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Evaluation of an WPC edible film added with a cocktail of six lytic phages against foodborne pathogens such as enteropathogenic and Shigatoxigenic *Escherichia coli*

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ABSTRACT

Active packaging materials with antimicrobial properties have gained significant interest over the last decade. Furthermore, phages are potentially useful as antimicrobial agents in food. Phage stability assays were conducted in the whey protein concentrate (WPC) matrix over 5 weeks of storage. In addition, the phages incorporated in the WPC matrix were characterized by their release from films into an aqueous system and solid food, as well as by their antimicrobial activity against pathogenic *E. coli* in a liquid medium and in meat at refrigerated, room and abusive temperatures. The results demonstrate that phages are highly stable in WPC films within 5 weeks of storage. Moreover, WPC films can release a significant number of phages into an aqueous system and onto a meat surface at similar concentrations. Phage-added films produced a significant reduction in *E. coli* cells to non-detectable levels in antimicrobial assays. Similar results, such as a complete inactivation achieved for DH5α and O157:H7 STEC strains, were obtained when antimicrobial assays were performed on meat. As a conclusion, the six-phage cocktail added into WPC films was highly stable, effectively released from films and proved highly effective as a biocontrol tool, though not under all the conditions evaluated.

1. Introduction

Shiga-toxigenic *Escherichia coli* (STEC) is the virotype responsible for most cases of hemolytic uremic syndrome (HUS) (Johnson & Taylor, 2008). Also, enteropathogenic *E. coli* (EPEC) strains are responsible for human outbreaks worldwide (Varela et al., 2007; Viljanen et al., 1990). These pathogens are the main causes of diarrhea and HUS in our country, Argentina being the country with the world's highest incidence of HUS (Rivas et al., 2008). STEC infections are transmitted to humans through contaminated foods such as meat (Rivas et al., 2003) and water (Swerdlow et al., 1992), while infection by EPEC is related to fecal contamination of food (Hernandes, Elias, Vieira, & Gomes, 2009).

Bacteriophages have proven to be effective as biocontrol agents against several foodborne pathogens (Bigwood, Hudson, Billington, Carey-Smith, & Heinemann, 2008; Mukhopadhyay & Ramaswamy, 2012; O'Flynn, Ross, Fitzgerald, & Coffey, 2004). Several studies have reported on their efficacy to control bacteria in food, animals and humans (Bruttin & Brussow, 2005; Carlton, Noordman, Biswas, de Meester, & Loessner, 2005; Greer, 2005; Hudson, Billington, Carey-Smith, & Greening, 2005), as well as along the food chain (Gill, Sabour, Leslie, & Griffiths, 2006; Kim, Klumpp, & Loessner, 2007; Modi, Hirvi, Hill, & Griffiths, 2001; Raya et al., 2006). In addition, the use of phage cocktails to control foodborne pathogens has been explored both in meat (O'Flynn et al., 2004) and in other foods (Garcia, Madera, Martínez, & Rodríguez, 2007; Zuber et al., 2008) and vegetables (Leverentz et al., 2003; Viazis, Akhtar, Feirtag, & Diez-Gonzalez, 2011). Furthermore, the six-phage cocktail proved to be effective at reducing bacterial populations on food matrices at refrigerated, room and abusive temperatures (Tomat, Casabonne, Aquili, Balague, & Quiberoni, 2018).

Whey proteins have been used to obtain edible films (Oses et al., 2009; Perez-Gago & Krochta, 2000; Talens & Krotcha, 2005) with antimicrobial purposes (Hosseinidoust, Olsson, & Tufenkji, 2014). Whey protein is usually commercialized as whey protein concentrate (WPC)

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containing between 25 and 80% w/w of proteins. WPC is a low-cost and readily available product (Cecchini, Spotti, Piagentini, Milt, & Carrara, 2017). Besides, phages are easy to isolate as well as economical to propagate. Different studies have demonstrated that active packaging formulations including cellulose, whey protein isolate (WPI), poly lactic acid (PLA) and alginate coatings added with phages have proved to have antimicrobial properties against several pathogens such as *E. coli* (Anany, Chen, Pelton, & Griffiths, 2011; Vonasek, Choi, Sanchez, & Nitin, 2018), *Salmonella* (Radford et al., 2017) and *Listeria monocytogenes* (Anany et al., 2011). Thus, WPC films combined with phages are a good and low-cost antimicrobial alternative against foodborne pathogens in order to improve the safety of meat and other food products as well. Moreover, coliphages are practically unexplored as antimicrobials when combined with active packaging materials such as WPC.

A major drawback of using phages as biocontrol agents is their limited host range, which may be bypassed by using a phage cocktail (O'Flynn et al., 2004). On the other hand, limited host range may be an advantage, since the high specificity of phages can be used to develop a specific antimicrobial packaging. In addition, these packaging materials will only kill the targeted pathogen, leaving commensal bacteria unaffected (Khalifa et al., 2015; Mayer, Narbad, & Gasson, 2008) without altering the organoleptic properties of foods that require further microbial action. Therefore, phages added to films could be used as an additional hurdle in applications where broad-spectrum antimicrobials are inappropriate, enhancing phage stability and improving packaging specificity against foodborne pathogens. Thus, the aim of the present work was to evaluate the potential utility of six coliphages mixed in a cocktail and added into WPC edible films by testing the stability and release of phages as well as their efficacy against pathogenic *E. coli* strains.

