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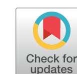
Bacteriophage ϕIBB-PF7A loaded on sodium alginate-based films to prevent microbial meat spoilage



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ABSTRACT

Despite the recent advances achieved in food industries to fulfil the growing consumer demand for high quality and food safety, microbial contamination remains a serious issue. This study aimed to incorporate ϕIBB-PF7A bacteriophage (phage) onto sodium alginate-based films crosslinked with calcium chloride, to prevent poultry spoilage caused by *Pseudomonas fluorescens*. Films were prepared by casting and characterized in terms of phage loading, distribution, stability, release profile and antimicrobial performance. Results showed that phages were successfully incorporated as evidenced by their viability and homogeneous distribution within the films as assessed by microscopy. A decrease in phage viability was only detected after 8 weeks when stored under refrigerated conditions. Antimicrobial activity demonstrated that incorporated phages significantly impaired *P. fluorescens* growth. Films' antimicrobial efficacy was further demonstrated on chicken breast fillets artificially inoculated, decreasing 2Log *P. fluorescens* viable cell counts in the first two days and reductions were maintained up to 5 days of exposure (1 Log). These results highlight that phage incorporation onto sodium-alginate-based films constitutes a simple approach of preserving the antimicrobial activity of phages in a dried and insoluble format, that can further be applied in food industry for the prevention of microbial spoilage.

1. Introduction

Food industry is one of the most important industries in the world, as it provides a basic requirement of everyday life. Food products, however, are prone to microbial contamination, which may compromise their safety and quality (Chmielewski and Frank, 2003). Microbial contamination associated to foodborne diseases represent a great concern to public health, but also play a crucial role in the food industry in terms of food spoilage. Despite the recent advances achieved within the scope of preservation methods, manufacturing practices, and quality and hygiene control, between 1/3 to 1/2 of the world food production is not consumed (Batt, 2016; Endersen et al., 2014). The sectors that contribute the most to food waste are households and processing (Stenmarck et al., 2016). Among the several reasons for this food loss, microbial spoilage plays a major role (Leyva Salas et al., 2017). A promising approach to deal with these challenges relies on the application of antimicrobial compounds to prevent the growth of pathogenic and/or spoilage microorganisms that may be present on food surfaces. This strategy also contributes to maintain the quality and safety of food products, and to improve shelf-life (Lone et al., 2016; Moreira et al., 2011; Valdés et al., 2017).

Among the antimicrobials used to be applied in food context, bacteriophages (phages) have been recognized for their great effectiveness in controlling bacterial pathogens in agro-food industry (Sillankorva et al., 2012). Phages exhibit important features that make them promising antimicrobial candidates, such as their ubiquity, high specificity against a target host or host range, self-replication capacity while their hosts are present, low inherent toxicity, easy and economical isolation and production, and a long shelf life (Abedon et al., 2017). In a food safety perspective, strictly lytic phages are possibly one of the most harmless antibacterial approaches available. Lytic phages infect and multiply inside of their specific bacterial host(s), causing lysis and the release of the newly formed virus particles, starting a new infection cycle in the non-infected hosts that were not infected in the previous round (Pires et al., 2017). In the last years, the use of phages has been a subject of great interest and research, prompting FDA to proclaim some phage-based products the status of generally recognized as safe (GRAS) (FDA, 2012).

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Antimicrobials can be applied on solid food surfaces using techniques such as spraying, dipping or brushing. However, direct application of antimicrobials on food products faces important challenges, such as poor solubility in aqueous systems, limited stability against chemical or physical degradation, uncontrolled release and possible adverse effects on food sensory qualities (Yezhi et al., 2016). Protecting antimicrobials within a matrix is, therefore, an important strategy to address these issues. This matrix can act as a delivery system which can be classified into three major groups, according to their structural and physicochemical properties: emulsion-based, micro or nanosized carrier-based, and film/coating-based (Yezhi et al., 2016). In the last years, several delivery systems have been developed using phages but most of them are mainly directed to control the growth of pathogenic species in food products (Colom et al., 2015; Korehei and Kadla, 2014; Vonasek et al., 2014). Only a few studies, using other antimicrobial agents, have tested their antimicrobial effect against spoilage microflora (Zinoviadou et al., 2010).

