysate (5×10^8 PFU ml^{-1}) in LB medium were added to 180 μl of sterile SM buffer (100 mmol l^{-1} NaCl; 8 mmol l^{-1} $MgSO_4.7H_2O$; 50 mmol l^{-1} Tris-HCl; 0·01% (w/v) gelatin; pH 7·5) with 0·1 mol l^{-1} sucrose.
MM vials were prepared by adding 20 μl of sterile LB to 180 μl of MM in skim milk. The mixture of phage and MM (MM + P) was prepared by adding 20 μl of phage lysate to 180 μl of MM in skim milk.
Vials were kept for 24 h at −80°C and frozen samples were dehydrated for 48 h in a Heto FD4 (LabEquipment, Allerød, Denmark) freeze-drier. Vials were flame-sealed and stored at 4°C.

The stability of the freeze-dried mixture of phage and MM during storage at 4°C was determined by resuspending two MM + P ampoules with 200 μl of SM buffer at different storage times from 0 to 120 days. The phage titre was determined by the soft agar overlay method in LB medium (Kropinski *et al.* 2009). The titre of the lactobacilli component of the MM was determined by plate count in MRS Agar (Difco), the yeast titres were obtained using YGC agar (Biokard Diagnostic, Beauvais, France) and lactococcus plate count was performed in 1 : 1 : 1 medium added with 1·5% w/v agar–agar (Merck, Darmstadt, Germany).

Hep-2 cells culture

Hep-2 cells were cultured at 37°C for 48 h in a 5% CO_2-enriched atmosphere in 48-well (for cell detachment experiments) or 24-well (for EHEC adhesion experiments) tissue culture plates (Greiner Bio One, Frickenhausen, Germany) using DMEM supplemented with 10% (v/v) of foetal calf serum (PAA Laboratories, GmbH, Pasching, Austria), antibiotics (12 IU ml^{-1} penicillin and 12 μg ml^{-1} streptomycin) and 1% (v/v) nonessential amino acids (GIBCO BRL Life Technologies) according to Hugo *et al.* (2008).

Fresh phage and MM treatments on the cytotoxicity of Hep-2 cells infected with EHEC

For cell infection, 10 ml of EHEC culture (OD600nm = 0·63) were centrifuged for 5 min at 15 000 ***g*** and the supernatant was separated in a sterile tube (supernatant tube). The bacterial pellet was resuspended in the same volume of fresh LB medium. A 200-μl volume of the resuspended bacteria was added to the supernatant tube and incubated for another 2 h at 37°C giving a final concentration of 3×10^8 CFU ml^{-1} (OD600nm = 0·36). Serial twofold dilutions of this culture in LB medium were used for cell detachment assays.

Hep-2 cells cultured in 48-well plates were washed twice with PBS and wells were divided into infected treated cells, infected untreated cells and uninfected control. Wells were prepared in triplicates as follows:

Infected treated cells: 20 μl of phage lysate (5×10^7 PFU ml^{-1}) or 20 μl of MM, for individual treatments, or a mixture of phage and MM (20 μl each) for a combined treatment were added to the respective wells. A 200-μl volume of the corresponding dilution of EHEC culture was added to each well, and the volume was adjusted to 300 μl with DMEM. Treatments were also run on uninfected cells (adding 200 μl of DMEM instead of the EHEC culture) to verify whether cell detachment was not produced by the addition of phage and/or MM. Controls of cell detachment with skim milk without probiotics and SM buffer with 0·1 mol l^{-1} sucrose without phage were also prepared.
Infected untreated cells: 200 μl of the corresponding dilution of EHEC culture were added to the respective wells and the volume of each well was adjusted to 300 μl with DMEM.
Uninfected control: For untreated uninfected controls, the respective wells were filled with 300 μl of DMEM.

Plates were incubated for 16 h as described above, then supernatants were extracted, wells were washed twice with PBS, fixed with 2% (v/v) formaldehyde and stained with Crystal Violet following the protocol of Minnaard *et al.* (2001). The dye was extracted with 50% (v/v) ethanol and the OD was measured at 540 nm. Cell monolayer integrity after each treatment was evaluated by calculating the percentage of cells detached using the following expression:

Cell detachment (%) = $100 \times (1 - (A_m - A_o)/(A_b - A_o))$ where A_m is the sample absorbance; A_o is the absorbance of a well without cells (control of stain adsorption by the well); and A_b is the absorbance of the uninfected untreated control.