2. Materials and methods

2.1. Bacterial strains and phages

E. coli DH5α was used as the sensitive host strain to enumerate phages in the stability and release assays. Three additional strains were used in the biocontrol experiments. Two of them, an enteropathogenic *E. coli* (eae+) (EPEC) and a Shiga toxigenic *E. coli* O157:H7 (stx2+ and eae+) (O157 STEC), were previously isolated from stool samples, identified by using API-20E system (Biomerieux, Buenos Aires, Argentina), and further characterized by PCR. The third strain was *E. coli* Shiga toxigenic non-O157:H7 (ARG4827; serogroup O18; stx1+ and stx2+) (non-O157 STEC) (Balague et al., 2006). All the strains were kept as frozen (−70 °C) stock cultures 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 15% (v/v) glycerol and routinely reactivated overnight at 37 °C in Hershey broth supplemented with $MgSO_4$ (5 mmol L^{-1}) (Cicarelli, San Lorenzo, Santa Fe, Argentina) (Hershey-Mg).

Myoviridae bacteriophages (T-even type) (Tomat, Migliore, Aquili, Quiberoni, & Balague, 2013a), DT1 to DT6, were previously isolated from stool samples of patients with diarrhea treated at the Hospital Centenario, Rosario (Tomat, Mercanti, Balague, & Quiberoni, 2013b) and characterized by electron microscopy, host range and PCR assays (Tomat et al., 2013a). Phages were grown to high titers as previously described (Tomat et al., 2013b). Phage stocks were enumerated (plaque-forming units per millilitre; PFU mL^{-1}) by the double-layer plate titration method (Jamalludeen et al., 2007) by mixing 0.1 mL of decimal dilutions with 0.1 mL of a log-phase culture of *E. coli* DH5α and 3 mL of warm (45 °C) Hershey soft agar (0.7% w/v) and by pouring the mixture onto Hershey-Mg agar (1.5% w/v). Stocks were stored in Tris-magnesium-gelatin buffer (0.05 mol L^{-1} Tris, 0.008 mol L^{-1} MgSO4, 0.01% w/v gelatin, pH = 7.5) (TMG) at 4 °C.

2.2. Whey protein concentrate (WPC)-based edible films added with bacteriophages

2.2.1. Phage stock preparation

The propagation of all the bacteriophages used in this study was accomplished in Hershey-Mg broth with *E. coli* DH5α as the sensitive host strain. Namely, an exponential phase culture of DH5α was infected with the phage to be amplified and incubated for 18 h at 37 °C. Then, 0.5 mL of chloroform was added and centrifuged for 10 min at 5000 rpm. The supernatant was stored at 4 °C. Next, concentrated phage stocks were obtained by centrifugation of phages in centricon tubes (*molecular weight cut off*, MWCO, 10 kDa) (Sigma-Aldrich S.A., Buenos Aires, Argentina) at 3000 rpm until obtaining a volume of phosphate-buffered saline (PBS) of 50 μL with a phage concentration of ~10^{10} PFU mL^{-1}. Phage enumeration (PFU mL^{-1}) was carried out by the double-layer plate titration method (Jamalludeen et al., 2007). The phage cocktail was composed by six phages, namely DT1 to DT6 in equal proportions.

2.2.2. WPC based edible film preparation

Edible whey films (total solids = 11.5%) were prepared according to Soazo et al. (Soazo, Rubiolo, & Verdini, 2011) with some modifications. Briefly, the whey protein concentrate (WPC) (Arla Foods Ingredients S.A., Bs. As., Argentina) was dissolved in sterile distilled water containing sterile glycerol (WPC:Glycerol ratio = 3:1) and stirred vigorously for 20 min. The resulting solution was placed in a water bath (90 °C, 30 min, without agitation) to denature the whey proteins and promote their cross-linking. The solution was then homogenized with an Omni GLH homogenizer (4 min at 20000 rpm) (Omni International Inc., Warrenton, Virginia, U.S.A.) and cooled to room temperature in an ice bath to prevent further protein denaturation. The suspension was degassed at room temperature under vacuum. The phage cocktail (1 mL) was added and mixed to ensure a uniform distribution within the film-forming solution. Subsequently, the suspension obtained (8 g/plate) was placed in sterile petri dishes of 90 mm diameter and dried for 24 h at 25 °C with a relative humidity of 58 ± 2%. Finally, the films were carefully removed from the plates.