Between the several biopolymers used for the preparation of films as delivery systems, sodium alginate has received great attention due to its ability to react with polyvalent metal cations, specifically with calcium ions, to form strong gels or insoluble polymers (Russo et al., 2007). Furthermore, according to FDA (21CFR184.1724 revised on April 1st, 2018), this biopolymer has GRAS status for food applications as stabilizers, thickeners, texturizers, emulsifiers (Rhim, 2004). Sodium alginate is a linear water-soluble polysaccharide consisting of monomeric units of 1–4 linked β-D-mannuronate (M) and α-L-guluronate (G) at different proportions in the chain (Ko et al., 2013). It has been demonstrated that crosslinking results from the ionic interactions between calcium ions and the guluronic acid residues' carboxyl groups of the two neighboring alginate chains, causing the formation of a three-dimensional network, designated by “egg-box” structure. This calcium-induced gelation process increases the tensile strength and the structure cohesion, resulting in stronger films that become insoluble in water (Li et al., 2007). There are several studies reporting calcium crosslinking in the production of alginate films or coatings to extend the shelf-life of food products such as cheese, fruits and vegetables (Del Nobile et al., 2010; Mitrakas et al., 2008; Rojas-Graü et al., 2007).

Pseudomonas fluorescens is a known spoilage causing agent of freshly poultry and refrigerated foods, in particular refrigerated meats (Arnaut-Rollier et al., 1999; Doulgeraki et al., 2012). Strategies to control its growth on food matrices include, for instance, the incorporation of flavor enhancers such as lactate salts or food additives such as ε-polylysine (Zinoviadou et al., 2010). Phage ϕIBB-PF7A, a broad-host range phage, was previously shown to be effective in removing *P. fluorescens* biofilms including young and mature biofilms formed under different conditions (Sillankorva et al., 2008a).

The main purpose of the present study was to use sodium alginate films to preserve phage ϕIBB-PF7A's activity, so it can be applied, in a solid and easy to handle format, to prevent poultry spoilage caused by *P. fluorescens*. Sodium alginate films were prepared as previously optimized (Costa et al., 2018) and phage entrapment on films was performed using a simple strategy, which does not require the use of organic solvents that may compromise phage viability.

2. Materials and methods

2.1. Materials

Alginate CR8223 (FMC BioPolymer) with M/G ratio of 65/35 and a molecular weight (MW) of 300 kDa was kindly provided by FMC Health and Nutrition. Glycerol 99% was purchased from Himedia® (India). Tris base and PEG 8000 were purchased from Fisher BioReagents™ (USA). Calcium chloride, magnesium sulfate and ferrous ammonium sulfate were obtained from Panreac Applichem (Spain) and the sodium chloride was acquired from Sigma-Aldrich (Portugal).

2.2. Bacteria and phage ϕIBB-PF7A

P. fluorescens PF7A, isolated in a previous work (Sillankorva et al., 2008b) was grown at 30 °C in liquid LB broth (Liofilchem®) or solid LB medium containing 1.2% (w/v) of agar (LBA, Acros Organics™). Phage ϕIBB-PF7A has been previously isolated and characterized (Sillankorva et al., 2008b).

2.3. Phage ϕIBB-PF7A production and titration

Phage ϕIBB-PF7A was produced using the plate lysis and elution method previously described with some modifications (Sambrook and Russel, 2001). Briefly, 10 μL of phage suspension was spread on *P. fluorescens* lawns using a paper strip and was incubated overnight at 30 °C. Afterwards, 3 mL of SM buffer [100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris/HCl (pH 7.5)] were added to each plate and were incubated for 6 h, at 4 °C and 90 rpm. The liquid and top-agar were collected, centrifuged (10 min, 10,000 × *g*, 4 °C), further concentrated with 0.1 M NaCl, and incubated for 1 h at 4 °C. The lysate was centrifuged (10 min, 10,000 × *g*, 4 °C) and the supernatant was further concentrated with 10% (w/v) PEG 8000, purified with chloroform 1:4 (v/v), and filtered (PES filter, GE Healthcare, 0.2 μm). Purified samples were stored at 4 °C until further use. Phage titration was performed according to Adams (1959). Briefly, 100 μL of diluted phage solution, 100 μL of *P. fluorescens* overnight culture, and 3 mL of molten agar were poured into a petri dish containing a thin layer of LBA. Plates were incubated at 30 °C overnight and plaque forming units (PFU) were enumerated.