Freeze-dried phage and MM treatments on the cytotoxic effect of EHEC-infected Hep-2 cells

Vials of freeze-dried phage, MM and MM + P were resuspended with 200 μl of SM buffer.

Wells were prepared as described for fresh treatments: for individual treatments of phage (P) or MM, 20 μl of the resuspended vial were added to the respective wells. For combined treatments, 20 μl of phage + 20 μl of MM (individually lyophilized), or 20 μl of MM + P lyophilized together were added to respective wells. All wells were then infected with 200 μl of EHEC culture ($7{\cdot}5 \times 10^7$ CFU ml^{-1}) prepared as described above, or 200 μl of DMEM for uninfected treated controls, and the

volume was adjusted to 300 μl with DMEM. Cell detachment controls with skim milk without probiotics and SM buffer + 0·1 mol l^{-1} sucrose without phage were prepared.

Preincubation with MM

For MM preincubation assays on Hep-2 cells, 20 μl of fresh or freeze-dried MM were added to the respective wells containing 280 μl of DMEM, and plates were incubated 1 h at 37°C in a 5% CO_2 atmosphere. Wells were then washed three times with PBS, filled with 280 μl of DMEM and 20 μl of fresh or freeze-dried phage were added to respective wells. The assays were then conducted as described above for the phage treatment.

EHEC adhesion, Hep-2 cell viability and monolayer integrity after individual and combined treatments with phage and MM

Hep-2 cell monolayer was infected with EHEC at 1×10^8 CFU per well in a 24-well tissue culture plate. For phage and MM treatments, 20 μl of the resuspended lyophilized phage or MM were added to the respective wells. For the combined treatment, lyophilized phage and lyophilized MM (20 μl each) were incorporated into respective wells. Wells were adjusted to a final volume of 500 μl with DMEM and the plate was incubated for 2·5 h at 37°C and 5% CO_2. For EHEC adhesion assays, cells were washed three times with PBS, lysed with sterile distilled water, and EHEC attachment to cells was determined by plate count in mEC agar with Novobiocin (Merck KGaA, Darmstedt, Germany).

The viability and monolayer integrity were evaluated by fluorescent dye staining of Hep-2 cells after incubation with EHEC with and without P, MM and MM + P treatments as described previously. Results were compared with a control of Hep-2 cells without EHEC and without further treatment. Controls of Hep-2 cells with MM, P and MM + P treatments without EHEC were also run. After the incubation, wells were washed three times with PBS and stained with 250 μl of a 2 μg ml^{-1} solution of acridine orange–ethidium bromide in PBS following the protocol described by Ching *et al.* (2002). Stained cells were observed by fluorescence microscopy. A total of 200 cells were counted in multiple randomly selected fields, and the apoptotic (red) cell percentage was calculated according to Grossmann *et al.* (1998).

Statistical analysis

All assays were performed in triplicate. Results were analysed by the one-way analysis of variance (ANOVA) with a significance level of 5% ($P < 0·05$) followed by Fisher's least significant difference test at a $P < 0·05$, $P < 0·01$ or $P < 0·001$ as specified for each result presented.

Results

Effect of combined treatment (phage and MM) of EHEC-infected Hep-2 cells on the cytotoxic effect

Treatment with probiotic microbial mixture (MM) and phage (individually or in combination) were assayed in their protective effect of Hep-2 cells infected with EHEC after incubation for 16 h.

The percentage of Hep-2 cell detachment after infection with different concentrations of EHEC with and without treatment with probiotic microbial mixture (MM) and phage (individually or in combination) are shown in Fig. 1.

Probiotics exhibited a protective effect on Hep-2 cells against EHEC infection, leading to a significant reduction ($P < 0·05$) of cell detachment with respect to the untreated control in the entire range of EHEC concentrations tested. In contrast, no reduction ($P > 0·05$) of cell detachment percentage was observed with the addition of phage to infected Hep-2 cells (Fig. 1). Moreover, for an EHEC infective dose of $7·5 \times 10^7$ CFU ml^{-1}, phage treatment significantly increased cell detachment percentage ($P < 0·05$) compared with the untreated control.