2.3. Phage stability in WPC edible films

The stability of phages was tested by storing the films in the dark under refrigeration conditions (4 °C) and in the presence of light at room temperature (24 °C) (Vonasek, Le, & Nitin, 2014). A quarter of the circular film was placed in 50 mL of sterile water at several incubation times, between 1 and 5 weeks. Samples were incubated with agitation at 150 rpm at 24 °C for 4 h to release phages from the film. Then, phages were counted as previously described by Jamalludeen et al. (2007). Three independent experiments and two replicates per each storage condition evaluated were carried out. As a control, 1 mL containing 1 × 10^9 PFU mL^{-1} in PBS buffer was dried in a Petri dish. At each sampling time, Petri dishes inoculated only with phages were incubated in sterile water at 24 °C for 1 h to recover the phages from the surface and to determine the number of particles that remained active. To set the maximum stability (100%) of phages in the films, freshly prepared films were tested, and the number of initial phage particles (initial time = week 0) was also evaluated.

2.4. Phage release from edible films into aqueous systems and solid foods

To evaluate the release of phages from WPC edible films into different media such as water and solid foods, phage-added films were placed in water and on meat as aqueous environment and food surface, respectively. In order to determine the passive release profile of the phages from films into an aqueous solution, disks (diameter = 15 mm) were cut and placed in water (1 mL) on 24 well plates without mechanical agitation (Vonasek et al., 2014). After 1, 2, 4, 24 and 48 h of

incubation at 4 and 24 °C, the films were removed from each well and the resulting solution was tested to determine the number of phages (PFU) released. To evaluate phage release on solid foods, squares of meat (~2 cm^2) and disks (diameter = 15 mm) of phage-added films were cut and placed together. After 1, 2, 4 and 24 h of incubation at 4 and 24 °C, each meat sample was separated from the disk (film added with the phage cocktail) and washed with 5 mL of sterile water with agitation for 10 min at 250 rpm. The wash water was assayed to determine the number of phages released from the films. Three independent experiments and two replicates per assay were carried out.

To calculate the percentage of phages released from the disks, the total load of phages per cm^2 in the film was first calculated. Next, considering the surface of the film disk used, the phage load per disk was determined. Finally, the cumulative percentage of phages released during a 48 h (water) or 24 h (meat) period was calculated by dividing the total phage concentration measured at each sampling time by the total phage load per disk.

2.5. Antimicrobial effect of phage-added edible films

2.5.1. Growth inhibition assay

The antimicrobial efficacy of the films was tested by means of a growth inhibition assay (Min, Harris, & Krochta, 2005). Film disks (diameter = 15 mm) containing or not the phage cocktail were placed in duplicate in a 24-well plate. Then, a log phase culture of each *E. coli* strain evaluated (DH5α, EPEC, non-O157 STEC and O157 STEC) was diluted in Hershey-Mg broth and placed in each well (1 mL, ~3 $\log_{10}$ CFU). The plate was then sealed to prevent evaporation of the culture medium and placed at refrigeration (4 °C), room (24 °C) and abusive temperature (37 °C) for 24 h. Samples (100 μL) were plated on MacConkey agar (18 h at 37 °C) for CFU count at the beginning (0 h) and after a 24-h incubation. Cultures containing only bacteria and Hershey-Mg broth with phages were used as a control of cell viability and to verify the absence of contamination, respectively. Three independent experiments and two replicates per assay were carried out.

2.5.2. Inhibition zone assay in agar medium

Disks (15 mm diameter) were aseptically cut from edible films with and without phages. The disks were aseptically transferred to Petri dishes containing solidified Mueller-Hinton agar previously inoculated with each bacterial (DH5α, EPEC, non-O157 STEC and O157 STEC) suspension (0.5 McFarland scale) to be evaluated. After a 24-h incubation at 37 °C, the diameter of the zone of inhibition around the edible film disk was determined. Films without phages were evaluated as controls. Three independent experiments and two replicates per assay were carried out.

2.5.3. Biocontrol assay on meat

The biocontrol activity of phage-added films was tested on artificially contaminated meat according to Anany et al. (Anany et al., 2011) with some modifications. A log phase culture of each *E. coli* strain evaluated (DH5α, EPEC, non-O157 STEC and O157 STEC) was diluted in Hershey-Mg broth and used to inoculate (~2–3 $\log_{10}$ CFU) pieces of meat (~2 cm^2) placed on Petri dishes. Then, film disks (diameter = 15 mm) with (treatment) or without (control) the phage cocktail were placed in duplicate on top of the meat artificially contaminated and incubated at refrigeration (4 °C) and room (24 °C) temperature for 24 h, and at abusive temperature (37 °C) for 1 h. After incubation, each meat sample was separated from the disk and washed with 1 mL of sterile water with agitation for 10 min at 250 rpm. Aliquots (100 μL) of the wash water were plated on MacConkey agar (18 h at 37 °C) for CFU count. Samples containing only meat, meat with phages (both without WPC films) and meat with WPC films (without bacteria and phages) were used as a control to verify the absence of contamination. Three independent experiments and two replicates per assay were carried out.

