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Effectiveness of bacteriophages incorporated in gelatine films against *Staphylococcus aureus*

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Highlights:

- Bacteriophage phiIPLA-RODI in gelatine films remained active against *S. aureus*.
- Films' physical properties were unaffected by incorporation of bacteriophages.
- The use of a gelatine-free solution of phages was the least effective treatment.
- Coatings showed better antimicrobial performance than previously dried films.

ABSTRACT

The use of antibiotics in the food industry is declining due to the emergence of resistant bacteria. Thus, the use of bacteriophages may provide a suitable alternative, since it allows the selective elimination of microorganisms. Another issue that faces the industry is concern about the difficulty of removing petroleum-derived plastics from the

environment. This problem makes the search for new food packaging materials with extended properties a necessity. In this study, edible gelatine films containing bacteriophages were produced, and the effects of increasing concentrations of bacteriophages on the light transmission, water vapour permeability, solubility and mechanical properties of the films were characterised; in addition, micrographs of transverse film sections were made and analysed. Finally, with the purpose of assessing the influence of the manner of application and the antimicrobial properties of the prepared packaging materials, pieces of cheese previously contaminated with *Staphylococcus aureus* were either coated by immersion in the film-forming solution or wrapped directly with gelatine films loaded with bacteriophages. According to the results obtained, the physical properties of the films remained unaltered, irrespective of the bacteriophage concentration. The pieces of cheese immersed in the film-forming solution showed higher microbial reduction than the pieces of cheese wrapped with previously dried films. Overall, the packaging materials prepared possessed concentration-dependant antimicrobial properties, but the results obtained underline the importance of the manner of application of the bacteriophages on the foodstuff in maximizing their antimicrobial properties.

Keywords: packaging, edible films, antimicrobial properties, bacteriophages, *Staphylococcus aureus*, cottage cheese.

1. INTRODUCTION

Edible films in the food industry must provide a barrier against humidity and oxygen, and prevent the movement of solids out of the food matrix (Guilbert, Gontard, & Cuq, 1995). These edible films are usually prepared with natural, totally biodegradable polymers, and they must meet certain requirements to be useful in this role. Thus, in addition to their barrier properties, edible films must maintain the colour and appearance of the food they wrap, they must have the mechanical strength to be handled and to support the pieces of food they contain, and they must be harmless

when they are consumed together with their contents. These properties depend on the raw materials selected to prepare the films, their fabrication process and finally, the mode of application (Guilbert, Gontard, & Gorris, 1996). Among the biopolymers that have been considered for preparing these films, polysaccharides and proteins must be highlighted, and among them, edible films based on gelatine occupy an important position, as is clear from the extensive research reported in the literature on this subject (Etxabide, Uranga, Guerrero, & de la Caba, 2017).

Gelatine is produced by the thermal hydrolysis or physical and chemical degradation of collagen, a protein widely present in the bones and skin of animals (Avila-Rodríguez, Rodriguez-Barroso, & Sánchez, 2018). Gelatine possesses a high degree of biocompatibility and biodegradability, and it has been widely used in the food industry as a gelling agent and as an ingredient that stabilises foams and emulsions. Furthermore, films prepared using gelatine are transparent and mechanically strong enough to coat pieces of food effectively; moreover, the properties of these films can be easily improved by introducing bioactive compounds in their formulation. To this end, Sáez-Orviz et al. (2020) prepared gelatine films loaded with polylactic acid nanoparticles with antimicrobial properties, Kanmani et al. (2014) blended gelatine with silver nanoparticles to prepare antimicrobial composite films, and Neira et al. (2019) prepared edible fish gelatine films with added carvacrol, increasing the storage period of breaded hake medallions. Other authors have added red cabbage extracts (Musso, Salgado, & Mauri, 2019), eugenol (Dammak & Sobral, 2019), pomegranate peel powder (Hanani, Yee, & Nor-Khaizura, 2019) or tea extracts (Wu, et al., 2013) to gelatine films to give them different functional properties. In this regard, bacteriophages are an antimicrobial agent gaining interest among the scientific community. They are a group of viruses that are able to infect and to lyse bacteria with high specificity, so they are harmless to animals, plants or any bacteria other than the specific bacterial strain recognised by each particular species of bacteriophage. Because of this,

bacteriophages have been used in a wide range of biotechnology applications since they were discovered in 1915 (Sillankorva, Oliveira, & Azeredo, 2012). Furthermore, bacteriophages occur naturally on the surface of many foodstuffs, so they are frequently consumed by humans with no health risk, and moreover, there are already several commercial phage cocktails such as EcoShield™, Salmo Fresh™, and ListShield™ approved by the FDA for their application directly on foodstuffs (Sadekuzzaman, Yang, Mizan, Kim, & Ha, 2017). In this sense, several authors have taken advantage of the antimicrobial properties of bacteriophages to prepare different films and coatings to cover foodstuffs. The biopolymers that have been used for this purpose are sodium alginate (Alves, Cerqueira, Pastrana, & Sillankorva, 2020; Alves, et al., 2019), xanthan gum coating a polylactic acid film (Radford, et al., 2017), chitosan (Amarillas, et al., 2018; Cui, Yuan, & Lin, 2017), whey protein (Vonasek, Le, & Nitin, 2014), methylcellulose (Kalkan, 2018), and acetate cellulose (Gouvêa, Mendonça, Soto, & Cruz, 2015). Although all these papers are relevant to the food technology field, none of them assess how an increasing concentration of bacteriophages might affect the film matrix, and furthermore, regarding the antimicrobial properties of the packaging materials, neither do they seek to investigate whether wrapping food with a previously dried film is more or less effective than a coating prepared by submerging the piece of food in the same film-forming solution used to create the dried film.