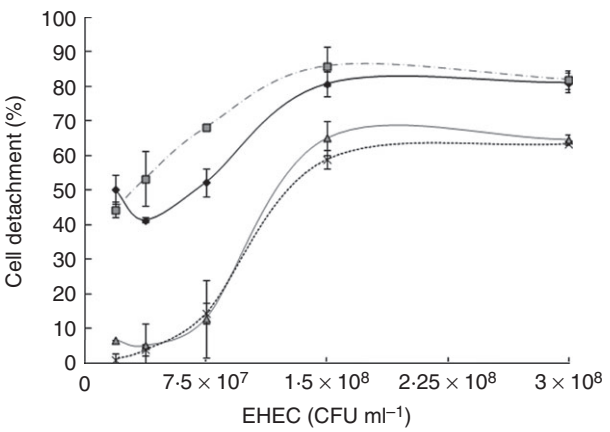

Figure 1 Percentage (relative to noninfected cells) of Hep-2 cells detachment after infection with different concentrations of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 without further treatment (EHEC) or treated with phage (P, 10^6 PFU per well) and MM (2×10^7 CFU of bacteria and 2×10^4 CFU of yeasts respectively**)** individually or in combination (P + MM). Symbols: EHEC (♦); P (□); MM (△) and P + MM (×). After Crystal Violet staining of uninfected untreated cells control and uninfected cells with phage, no significant difference ($P < 0·05$) was observed in OD 540 nm values (0·456 ± 0·176 and 0·517 ± 0·087 respectively) indicating no cell detachment caused by phage without EHEC.

The addition of phage along with MM (MM + P) did not significantly modify ($P < 0{\cdot}05$) MM protective activity (Fig. 1). Particularly, no difference was observed among MM and MM + P treatments for an EHEC infective dose of $7{\cdot}5 \times 10^7$ CFU ml^{-1}, indicating MM is able to compensate for the detrimental activity of phage treatment on cell monolayer integrity.

The maximum difference between probiotic treatments (MM and MM + P) and the untreated control was observed on cells infected with an EHEC concentration of $7{\cdot}5 \times 10^7$ CFU ml^{-1} (Fig. 1), thus this pathogen concentration was selected for further assays.

Effect of combined treatment (phage and MM) of EHEC-infected Hep-2 cells on the cytotoxic effect: effect of freeze-drying and preincubation with probiotics

Preincubation of Hep-2 cells with MM was assayed in the effectiveness of the combined MM + P treatment to study if there is competition for adhesion to epithelial cells between the probiotic micro-organisms and EHEC. This competition would be expected to be enhanced by the contact of probiotics with cells prior to the infection (Jankowska *et al.* 2008).

Incubation with MM prior to the addition of EHEC and phage did not improve Hep-2 cells response to EHEC cytotoxic effect (Table 1), suggesting MM antipathogenic activity is not related to probiotic micro-organisms completely covering the eukaryotic monolayer surface, which would lead to a decrease in EHEC adhesion to cells by unspecifically blocking the adhesion sites of the pathogen.

The effect of the lyophilization of the MM + P mixture on its antipathogenic activity was also studied in order to analyse the possibility of extending its shelf life and explore the chance of formulating a product suitable for commercialization.

Hep-2 cell culture infected with EHEC at $7{\cdot}5 \times 10^7$ CFU ml^{-1} treated with fresh MM + P exhibited no significant difference ($P > 0{\cdot}05$) on cell detachment percentage with respect to those treated with the lyophilized mixture (Table 1). Preincubation assays using fresh or freeze-dried MM and P also showed no significant difference ($P > 0{\cdot}05$) on cell detachment (Table 1).

Table 1 Percentage of detachment of enterohemorrhagic *Escherichia coli* (EHEC)-infected Hep-2 cells after different treatments

Treatment	Fresh	Freeze-dried
None (control)	49·1 ± 9·2	
P	65·0 ± 2·3	57·9 ± 8·5
MM	10·6 ± 7·8	28·6 ± 11·8
MM + P*	23·1 ± 4·4	17·4 ± 12·3
Preinc. MM and P†	32·3 ± 3·9	29·0 ± 1·4
MM and P‡		20·8 ± 2·7

Percentages were calculated with respect to the uninfected control. The control of cells with P and without EHEC showed no significant difference ($P > 0{\cdot}05$) with the uninfected control.