2.6. Statistical analysis

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

3. Results and discussion

3.1. Phage stability in WPC edible films

The stability, release and activity of six coliphages in a cocktail combined with WPC edible films were evaluated under several conditions with the aim of applying these technologies together to improve food safety. Both technologies have great potential and have been extensively studied separately (Perez, Soazo, Balagué, Rubiolo, & Verdini, 2014), though characteristics of films added with phages have been scarcely documented in food applications (Hosseinidoust et al., 2014).

Taking into account that a high stability of phages in active packaging formulations is a critical requirement for their application in food systems (Vonasek et al., 2014), further studies are necessary due to limited understanding of the stability of phages on these formulations (Jepson & March 2004; Schaper, Duran, & Jofre, 2002). Moreover, stability of coliphages (cocktail) on WPC edible films have not been previously evaluated. Fig. 1 shows the results of stability testing of phages in WPC-based edible films at refrigerated (4 °C) and room temperature (24 °C) over a five-week period. At 4 °C and after a 5-week incubation, a significant reduction in phage counts was observed, though the phage titre was always close to 7 $\log_{10}$ PFU mL^{-1}. At 24 °C, higher reduction values (up to ~ 1 $\log_{10}$ PFU mL^{-1}) were observed after 5 weeks of storage. Similar results were found when one coliphage was added in whey protein isolate (WPI) films at 4 °C and 22 °C over a period of one month (Vonasek et al., 2014). Regarding other matrices, chitosan films containing liposome-encapsulated phages showed an increased stability of phages (Cui, Yuan, & Lin, 2017). On the contrary, the effect of dryness on cellulose membranes resulted in a reduced phage stability in most cases evaluated (Anany et al., 2011). In addition, when phage viability was assessed on food pads at 10 °C and 15 °C, counts of *Salmonella*-phages remained constant (Gouvea, Santos Mendonça, Soto Lopez, & Silva Batalha, 2016). Such contrasting results mentioned here may arise from the different properties of the matrix used to contain the phages. Several factors may increase phage stability

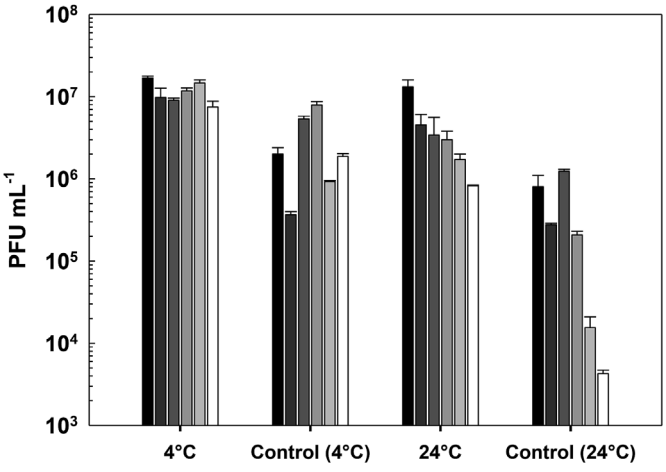

Fig. 1. Stability of phages (phage titre; PFU mL^{-1}) in WPC edible film at the beginning (■) and after 1 (■), 2 (■), 3 (■), 4 (■) and 5 (□) weeks of storage at refrigerated (4 °C) and room (24 °C) temperature. Controls with phages placed on petri dishes at both temperatures evaluated were also carried out. The total phage load on each WPC edible film (diameter = 90 mm) was 1.7×10^9 PFU $film^{-1}$. Error bars represent the standard deviation of three independent determinations ($p < 0.05$).

on active packaging materials such as WPC. Namely, the oxygen barrier of films may limit oxidative damage (Janjarasskul & Krochta, 2010), the presence of the plasticizer glycerol may stabilize the viral capsid upon drying of WPC films (Mendez et al., 2002), and the protein environment plays a role in stabilizing viral capsid proteins (Vonasek et al., 2014).

Controls with phages placed on Petri dishes at both temperatures evaluated were also carried out. Phages were air dried in Petri dishes, stored at 4 °C and at 24°, and then reconstituted in PBS buffer before counting the number of active phage particles (Fig. 1). Phage titers at the end (5 weeks) of each experiment were significantly affected at 24 °C since they decreased with time, showing a significant loss in activity during storage. Accordingly, Vonasek et al. (2014) found similar results of stability when phages were deposited on the surface of polystyrene plastic as an additional control. In contrast, after storage at 4 °C, reduction in phage counts was very variable between weeks, though the phage titers at the end of the experiments were similar to the initial values (week 0 = phage inoculum was ~6.1 $\log_{10}$ PFU mL^{-1}). Another study suggests that desiccated phages irreversibly lose infectivity (Anany et al., 2011). However, high titers were observed (~10^3 to 10^4 PFU mL^{-1} at 24 °C and ~10^5 PFU mL^{-1} at 4 °C) in our experiments after several weeks of storage. Furthermore, the stability of phages in edible WPC films was compared against the air-dried phages in the plastic surface. Overall, stability was significantly improved when phages where included in the WPC film, specially at the higher temperature (24 °C) tested. Previous studies showed that several active packaging materials improved phage stability (Cui et al., 2017; Vonasek et al., 2014), while others suggest that the incorporation technique of bacteriophage into the materials should be improved (Gouvea et al., 2016).