2.4. Preparation of sodium alginate films and phages incorporation

Sodium alginate-based films were prepared as previously described by Costa et al. (2018). In short, a film-forming solution was first prepared by slowly adding sodium alginate powder [1 % (w/v)] to distilled water that was stirred (magnetic stirrer, 350 rpm) at room temperature for 18 h. After, glycerol was added at a concentration of 0.5% (v/v) and the solution was stirred (350 rpm) for 12 h at room temperature. Phage was added in order to have a final concentration of approximately 10^8 PFU/mL in the films, and the solution was stirred for 30 min at room temperature. The amount of phage was chosen based on preliminary studies to guarantee an initial multiplicity of infection above 1. To produce the films, 28 mL of film-forming solution was cast onto a 9.2 cm diameter Petri dish and dried at 30 °C for 48 h. Films' crosslinking with $CaCl_2$ solution was performed as previously described, using the “immersion film” method (Costa et al., 2018; Rhim, 2004), in which dried films in Petri dishes were soaked for 5 min in a solution of $CaCl_2$ [1% (w/v)]. After this period, the excess of $CaCl_2$ solution was discarded and films were put in desiccators containing a saturated solution of $Mg(NO_3)_2{\cdot}6H_2O$, at 53% of relative humidity and 20 °C. Phage-containing films were immersed in a $CaCl_2$ solution [1% (w/v)] supplemented with phage at a concentration of approximately 10^8 PFU/mL. This step of the procedure was performed to prevent phage diffusion from sodium alginate films in contact with the aqueous solution of $CaCl_2$.

The films were afterwards peeled from the Petri dishes, cut into 2 × 2 cm^2 square pieces with an average weight of 0,042 ± 0,0098 g, and stored at 4 °C for further experiments.

2.5. Phage titre after incorporation in sodium alginate-based films

The titre of incorporated phages in the films was determined by placing the 2 × 2 cm^2 square films in 15 mL tubes containing 2 mL of SM buffer, and were subjected to vigorous agitation (250 rpm, Heidolph Unimax 1010 orbital shaker) for 45 min, at room temperature, in order to promote phages' total release. The number of active phage particles was determined by PFU enumeration.

2.6. Phage distribution within the sodium alginate-based films

To evaluate the spatial distribution of phages incorporated within the films, fluorescently labelled samples were observed by confocal microscopy. Phages were stained with 100 × SYBR gold (Molecular Probes™) by adding 2 μL of SYBR gold to 1 mL of phage solution (~10^{10} PFU/mL). After 1 h of incubation in the dark, the fluorescently labelled phages were incorporated on sodium alginate solution. Confocal z-stack images of samples were acquired on a Confocal Scanning Laser Microscope (Olympus® BX61, Model FluoView 1000, Tokyo, Japan) equipped with 488 and 505–605 nm laser lines. Images were obtained with the program FV10-Ver 4.1.1.5 (Olympus®, Tokyo, Japan).

2.7. Phage stability in sodium alginate-based films and phage inactivation with temperature

The phage stability inside the films was evaluated at different time points after storage of the 2 × 2 cm^2 sodium alginate-based films with incorporated phages under refrigerated conditions (4 °C). Measurement of viable phage particles in the films was determined as previously described by PFU counting. Phage inactivation with temperature was evaluated by exposing phage solutions with a titre of approximately 10^9 PFU/mL for 15 s and 30 s, in a heating block. Phage activity after exposure to temperature was afterwards investigated by verifying the presence (+) or absence (−) of plaques on a bacterial lawn.

2.8. Phage release profile from sodium alginate-based films

To determine the release profile of phage particles from the sodium alginate-based films, the square pieces (2 × 2 cm^2) were placed in 60 mL of SM buffer constantly stirred at refrigerated conditions (4 °C). Samples (0.5 mL) were taken at several time points under sterile conditions. The buffer was refreshed after sampling in order to evaluate phage release without altering the initial sample volume. The number of phage particles released was determined by PFU counting.