Fresh and freeze-dried treatments were equivalent ($P > 0{\cdot}05$) except for MM, whose fresh form exhibited a better performance ($P < 0{\cdot}05$) than the lyophilized one. All MM treatments (alone and combined) were significantly different ($P < 0{\cdot}05$) from P and the control, which were equivalent ($P > 0{\cdot}05$) to each other.

*Freeze-dried mixture of MM and phage.

†1-h preincubation of cells with MM before EHEC infection and further treatment with phage (P).

‡Mixture of individually freeze-dried therapeutic agents. Concentrations per well: EHEC $1{\cdot}5 \times 10^7$ CFU; fresh P 10^6 PFU; freeze-dried P $4{\cdot}3 \times 10^5$ PFU; fresh or freeze-dried MM 2×10^7 CFU of bacteria and 2×10^4 CFU of yeasts; freeze-dried P + MM $3{\cdot}2 \times 10^5$ UFP, 2×10^7 CFU of bacteria and 2×10^4 CFU of yeasts. The same concentration of P and MM used in the individual treatments was applied for the combined treatments.

Stability of the lyophilized mixture of phage and MM

Stability of the lyophilized MM + P mixture stored at 4°C was examined in order to evaluate the shelf life of the freeze-dried product. No significant loss in the titre of any of the micro-organisms composing the probiotic mixture was observed after 120 days of refrigerated storage of the lyophilized MM + P (data not shown). Therefore, the addition of phage did not modify the stability previously reported for MM ($P < 0{\cdot}05$) during freeze-drying in skim milk and refrigerated storage of the lyophilized product (Bolla *et al.* 2011). Conversely, phage was progressively inactivated during refrigerated storage (4°C) of the lyophilized mixture. A decrease of 0·5 log PFU in phage titre was observed immediately after the freeze-drying process, followed by a progressive decrease in phage titre during the 120 days of storage, resulting in a total loss of 2·1 log PFU with respect to the initial phage count. This is in agreement with the previously reported instability of phage CA933P during freeze-drying and storage in skim milk (Dini and de Urraza 2013), thus the more complex matrix of milk plus MM micro-organisms did not improve phage stability.

Therapeutic effectiveness of individually freeze-dried phage and MM alone or in combination

Due to the instability of the phage in the lyophilized mixture of P + MM during refrigerated storage, the combined treatment was assayed using individually lyophilized phage in SM buffer + 0·1 mol l^{-1} sucrose and MM in skim milk. Both media, SM buffer with 0·1 mol l^{-1} sucrose and skim milk, have been reported to stabilize the titres of freeze-dried phage and MM,

respectively, during refrigerated storage (Bolla *et al.* 2011; Dini and de Urraza 2013).

Freeze-dried phage and MM treatments were assayed individually or in combination. Results are shown in Table 1.

A decrease of 0·37 log PFU in the phage titre was observed immediately after the freeze-drying process ($P < 0{\cdot}05$), which agrees with the results reported in a previous work (Dini and de Urraza 2013). However, lyophilized phage treatment did not significantly modify cell detachment percentages with respect to the fresh phage treatment ($P > 0{\cdot}05$), with results similar to those observed for the untreated cells ($P > 0{\cdot}05$). Conversely, incubation with the fresh and the lyophilized probiotic mixture (MM) significantly reduced cell detachment ($P < 0{\cdot}05$) with respect to the untreated cells, and the fresh mixture showed a better performance ($P < 0{\cdot}05$) than the lyophilized one.

No significant difference was observed between cell detachment percentages on Hep-2 monolayer infected with EHEC and treated with individually lyophilized phage and MM with respect to those obtained with the treatment using the freeze-dried MM + P mixture (Table 1).

Effect of P, MM and MM + P treatments on infected Hep-2 cell monolayer integrity and EHEC adhesion

MM probiotic mixture has proved to play a decisive role in the effectiveness of the combined MM + P treatment regarding the cytotoxic effect of EHEC infection on Hep-2 cells. Conversely, phage treatment showed no improvement on cell cytotoxic effect after incubation with EHEC for 16 h, probably due to the burst release of toxins during bacterial lysis during phage multiplication cycles, which counteracts the reduction in the number of pathogens available for the infection.