3.2. Phage release from WPC edible films

Besides having high stability in the active packaging materials, phages should be efficiently released into the environment to control the growth of the target pathogen on the food surface (Lone et al., 2016). In this regard, phages coated on WPC edible films represent a good alternative to the application of phages as a suspension (Anany, Brovko, El-Arabi, & Griffiths, 2015) by dipping and/or spraying.

Fig. 2 shows the pattern of phage release from WPC edible films upon exposure to an aqueous solution at 4 °C and 24 °C. Within an hour period and until 24 h of incubation, about 2×10^4 PFU mL^{-1} to 3×10^4 PFU mL^{-1} phage particles of the coated phages were released from the film into the aqueous medium at 4 °C, whereas after a 48-h incubation more than 1×10^5 PFU mL^{-1} particles were released. The total phage load per disk used in our trials was 2.3×10^7 PFU. The amount of phages released in the aqueous medium assays represents 0.09% (after 1–24 h of incubation) and 0.5% (after 48 h of incubation) of the initial phage load per disk. In contrast, almost a complete release of phages, namely 99.99%, was accomplished only after a 30-min incubation at 4 °C for *Salmonella* and *Listeria* phages in poly lactic acid (PLA) films (Radford et al., 2017), as well as for smaller molecules (potassium sorbate; release of 90% after 1 h incubation) from WPC films (Perez et al., 2014). Although no bibliography was found for coliphage release assays in formulations of edible films made with WPC, release of a coliphage cocktail from paper coated with phage-containing alginate beads after storage at 4 °C showed similar results since a 2.6 $\log_{10}$ PFU mL^{-1} reduction in the phage count was observed when compared with the initial amount used (Lone et al., 2016). At 24 °C, WPC films were able to release a significant level of phages into an aqueous solution within 24 h of incubation. Vonasek et al. (2014) have found similar results using WPI films at ambient conditions. In addition, they found a non-significant increase in the level of phage release only after a 3-h period (Vonasek et al., 2014), while the increase in phage counts was extended for about 24 h in the trials. This observation suggests a slow and controlled release of phages to avoid unnecessary virus release into the environment (Lone et al., 2016). In accordance, several observations showed the need to optimize the formulations of edible films containing antimicrobials for a controlled release (Guillard, Issoupov, Redl, & Gontard, 2009).

To determine whether bacteriophages can be efficiently released into solid foods, tests with the WPC films added with the phage cocktail were carried out on meat pieces at refrigerated (4 °C) and room (24 °C) temperature (Fig. 3). A similar release behavior was found at both temperatures evaluated. The total phage load per disk used in meat trials was 1.8×10^8 PFU disk^{-1} and the number of phage particles released from edible films onto the meat surface was about 3×10^4 PFU mL^{-1} at 4 °C (~0.02%) and 5×10^4 PFU mL^{-1} at 24 °C (~0.03%). At both temperatures, no significant increase in phage titre was observed after 1 h of incubation. In addition, a slightly higher yet not significant number of phages were released at 24 °C. In contrast to our results, Radford et al. (2017) found that over 99.99% of the phages lytic against *Salmonella* present in PLA films were released onto the meat samples within 30 min at 4 °C and 10 °C. This may be likely due to properties of the phage or coating formulation (Radford et al., 2017),

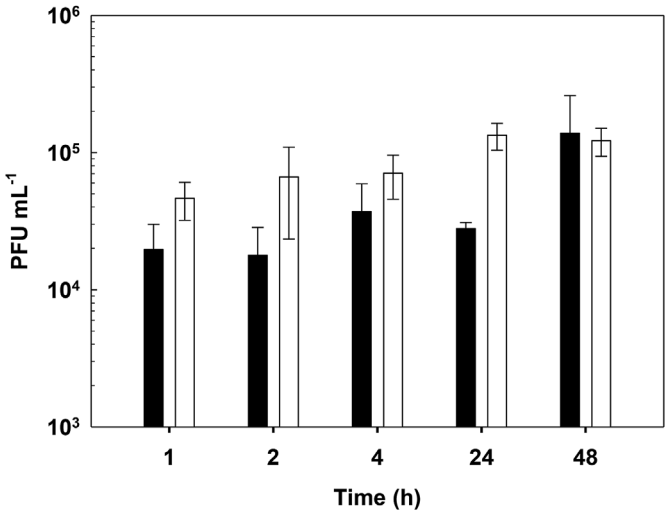

Fig. 2. Release of phages (phage titre; PFU mL^{-1}) from WPC edible film upon exposure to an aqueous system (water) at 4 °C (■) and 24 °C (□). The total phage load was 2.3×10^7 PFU disk^{-1} (diameter = 15 mm). Error bars represent the standard deviation of three independent determinations ($p < 0.05$).