2.9. Swelling index

The films' swelling index (SW) was determined as previously described (Cao et al., 2007; Costa et al., 2018) with some modifications. Films were cut into squares with 2 × 2 cm^2 size and their weight was measured. Afterwards, the previously weighted films were immersed in distilled water for 24 h at room temperature. Samples were wiped with paper filter to remove liquid excess, and the final weight was measured. The amount of absorbed water, in percentage, was calculated using Eq. (1), in which S_1 is the weight of the film after immer and S_0 is the initial weight of the film. All measurements were performed in triplicate for each type of film.

$$SW\ (\%) = \frac{(S_1 - S_0)}{S_0} \times 100 \tag{1}$$

2.10. In vitro antimicrobial activity

The antimicrobial activity of phage-entrapped sodium alginate-based films was first performed according to the Standard 206 JIS 2801 (Japanese Standards Association, 2000), with some modifications. Briefly, a bacterial suspension of *P. fluorescens* adjusted to a final concentration of 10^6 CFU/mL was prepared in LB, from which 50 μL were added on top of each 2 × 2 cm^2 films. Sodium alginate films without phages were used as control. Samples, in duplicate, were incubated for 24 h under refrigerated conditions (4 °C). Films were placed in saline solution [NaCl 0.9% (w/v)], being afterwards subjected to vigorous agitation (250 rpm, Heidolph Unimax 1010 orbital shaker) for 15 min at room temperature, in order to promote bacterial detachment from the films. The number of colony forming units (CFU) was determined by plating serial dilutions. Antimicrobial activity was also assessed using the immersion method. In brief, to a 6-well microtiter plate, sodium alginate-based films with and without phage were placed in duplicate. Three milliliters of *P. fluorescens* bacterial suspension adjusted to 10^6 CFU/mL were added to each well. The plates were incubated for 24 h under agitation at refrigerated conditions (4 °C, 90 rpm, Orbital Shaker ES-20/60, BIOSAN). After this period, samples were collected and the number of CFU determined by plating serial dilutions. In order to avoid phages to continue infecting cells once diluted and plated, samples in contact with entrapped-phage sodium alginate-based films were diluted in ferrous ammonium sulfate (2 mM prepared in saline solution) (Park et al., 2003). Three independent assays with two replicates were performed.

2.11. In vivo antimicrobial activity of bacteriophage-loaded sodium alginate-based films

Skinless chicken breast fillets were purchased in a local retail store. The fillets were aseptically cut into small pieces with 2 × 2 cm^2 size and an average weight of 3,4 ± 0,81 g. To reduce bacterial levels prior to inoculation, samples were irradiated with UV-C light for 30 min (15 min on each side) at a distance of 10–15 cm. Chicken samples were artificially inoculated with 100 μL of *P. fluorescens* inoculum (~10^6 CFU/cm^2) and kept for 30 min at room temperature for proper bacterial attachment. Afterwards, samples were placed on top of entrapped-phage sodium alginate-based films with 2 × 2 cm^2 size, previously added to a 6-well microtiter plate, which was incubated at 4 °C for 7 days. As a control, sodium alginate-based films without phage were also used. Every 24 h, samples were transferred aseptically to a sterile Stomacher bag (Seward (Fermion X) Ltd., UK), were diluted with 20 mL of saline solution and blended in Stomacher for 10 min at 175 rpm and room temperature. Ten-fold dilution series in saline solution of the obtained suspensions were plated in LBA containing Petri dishes, which were incubated overnight at 30 °C, and finally the numbers of CFU were counted. As described above, samples in contact with entrapped-phage sodium alginate-based films were diluted in ferrous ammonium sulfate (2 mM prepared in saline solution) (Park et al., 2003). The samples were, afterwards, put through a Stomacher blending and were further serial-diluted and plated. Three independent assays were performed.

2.12. Statistical analysis

Results are presented as a mean ± standard deviation (SD). Statistical analysis was performed using Graph Pad Prism 7.0. To compare the films stability throughout the time points assessed, one-way ANOVA followed by Dunnet's test was implemented. Antimicrobial activity of films with and without phages was determined using a Two-way ANOVA, followed by a Tukey's test. In all the analysis, the used confidence interval was 95%.

3. Results and discussion

3.1. Incorporation and distribution of ϕIBB-PF7A phage on sodium alginate films

Films with ϕIBB-PF7A phage were produced by adding a concentrated phage suspension to the aqueous solution of sodium alginate containing glycerol. The titre of phages found inside these soluble films was 1.52 ± 2.12 × 10^5 PFU/cm^2. Crosslinking of the films with a solution of calcium chloride, as previously described (Costa et al., 2018; Russo et al., 2007), led to a leaching of the phages, since no viable phages could be detected inside the films. To prevent phage release, crosslinking was performed with calcium chloride containing a concentrated phage suspension. Crosslinked films presented 10-fold higher phage concentrations, (1.01 ± 0.92) × 10^6 PFU/cm^2, as a result of