The effects of individually lyophilized phage and MM alone or in combination were also evaluated by their ability to reduce EHEC attachment to Hep-2 cells.

Figure 2 shows a significant reduction ($P < 0{\cdot}05$) in EHEC adhesion to epithelial cells after individual treatments with MM or phage with respect to the untreated control, but the maximum reduction in EHEC attachment was observed for the combined treatment of phage and MM, resulting in a decrease in bacterial attachment of 1·2 log CFU per well ($P < 0{\cdot}05$) with respect to the untreated control. Combined treatment resulted in a significantly lower ($P < 0{\cdot}05$) count of attached bacteria than the treatment with MM alone. Additionally, Hep-2 monolayer integrity and cell viability was evaluated by fluorescence microscopy (Fig. 3) after staining cells with acridine orange and ethidium bromide. The percentage of apoptotic (red stained) cells was calculated from a total of 200 cells from randomly selected fields (Table 2).

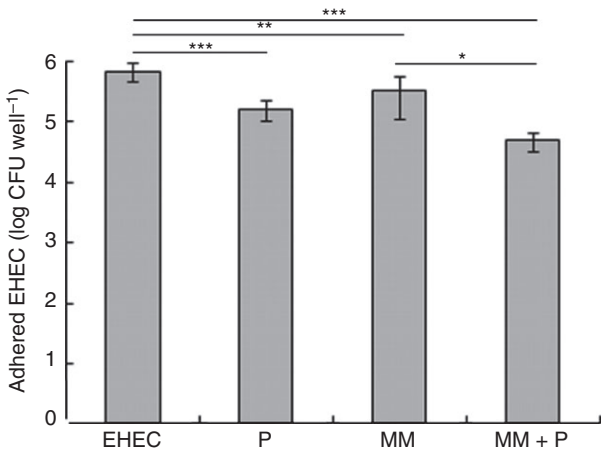

Figure 2 Enterohemorrhagic *Escherichia coli* (EHEC) (CFU per well) adhered to Hep-2 cells after 2·5 h infection without further treatment (EHEC) or treated with freeze-dried phage (P), freeze-dried MM or the mixture of individually freeze-dried phage and MM (MM + P). Asterisks indicate statistical differences: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

As observed in Fig. 3, the monolayer integrity of the uninfected control (Fig. 3a) does not differ from that of infected cells treated with lyophilized phage, MM or a mixture of individually freeze-dried phage and MM (Fig. 3c–e), while uninfected and infected treated cells (Fig. 3a and c–e) considerably differ from the untreated infected cells (Fig. 3b), the latter exhibiting a greater ratio of cell rounding and disorganization of the actin network, these morphological changes being typically triggered by EHEC infections (Hugo *et al.* 2008).

MM treatment exhibited similar apoptotic counts ($P > 0{\cdot}05$) to those of the control without EHEC, but exhibited significantly lower counts ($P < 0{\cdot}05$) than those of the infected cells without treatment (Table 2). The phage treatment exhibited a significant reduction in the number of apoptotic cells with respect to the control with EHEC, but was significantly higher than that observed for the MM treatment and the uninfected control (Table 2). The combination of both phage and MM decreased the number of apoptotic cells ($P < 0{\cdot}05$) with respect to the infected cells without treatment, resulting in no significant difference ($P > 0{\cdot}05$) from that of the uninfected control.

No significant difference was observed for the uninfected treated cells with respect to the uninfected untreated control for any of the treatments assayed.

Discussion

In this work, the antimicrobial activity of EHEC phage CA933P in combination with a microbial mixture (MM) of probiotic micro-organisms isolated from kefir grains