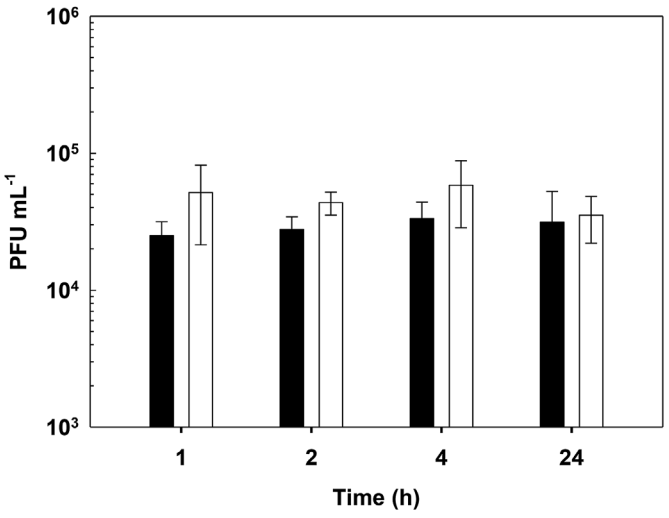

Fig. 3. Release of phages (phage titre; PFU mL^{-1}) from WPC edible film upon contact with a solid food (meat) at 4 °C (■) and 24 °C (□). The total phage load was 1.8×10^8 PFU disk^{-1} (diameter = 15 mm). Error bars represent the standard deviation of three independent determinations ($p < 0.05$).

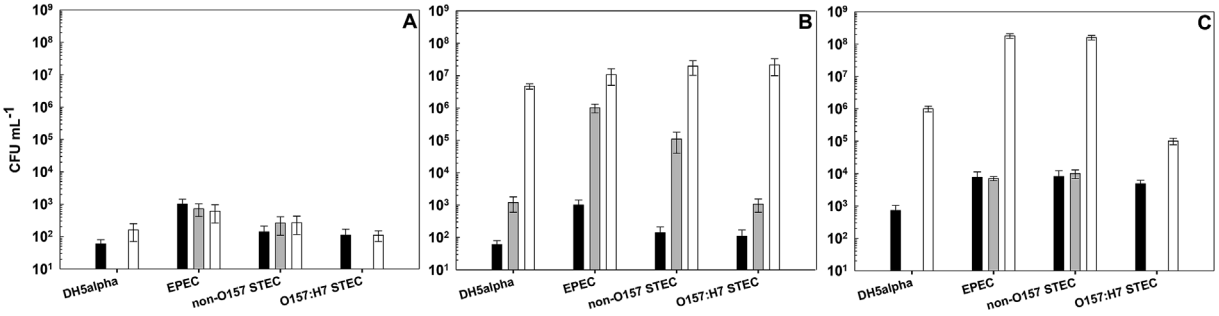

Fig. 4. Reduction of *Escherichia coli* viable cells (cell count; CFU mL^{-1}) treated with WPC edible film added with the six-phage cocktail after 24 h at 4 °C (A), 24 °C (B) and 37 °C (C) in aqueous solution. Viable cell counts at the beginning (■), after treatment (WPC film with phages; ■) and in the controls (WPC film without phages; □). The phage load used was 5.2×10^7 PFU $disk^{-1}$ (diameter = 15 mm). Error bars represent the standard deviation of three independent determinations ($p < 0.05$).

though other authors found that dip coating in WPI improves phage loading efficiency and stability but not phage release (Vonasek et al., 2018). To sum up, results in meat were almost alike to those obtained in the aqueous system, as it was observed in other foods previously evaluated (Vonasek et al., 2014).

3.3. Antimicrobial effect of phage-added WPC films

Next, the antimicrobial activity of phage-added films was evaluated through three different inhibition assays: growth inhibition assay (Fig. 4), inhibition zone assay in agar medium (Fig. 5), and a biocontrol assay on meat (Fig. 6).

First, the growth inhibition assay was carried out by challenging phages (the phage load used was 5.2×10^7 PFU $disk^{-1}$) in a 24-well plate with *E. coli* strains (DH5α, EPEC, non-O157 STEC and O157 STEC). Fig. 4 shows the results after 24 h of incubation at 4 °C (Figs. 4A), 24 °C (Figs. 4B) and 37 °C (Fig. 4C). Reduction of *E. coli* viable cells to non-detectable levels was achieved only at 4 °C and 37 °C for DH5α and O157:H7 STEC strains, while at 24 °C both strains grew though always to a lesser extent than in controls. At 4 °C, phages released at high counts may inhibit bacterial cells by the mechanism lysis from without, where phages kill bacteria, preventing cell multiplication without phage production (Abedon, 2011; Tarahovsky, Ivanitsky, & Khusainov, 1994). On the other hand, phages present a self-amplification response at 37 °C (Bourdin et al., 2013; Stewart et al., 1998) which could help eliminate the pathogen at this abusive temperature. Accordingly, a significant inhibition was obtained at 37 °C and a lower final bacterial density was detected when *Listeria* and *Salmonella* strains were exposed to PLA films added with phages (Radford et al., 2017). Regarding EPEC and non-O157 STEC strains, when incubation was accomplished at 4 °C and 37 °C, the concentrations of both pathogens in phage-treated samples were like the initial contamination level. However, in the challenge at 24 °C, bacterial cells almost reached the level of multiplication achieved in the phage free controls, probably due to a lesser self-amplification response under non-optimal bacterial growth conditions (Bryan, El-Shibiny, Hobbs, Porter, & Kutter, 2016). The antimicrobial effect was also evaluated by Vonasek et al. (2014) only at 22 °C in WPI films added with T4 phages; they found that the treatment reduced the level of bacteria by ~ 1 $\log_{10}$ PFU mL^{-1} below the initial level. From the above, the use of WPC films combined with phages against the four *E. coli* strains evaluated suggests that further research is necessary to determine the adequate film coating composition and phage concentration to obtain films with a satisfactory antimicrobial activity, especially when this technology is evaluated at non-refrigerated (24 °C) temperatures.