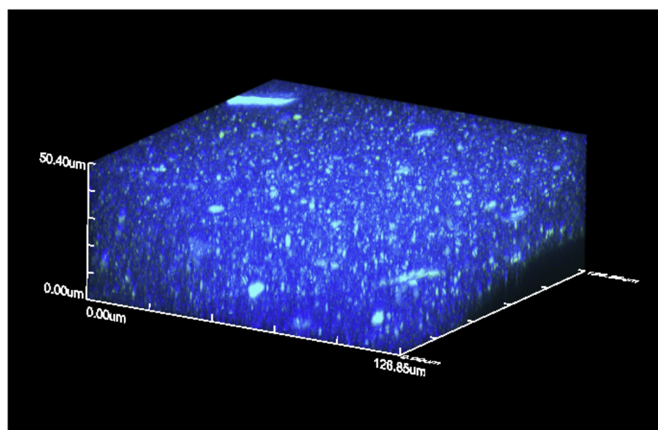

Fig. 1. 3D confocal microscopy image of SYBR gold-labelled ϕIBB-PF7A (green) incorporated in the sodium alginate film (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

additional physical absorption of phage from the solution.

Confocal microscopy was used to understand how phage particles are spatially distributed inside the films (Fig. 1). Using SYBR gold, it was possible to distinguish the phage particles (green) inside the autofluorescent alginate matrix (blue). For the z-stack analysis performed, a distance including the entire thickness of films was chosen to evaluate phage distribution. Phages were distributed relatively uniformly across the entire depth and surface of the film. Occasional phage agglomerates were also present. These could be the result of the film drying process or the presence of net opposite charges in the head and tail regions of the phage.

3.2. Phage stability inside sodium-alginate films

Once phages' homogeneous incorporation inside the films was established, their stability in these films was determined, as this is a crucial requirement for their successful application in food systems. Fig. 2 shows the stability results for ϕIBB-PF7A phage incorporated in sodium alginate-based films under refrigerated conditions (4 °C), over a period of 12 weeks. Results only showed a decrease in phage activity of approximately 2.4 Log after a period of six to eight weeks and a 4.2 Log after 12 weeks. Previous studies have demonstrated that the incorporation of phages in polymeric matrices may result in the loss of their activity over time (Velasco-barraza et al., 2006; Vonasek et al., 2014). This loss of activity may be attributed to the incorporation

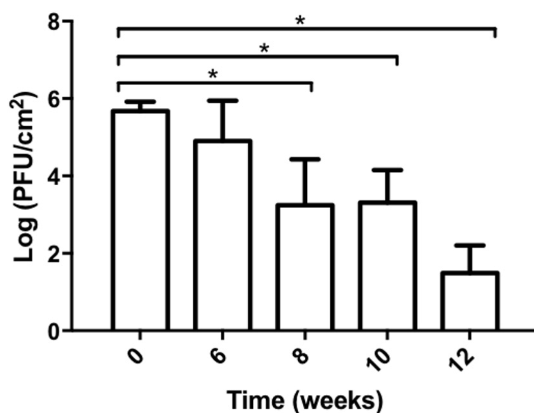

Fig. 2. Stability of phages in sodium alginate-based films under refrigerated conditions (4 °C) during time. * indicates significant differences ($p < 0.05$) between time zero and conditions tested.

process, during which phages are exposed to shear stresses through mixing and agitation, and further desiccation stress during drying (Malik et al., 2017). For instance, phages' incorporation in acetate cellulose films compromised their viability, since no viable phages were detected after two weeks of storage under refrigerated conditions (Gouvêa et al., 2015). Differences found between sodium alginate and acetate cellulose films may be credited to the use of organic solvents for cellulose film preparations, highlighting, once more, the advantage of sodium alginate as a polymeric matrix for phage entrapment.

The overall results emphasize the ability of sodium alginate-based films to keep phages viable under refrigerated conditions, allowing their storage up to eight weeks before their application onto package systems. Furthermore, taking into account that refrigerated meat must be consumed within, approximately, one week after packaged, the loss of stability found should not be a problem for the industrial application of films. The presence of phages on chicken fillets and their subsequent human consumption do not present a problem because they are completely inactivated during cooking. In fact, ϕIBB-PF7A phage was inactivated already after 15 s exposure at 77.5 °C and 30 s exposure to a lower temperature (67.5 °C) (Table S1 on Supplementary Material).