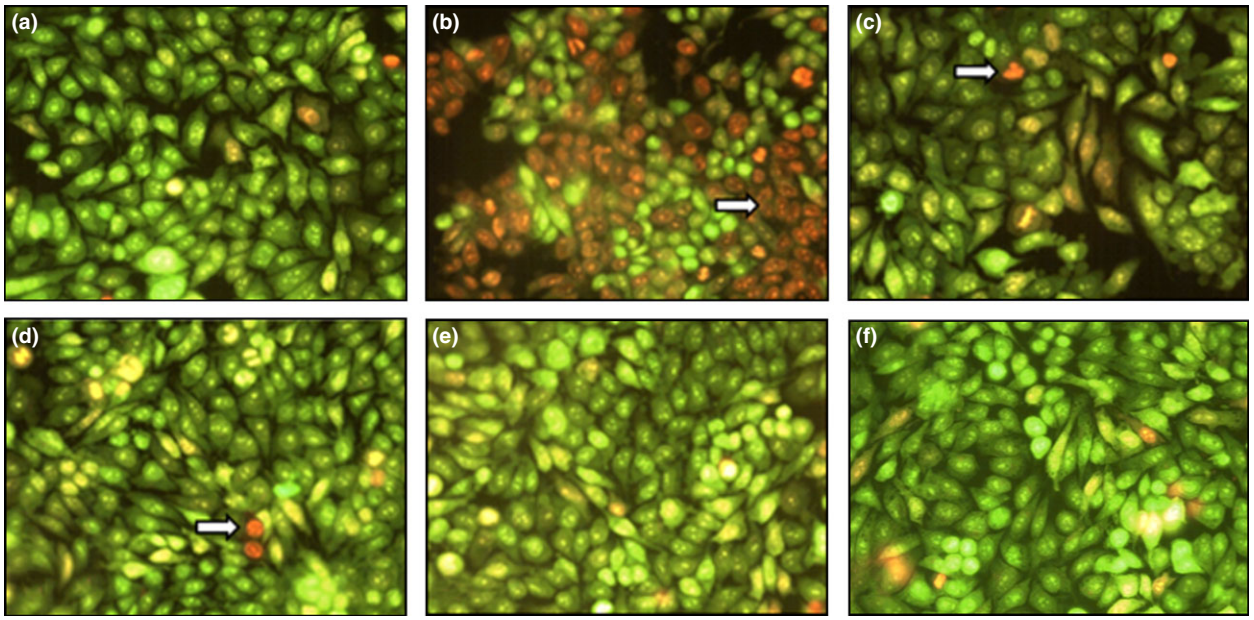

Figure 3 Micrographs of acridine orange–ethidium bromide-stained Hep-2 cells without infection (a), infected with enterohemorrhagic *Escherichia coli* (EHEC) at 1×10^8 CFU per well without further treatment (b), and infected with EHEC and treated with phage at $4{\cdot}3 \times 10^5$ PFU per well (c), MM at 2×10^7 CFU of bacteria and 2×10^4 CFU of yeasts per well (d) and MM + P both at the same concentration used for the individual treatments (e). Control of Hep-2 cells with phage and without EHEC (f). Percentage of apoptotic cells (arrows) was determined from a total count of 200 cells from randomly selected fields for each condition. Magnification 40×.

was evaluated in order to combine the lytic effect of phage and the ability of MM to reduce the pathogenesis and the cytotoxic effect of EHEC on epithelial cells *in vitro*.

The cytotoxicity produced by EHEC infection was evaluated by the measurement of Hep-2 cell detachment after 16 h of infection with EHEC concentrations ranging from $1{\cdot}87 \times 10^7$ to 3×10^8 CFU ml^{-1} without further treatment (untreated controls) or treated with phage and MM, individually or combined.

The phage treatment resulted in cell detachment percentages comparable to ($P > 0{\cdot}05$) or even higher ($P < 0{\cdot}05$) (for an EHEC concentration of $7{\cdot}5 \times 10^7$ CFU ml^{-1}) than those of the untreated infected control. The lack of effectiveness of phage treatment was attributed to a counterpoised effect between the partial reduction in the number of pathogens prior to the proliferation of phage-resistant bacteria, which reduces the contact time between the eukaryotic cells and the pathogen, and the burst release of toxins produced by EHEC lysis during phage replication (Matsuda *et al.* 2005). It is noteworthy that although the toxin load is released at once during bacterial lysis, it has been reported that phage infection does not increase the amount of toxins produced by bacteria (Viscardi *et al.* 2008).

Treatment with MM significantly reduced EHEC cytotoxic effect on Hep-2 cells, decreasing cell detachment percentages in the entire range of EHEC concentrations tested.

Preincubation with MM did not improve its antipathogenic activity against EHEC, suggesting that the MM protective activity to Hep-2 cells is not related to the assembly of a microbial 'layer' isolating cells from EHEC (Jandu *et al.* 2009). Therefore, other mechanisms are likely responsible for the reduction in the number of EHEC attached to Hep-2 cells observed after a short-term co-incubation of MM with the pathogen (2·5 h).