Fig. 5 shows the inhibition zone assays performed in agar medium inoculated with *Escherichia coli* strains. A representative picture is presented since similar results were obtained with all the *E. coli* strains tested. The freshly prepared WPC film disks (Figs. 5A-1) as well as film disks stored for a week (Figs. 5A-2) and for a month (Figs. 5A-3) at 4 °C, all of them added with phages, produced the same inhibitory effect. Namely, the zone of inhibition on the strains seeded in the agar was 1.5 ± 0.1 mm, whereas the freshly prepared WPC edible film disk without phages showed not inhibition around the film (Figs. 5A-4). Several studies have proved the efficiency of phage-added films against *Salmonella* and *Listeria* (Radford et al., 2017). However, variable results have been found against other pathogens such as *Listeria innocua, Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enteritidis* and *Pseudomona fragi* (Fernandez-Pan, Royo, & Mate, 2012; Seydim & Sarikus, 2006) with WPI films incorporated with several aromatic essentials oils. Furthermore, the agar diffusion method was used for the measurement of the antifungal effect of films against *Penicillium expansum* (Simonaitiene, Brink, Sipailiene, & Leskauskaite, 2015). However, no bibliography was found regarding the measurement of inhibition by the diffusion method in WPC films added with coliphages. Fig. 5B shows an amplified inhibition zone produced by a WPC film added with phages (six-phage cocktail). An inhibition perpendicular to the disk (in the form of a cut) was observed in addition to the radial inhibition on the bacterial lawn. This pattern of inhibition was also observed in pads added with phages by Gouvea in solid medium against *Salmonella* Typhimurium at 10 °C and 15 °C (Gouvea et al., 2016). From Gouvea experiments, it seems that perpendicular inhibition only took

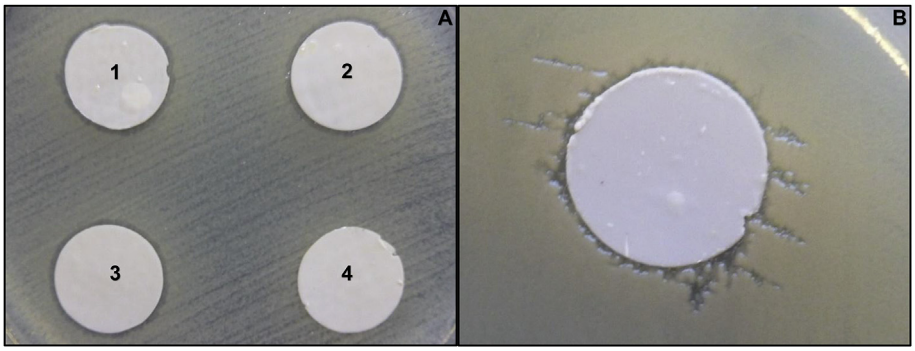

Fig. 5. Inhibition zone assay in agar medium inoculated with *Escherichia coli* strains. Freshly prepared WPC edible film disk added with phages (A1), WPC edible film disk added with phages and stored for 1 week at 4 °C (A2), WPC edible film disk added with phages and stored for 1 month at 4 °C (A3), freshly prepared WPC edible film disk without phages (A4). The phage load used was $6.4 \pm 2.7 \times 10^7$ PFU $disk^{-1}$ (diameter = 15 mm). Amplified inhibition zone produced by an WPC film added with phages (B).