3.3. Swelling properties

An important property of alginate-based films, in their dry form, is their ability, after being in contact with a fluid, to rehydrate, absorbing the fluid and undergo a swelling process. This process is mainly associated with the hydration of the hydrophilic groups of alginate (Sarheed et al., 2015). It is, therefore, a desired characteristic and it can determine their ability to absorb, similarly to the commonly used white absorbent pads found in packages, the exudates liquids that naturally ooze out of meats, and poultry. Table 1 shows a high rate of swelling of sodium alginate-based films, as previously reported (Costa et al., 2018). Most importantly, phage incorporation did not interfere with the swelling ability of the films. This high swelling ability and stability of the films after swelling is explained by the crosslinking process implemented. In this process, the number of G blocks linked with the calcium ions - which leads to the “egg-box” conformation - is sufficient to increase the films resistance. By doing so, these films no longer dissolve in water. However, there are still strands available to uptake water, resulting in the high values of swelling index obtained as already previously reported by other authors (Li et al., 2007).

3.4. In vitro release of phages from sodium alginate-based films

Another critical requirement for antimicrobial films application on food products is the release of the active compound from the films, so that these will be able to act on the microorganisms present in food. Fig. 3 presents the release profile of phages from the films in SM buffer during time. These results showed that, upon contact with an aqueous solution, most of the incorporated phage were released within the first 2–3 min. These outcomes are in accordance to the results found during films crosslinking optimization, in which after 5 min of exposure to calcium chloride solution, phage particles could no longer be found inside the films. A potential explanation for this fast release is the small size of ϕIBB-PF7A phage, a T7-like lytic phage with a head diameter of about 63 nm and a tail size of about 13 × 8 nm (Sillankorva et al.,

Table 1
Swelling index (%) of sodium alginate films before and after phage incorporation.

Sample	SW (%)[a]
Control	723 ± 121
Phage-entrapped film	720 ± 142

[a] Means in columns do not differ statistically ($p > 0.05$).

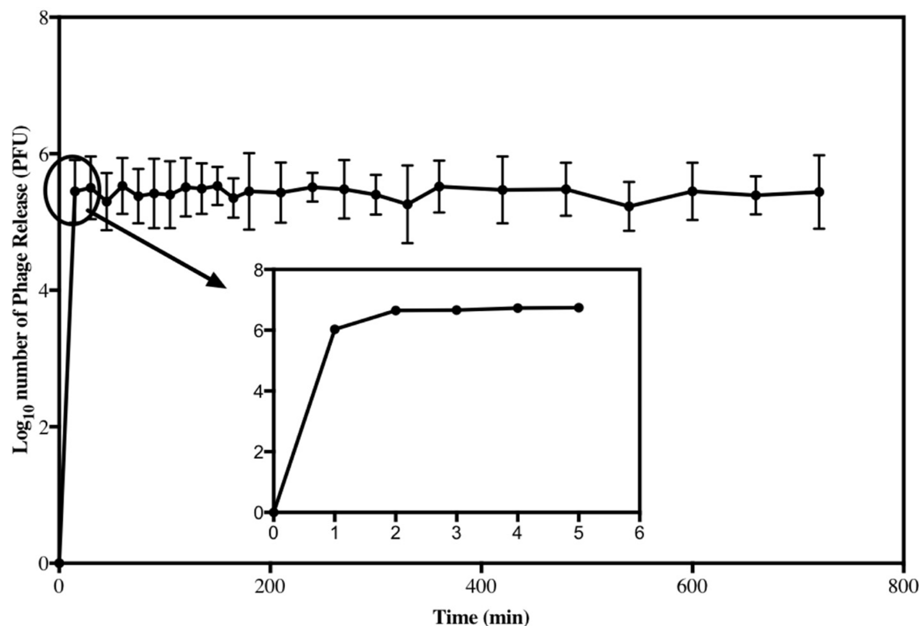

Fig. 3. *In vitro* release profile of ϕIBB-PF7A phage from sodium alginate-based films.

2008b). The fast release and the high swelling ability found in these sodium alginate-based films suggests that the majority of phages are released during the swelling period. This, however, will not be the situation in packages, since the films will not be soaked in liquid but rather in contact with food matrices, and this will most likely result in a slower kinetic release (Zactiti and Kieckbusch, 2009).