No interference was observed on the protective activity of MM with the addition of phage. Particularly, at the EHEC concentration for which phage produced a greater amount of cell detachment compared to the untreated control, combined treatment of phage and MM exhibited the same percentage of cell detachment as the treatment with MM alone. This indicates not only that the phage does not interfere with MM protective activity but also that MM is able to offset the detrimental effect produced by phage on the cell's monolayer integrity after EHEC infection. Based on these results, it would probably be more suitable to stagger the combined treatment by first applying probiotic treatment and then the phages for *in vivo* treatments.

Freeze-drying diminished MM protective activity on infected Hep-2 cells, probably due to the lag time needed for microbial antipathogenic activity to be restored after

Table 2 Percentage of apoptotic (red stained) Hep-2 cells after different treatments

	% red cells
Control	2·68 ± 1·58
Control P	4·26 ± 1·33
EHEC	60·7 ± 1·17
P	14·69 ± 6·98
MM	5·08 ± 0·78
MM + P	10·33 ± 2·17

Percentages were calculated from a total of 200 cells counted in multiple randomly selected fields. Control: uninfected untreated cells. Control P: uninfected cells treated with phage. EHEC: infected untreated cells (1×10^8 CFU per well of EHEC). P, MM and P + MM: infected cells (1×10^8 CFU per well of EHEC) treated with freeze-dried phage ($4{\cdot}3 \times 10^5$ PFU per well), freeze-dried MM (2×10^7 CFU of bacteria and 2×10^4 CFU of yeasts) and MM + P (mixture of the same concentrations of P and MM used in the individual treatments), respectively.

Phage treatment without EHEC (Control P) did not significantly modify the percentage of red cells compared to the uninfected untreated control ($P > 0{\cdot}05$).

All treatments significantly reduced red cells percentages with respect to the EHEC infected cells without treatment ($P < 0{\cdot}05$). MM treatments (alone and combined) showed no significant difference with the control without EHEC ($P < 0{\cdot}05$). P treatment significantly differed from MM and MM + P treatments and the control.

the reactivation of MM mixture. In contrast, phage performance was not affected by lyophilization in SM buffer with 0·1 mol l^{-1} sucrose, as it did not differ from that observed in the fresh treatment ($P > 0{\cdot}05$). In a previous work SM buffer with 0·1 mol l^{-1} sucrose proved to effectively stabilize phage CA933P during storage of the lyophilized product at 4°C for at least 110 days (Dini and de Urraza 2013). Lyophilized MM + P mixture showed a similar performance to that of the fresh combined treatment, but the phage was significantly inactivated during the storage of the freeze-dried mixture. Furthermore, the effectiveness of the combined treatment using a lyophilized mixture of phage and MM did not differ from the one obtained using the blend of the individually freeze-dried antimicrobial agents, enabling the possibility of using pharmaceutically acceptable freeze-drying media designed specifically for each type of therapeutic agent (phage or probiotic microbial mixture).

As mentioned, freeze-dried MM, besides reducing EHEC cytotoxic effect, was also able to significantly reduce EHEC adhesion to Hep-2 cells. With an EHEC infective dose of 10^8 CFU per well, untreated cells showed a bacterial attachment of 5·83 log CFU per well. Treatment with MM significantly reduced ($P < 0{\cdot}05$) EHEC adhesion to eukaryotic cells by 0·31 log CFU per well with respect the untreated cells (Fig. 2). This lowering effect on EHEC attachment to Hep-2 cells has been previously reported for *Lactobacillus* CIDCA 83114 (included in MM) but only at the lowest EHEC concentration assayed (10^6 CFU per well) and after preincubation with the probiotic (Hugo *et al.* 2008). In this work the reduction in EHEC attachment was also observed for higher infection doses, indicating a contribution of the other microorganisms composing the MM to the reduction of EHEC adhesion to the eukaryotic cells.

In addition to alleviating the negative effects derived from pathogen infections, such as Stx cytotoxic effect, probiotics also can control or attenuate bacterial infections by different mechanisms, such as: the modification of the pathogen's ability to infect cells (Ohland and MacNaughton 2010), the increase in the immune response of cells to the infection (Romanin *et al.* 2010) and the production of bacteriocins to kill the pathogens (Oelschlaeger 2010; Arqués *et al.* 2015). For its part, phage activity against bacteria is directly related to pathogen elimination during the lytic cycle.