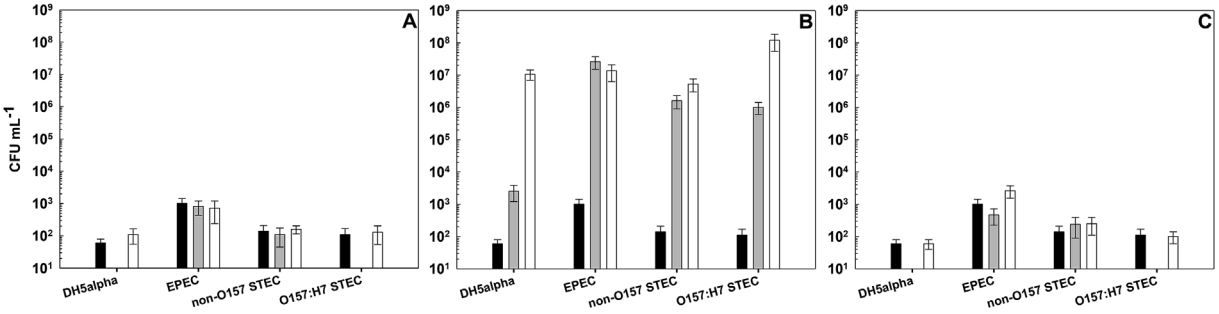

Fig. 6. Reduction of *Escherichia coli* viable cells (cell count; CFU mL^{-1}) treated with WPC edible film added with the six-phage cocktail after 24 h at 4 °C (A) and 24 °C (B), and after 1 h at 37 °C (C) in meat. Viable cell counts at the beginning (■), after treatment (WPC film with phages; ■) and in the controls (WPC film without phages; □). The phage load used was 5.2×10^7 PFU $disk^{-1}$ (diameter = 15 mm). Error bars represent the standard deviation of three independent determinations ($p < 0.05$).

place at the highest temperature evaluated (15 °C). However, in our trials, all the incubations were accomplished at 37 °C, thus further testing is needed to determine the nature of this inhibition pattern.

The last test was performed to evaluate the potential of the phage cocktail added in WPC films to control bacterial contamination on meat. For these assays, meat pieces were inoculated with *E. coli* ($\sim 10^2$–10^3 CFU). Then, each disk (5.2×10^7 PFU $disk^{-1}$) was placed above the meat preequilibrated at each temperature assayed. After that, meat samples were incubated at refrigeration (4 °C; Fig. 6A) and room (24 °C; Fig. 6B) temperature for 24 h, and at abusive (37 °C; Fig. 6C) temperature only for 1 h due to the spoilage of the meat. In biocontrol assays at 4 °C and 37 °C, the phage cocktail added in WPC films significantly reduced DH5α and O157:H7 STEC to non-detectable levels, while 10^2–10^3 bacterial cells of EPEC and non-O157 STEC remained viable after 24 h (4 °C) and 1 h (37 °C) of incubation, respectively. In accordance, an *E. coli* phage cocktail released from positively charged cellulose membranes produced a reduction of *E. coli* cells to undetectable levels when meat was stored at 4 °C (Anany et al., 2011). Also, significant reductions were observed when *Pseudomonas* cells were challenged on a meat surface against WPI films at 5 °C, though these films were added with sodium lactate or polylysine instead of phages (Zinoviadou, Koutsoumanis, & Biliaderis, 2010). Similarly, another study reported a significant reduction of *L. monocytogenes* on refrigerated smoked salmon samples coated with lactoperoxidase-added WPI films (Min et al., 2005). No bibliography was found on biocontrol assays carried out at 37 °C. Regarding our results at 24 °C, the level of *E. coli* cells than survived in meat after treatment was similar to those observed in the growth inhibition assay. Namely, a bacterial regrowth (10^3–10^7 CFU mL^{-1}) was observed in treated samples after a 24-h incubation. Nonsignificant reductions of *E. coli* cells were also found in alfalfa seeds and sprouts after a 5-days incubation at room temperature (Lone et al., 2016). Likewise, the same immobilized coliphages released from positively charged cellulose membranes that were effective at 4 °C, resulted in a nonsignificant reduction of *E. coli* cells in raw beef at 25 °C (Anany et al., 2011). This could be due to the fact that targeted bacteria may be embedded within the protein network on the meat, becoming unreachable to phage particles (Tomat et al., 2013a) and to the suboptimal growth conditions as well (Bryan et al., 2016). Furthermore, bacterial regrowth can also be facilitated by the contribution of proteins of WPC films as a supplemental carbon source as suggested for other edible films (Radford et al., 2017).

The six-phage cocktail together with the active packaging proved to be effective for reducing bacterial populations on meat specially at refrigerated and abusive temperatures. However, further research is necessary to determine adequate film composition and phage concentration to obtain films with satisfactory antimicrobial activity in order to reduce the total amount of preservatives in foods.

4. Conclusion

The findings presented in this work showed that the six coliphages mixed in a cocktail and added into WPC films were stable and effective against foodborne pathogens. WPC films released a significant number of phages that efficiently inhibited or killed pathogenic *E. coli* strains on the meat surface at refrigeration and abusive temperatures. Although several authors have found encouraging results with other phages (Radford et al., 2017; Vonasek et al., 2014, 2018), several studies performed in this work had never been previously carried out before. Namely, the stability and release of coliphages (cocktail), the growth inhibition assay by the agar diffusion method as well as the biocontrol assays in meat at 37 °C: studies conducted against *E. coli* with phages added in WPC edible films. The results of this study proved that this technology is useful, although, additional hurdles may be needed to significantly reduce or eliminate bacterial contamination by *E. coli*, especially at room temperature. In addition, further studies are necessary with the aim of enhancing the killing of bacteria in different foods in order to improve their safety.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2019.108316.

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