3.5. Antimicrobial activity of films

The antimicrobial activity of films was evaluated using two approaches. In the first approach, a bacterial suspension of *P. fluorescens* was added on top of each film, which were then incubated for 24 h under refrigerated conditions (4 °C). The second approach consisted in using an immersion method, in which the films were completely immersed in a bacterial suspension of *P. fluorescens*. Results presented in Fig. 4 showed that *P. fluorescens* grew on the control films (films without incorporated phage), especially when using the immersion method. Possibly this was due to the higher volume of bacterial suspension in LB added, that probably favored bacterial growth (Gouvêa et al., 2015). On the other hand, films with phages incorporated, impaired significantly the growth of *P. fluorescens*, as evidenced by the 1.9 and 4.0 Log reductions after 24 h, using the contact and immersion approaches, respectively. The higher efficiency exhibited for the immersion approach may be explained by the phages fast release when exposed to liquid solutions, as previously described herein in the release experiments. In the immersion method, there were more bacteria in the suspension since a higher volume was used. It is, therefore, easier for phage particles to find the host bacteria in this liquid environment, upon application, and also after progeny release leading faster to new cycles of infection (Gouvêa et al., 2016). The antimicrobial effect observed in our work is similar or better than the ones reported by other authors, using broad spectrum antimicrobial agents on food materials. For instance, in a study performed by Zinoviadou et al., in which whey protein isolate films functionalized with sodium lactate and 3-polylysise were used, a 2–3 Log reduction in the growth of *Pseudomonas* spp. after being in contact with meat surfaces was reached (Zinoviadou et al., 2010). Other study reported a 4.2 Log reduction of *Listeria monocytogenes* on smoked salmon surfaces in contact with whey protein isolate films, functionalized with an antimicrobial enzyme, lactoperoxidase (Min et al., 2005). Overall, the results presented in our work show an important reduction of *P. fluorescens* revealing that phages provide an efficient antimicrobial activity when incorporated in alginate films.

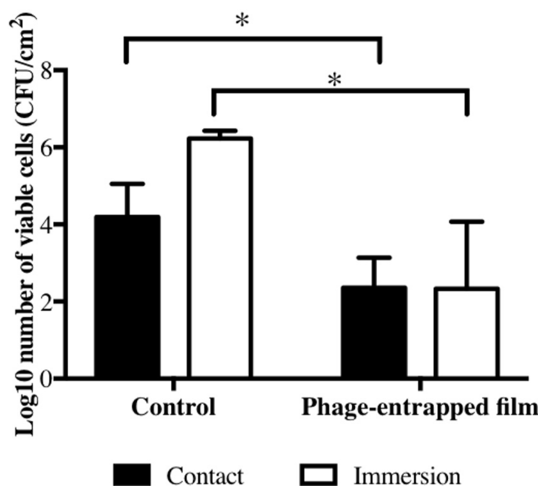

Fig. 4. Antimicrobial activity of ϕIBB-PF7A phage incorporated in sodium alginate-based films over a period of 24 h when in direct-contact with *P. fluorescens* (contact) or when completely immersed on a bacterial suspension (immersion). Sodium alginate-based films without incorporated phage were used as control. * indicates significant differences ($p < 0.05$) between control and phage-entrapped films.

3.6. Efficacy of films in reducing P. fluorescens on chicken breast fillets

Meat has been recognized as one of the most perishable food products. This feature is attributed to its chemical composition that stimulates microbial growth to levels responsible for meat deterioration and spoilage (Doulgeraki et al., 2012). Therefore, once the antimicrobial activity *in vitro* of alginate-based films with incorporated phages was established, the next step was to evaluate their efficacy when applied on chicken breast fillets, artificially contaminated with *P. fluorescens*. Before inoculation, samples were irradiated with UV light to reduce the natural microflora on chicken fillets that has been identified (Holck et al., 2014). UV applications comprise pasteurization of juices,

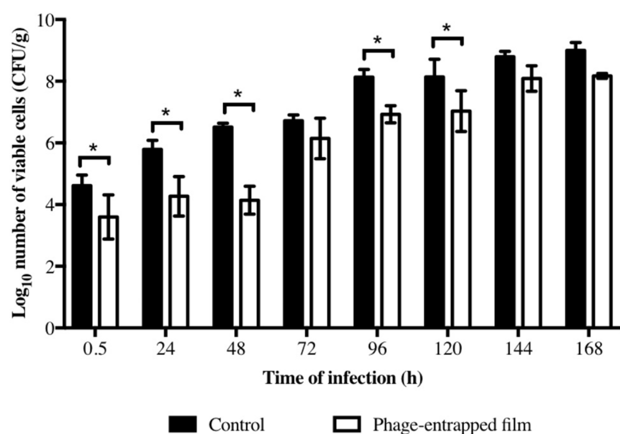

Fig. 5. Efficacy of ϕIBB-PF7A phage incorporated in sodium alginate-based films on reducing *P. fluorescens* when applied on chicken breast fillets for a period of 7 days. Sodium alginate-based films without incorporated phage were used as controls. * indicates significant differences ($p < 0.05$) between control and phage-entrapped films.