Lyophilized phage, CA933P, was also able to significantly reduce ($P < 0{\cdot}05$) EHEC adhesion to Hep-2 cells by 0·62 log CFU per well with respect to the untreated control (Fig. 2); possibly linked to a reduction in the total number of pathogens available for infection. Also, in the short-term incubation with the pathogen (2·5 h) no cytotoxic effect was observed on Hep-2 cells, probably due to the reduction in the number of pathogens with an incubation period not long enough to allow several phage replication cycles (which would enhance toxins release) or the proliferation of phage-resistant bacteria. It is worth mentioning that, unlike that observed in the *in vitro* infection, the control of the pathogens by the host's defences *in vivo* is considerably favoured when the number of pathogens decreases, thus the extensive proliferation of phage-resistant bacteria as observed *in vitro* would be unlikely. Likewise, cell damage is not expected to reach the extensiveness observed *in vitro* after the short-term incubation with phage and EHEC since the number of lytic cycles would be capped, for the hosts' defences would be controlling the proliferation of the pathogen. Nonetheless, as proposed in the work of Matsuda *et al.* (2005), bacterial toxins' release during the lytic cycle would be avoided by using lysis-deficient (LyD) bacteriophages, which could be an interesting alternative for being applied in combination with probiotics.

The combined treatment of individually lyophilized phage and MM exhibited the best performance in the decline of bacterial attachment, showing a significant reduction ($P < 0{\cdot}05$) of 1·15 log CFU per well in the number of adhered EHEC with respect to the untreated control, indicating that both antimicrobial agents can contribute to decrease the infectivity of EHEC without interfering in their antimicrobial activity. At longer incu-

bation times (16 h) the beneficial effect of reducing the number of pathogens produced by phages is masked by the increased release of toxins produced by the bacterial lysis during the multiplication cycle of the phage. In this case the protection offered by MM to Hep-2 cells is evident when applied together with the phage.

Additionally, all treatments showed similar cell morphologies to those of the uninfected control, and exhibited a marked improvement on cell monolayer integrity compared to the EHEC-infected cells without treatment. Shiga toxins are considered the main virulence factor associated with EHEC infection, and Stx-1 and Stx-2 have both been reported to induce Hep-2 cells apoptosis (Jones *et al.* 2000). Thus, both antimicrobial agents exert a protective effect on Hep-2 cells against Shiga toxins in short-term incubation periods, reducing the number of apoptotic (red stained) cells. MM treatment was the most effective one, showing no significant difference with the uninfected control ($P < 0{\cdot}05$).

Although MM was more efficient than phage in reducing apoptotic cell count, both antimicrobial agents showed a marked reduction in the number of red-stained cells with respect to the control of EHEC-infected cells without treatment, indicating that both agents are effective in reducing the cytotoxicity derived from EHEC infection. Furthermore, the combination of both reduced the total number of apoptotic cells resulting not significantly different ($P > 0{\cdot}05$) to that of the control without EHEC.

The results obtained show that the use of probiotics (MM) is effective in reducing the cytotoxic effect produced by EHEC O157:H7 infection *in vitro* on epithelial Hep-2 cells. Phage treatment of EHEC-infected cells resulted in similar or higher cell detachment percentages than the untreated control, but the addition of MM was able to offset that negative effect, restoring the protective effect observed for the MM treatment and indicating no interference of phage with MM activity. Individual treatments with phage or MM were able to reduce EHEC attachment to Hep-2 cells, exhibiting the combination of both the lowest EHEC adhesion, significantly lower than that obtained with the MM alone. The effectiveness of the combined treatment with individually freeze-dried phage and MM did not differ from that using both antimicrobial agents in a fresh form, enabling the possibility to extend the shelf life of this therapeutic mixture. Also, cell monolayer integrity was improved for all treatments of infected cells compared to the untreated infected cells, considerably reducing the counts of apoptotic cells compared to the infected untreated control.

The combined use of phage and probiotics proved to be potentially effective in the treatment of EHEC infections, by reducing the number of pathogens in the focus of infection and protecting epithelial cells from damage by cytotoxic effect.

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Conflict of Interest

The authors have declared no conflict of interest.

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