post lethality treatment for meats, treatment of food contact surfaces and to prolong the shelf-life of fresh produce. In recent years, there has been a growing interest in UV light for decontamination of poultry, as an alternative to traditional thermal processing (Gayán et al., 2012; Graça et al., 2017; Stermer et al., 1987; Yeh et al., 2018). After UV treatment a reduction of approximately 1 Log was achieved (data not shown), so it should be emphasized that it was not possible to control some background microflora associated with meat samples.

Results in Fig. 5 show that *P. fluorescens* was able to grow on the surface chicken breast fillets. The initial concentration of *P. fluorescens* increased by approximately 4 Log at the end of the experiment. Alginate-based films with incorporated phages were able to impair this growth by 2 Log, especially after the first two days of exposure, and these films still provided antimicrobial effect after 5 days, reducing by 1 Log the viable counts compared to non-phage exposed fillets. After 6 and 7 days stored at 4 °C, even though there was a slight reduction of viable cells in the samples in contact with active films, no statistical differences between treated and control samples were perceived. It should be emphasized, however, that the poultry expiry date had already been reached on the sixth day. Furthermore, as aforementioned, chicken samples used in this this assay where not completely sterile, comprising other bacteria against which this phage had no activity. Their presence will inevitable affect the action of phages, since these can grow in the same areas where *P. fluorescens* had adhered and, if these other strains are good EPS producing strains, they can mask ϕIBB-PF7A receptors present in the cell surface. Further experiments need to be performed to better assess the microbiome in commercially purchased chicken fillets. This can, for instance, be done using culture methods (*e.g.* selective media), multiplex PCR with specific primers for the commonly described bacteria isolated in poultry, among others. Nonetheless, being ϕIBB-PF7A a broad host range phage (Sillankorva et al., 2008b), if other *P. fluorescens* strains are present in the samples, this phage will probably be able to kill them. It needs to be highlighted that films performance was evaluated in a scenario that better mimics a real application, where other strains are unquestionably present.

In summary, phage containing films applied on commercial poultry fillets were able to control bacterial growth for a period up to 5 days. These results suggest that the phages released to a solid food product exhibited a different kinetics than the one obtained on an aqueous solution, prolonging in the first the release of the antimicrobial agent. Although further experiments should be performed to understand the release of phages upon contact with poultry, the results obtained evidence that a fast release upon contact with an aqueous solution should not be a problem for further applications of phage-entrapped films in food industry to prevent product spoilage. Furthermore, the contact with non-liquid samples maintains phages' antimicrobial proficiencies. Additionally, spoilage originated from microbial development is often associated to the release of volatile organic compounds that can lead to rejection of the meat (Casaburi et al., 2015; Cutter and Siragusa, 1994). Since these compounds are the results of microbial development and consumption of meat nutrients by bacteria such as *P. fluorescens*, reducing the microbial loads on food matrices should hypothetically also reduce the generation of such products.

4. Conclusions

This study shows that the application of alginate-based films crosslinked with calcium chloride is a promising delivery system of phages within the food context, using a simple and efficient approach. Phages incorporation did not compromise their viability, which was preserved for long periods of time (8 to 10 weeks), under refrigerated conditions. Films' ability to interact with water was not influenced by phages incorporation, as supported by the high swelling index obtained. Phage release from these films upon contact with chicken fillets prevented the growth of *P. fluorescens* up to 5 days. Overall, this study highlights the great potential of sodium alginate-based films to preserve phage activity in a dried format. This platform should be further explored to incorporate a cocktail of phages to increase the spectrum of action of these films, in order to prevent not only this spoilage bacterium but also to minimize pathogenic bacterial levels as well.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.11.026.

Author's contributions

DA and AM equally contributed to the paper and conducted the majority of the experiments. DA wrote the manuscript. CM contributed in the phage production experiments and MJC in the swelling experiments. LMP, MAC, and SMS reviewed and edited the manuscript writing and supervised the work.

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