In this study several concentrations of the bacteriophage pilIPLA-RODI, which was discovered in a sewage treatment plant in Asturias, Spain, and is able to lyse the food-poisoning bacteria *S. aureus* (Gutiérrez, et al., 2015), were mixed with gelatine and glycerol to prepare film-forming solutions. These film-forming solutions were used to prepare edible films, in which the effect of the bacteriophages on the film matrix was assessed; furthermore, in order to test the influence of the method of application of the bacteriophages on their antimicrobial activities in a real-case scenario, previously

contaminated pieces of cheese were alternatively coated by immersion in the film-forming solution or wrapped with previously dried films.

2. MATERIALS AND METHODS

2.1. Preparation of gelatine films with phiIPLA-RODI bacteriophage

The stock of bacteriophage phiIPLA-RODI (Gutiérrez, et al., 2015) in TSB (Tryptic Soy Broth, ref. 22902, Sigma-Aldrich, Germany) medium with a titre of 7×10^8 PFU/mL and the strain *S. aureus* IPLA1 isolated from contact surfaces of the dairy industry (Gutiérrez, et al., 2012) were kindly donated by the Dairy Research Institute of Asturias IPLA-CSIC (Asturias, Spain).

To prepare the film-forming solutions, gelatine (gelatine from porcine skin, G1890, Sigma-Aldrich) and glycerol in water were solubilised by heating the mixture at 50 ºC for 20 minutes under continuous stirring. Then, this solution was cooled to 40 ºC and filtered using a 0.45 µm pore size syringe filter under aseptic conditions. Afterwards, different volumes of the bacteriophage stock were added and gently stirred for 5 minutes, so as to obtain final concentrations of gelatine and glycerol for every film-forming solution with bacteriophages of 10% and 2% (w/v) respectively. The final concentrations of bacteriophages tested in the film-forming solution were 1.75×10^8 PFU/mL (GF1), 1.16×10^8 PFU/mL (GF2) and 6.35×10^7 PFU/mL (GF3). A control sample with no bacteriophages was also assessed. Finally, every film-forming solution was poured into a Petri dish in such a way that 0.11 mL was cast per cm^2 of Petri dish surface. The films, still in their Petri dish moulds, were dried in a laminar flow chamber for 2 days at room temperature and then completely removed from the dishes as single intact discs.

2.2. Physical properties of gelatine films loaded with increasing concentrations of bacteriophages

2.2.1. Light transmission and transparency

The barrier properties of the films against ultraviolet and visible light were assessed according to Dick et al. (2015). Briefly, films were cut into rectangular pieces and placed in a spectrophotometer test cell. The light transmission of the samples was tested using a Helios gamma spectrophotometer (Thermo Fisher Scientific, USA), from 280 to 800 nm, with an empty test cell as reference. The transparency of the films was calculated according to equation 1:

$$Transparency = A_{600}/x \quad (1)$$

Where A_{600} is the absorbance of the film sample at 600 nm and *x* is the film thickness (mm).

A digital micrometer (Mitutoyo C., Japan), with a precision of ± 1 µm, was used to measure the thickness of the films. This thickness was measured in five different areas, one of them in the centre of the film and the other four around the film perimeter.

2.2.2. Mechanical properties

The mechanical properties of the gelatine films loaded with bacteriophages were tested by means of a puncture test according to the methodology described by Sobral et al. (2001), and using a TA.XT.plus Texture Analyser (Stable Microsystems, UK) equipped with a 5 kg load cell and a 5 mm diameter probe (P/5S). For this purpose, the films were cut into 4 x 2 cm strips and placed in the texture analyser between two plates. These plates have a hole, allowing contact between the film sample and the probe, which can stretch the film to breaking. In this case, the probe speed was 1 mm/s and the puncture strength (PS) and puncture deformation (PD) values were obtained according to equations 2 and 3 (Otero-Pazos, et al., 2016):

$$PS = Fm/Th \quad (2)$$

$$PD = \left(\sqrt{D^2 + R^2} - R\right)/R \quad (3)$$

Where *Fm* is the maximum force applied before the film breaks, *Th* is the film thickness, *D* is the distance covered by the probe while it is in contact with the film until the film is broken, and *R* is the radius of the orifice in the plates.

2.2.3. Water vapour permeability and solubility

Polyvinyl chloride cups were filled with distilled water and sealed with films that had been cut into circles with the same diameter as the cup mouth. A height of 1 cm was left between the water surface and the gelatine films, and the thickness of the film samples was measured. The samples used in this experiment were visually checked and films with pinholes or breakages were discarded. Finally, the containers were weighed, placed in desiccators with silica gel, and the change in their weights was registered every hour for 7 h. The weight loss was plotted against time and the water vapour transmission rate (WVTR) was calculated according to equation 4:

$$WVTR = G/(t \times A) \quad (4)$$

Where *G/t* is the change in the weight of the cup per unit of time (g/h) and *A* (m^2) is the area of the cup mouth covered by the film.

These WVTR values can be used to calculate the water vapour permeability (WVP) by means of equation 5:

$$WVP = (WVTR \times Th)/\Delta P \quad (5)$$

Where *Th* (mm) is the film thickness and ΔP (kPa) is the difference in partial pressure between the two faces of the film.

The solubility measurement was conducted according to Marcet et al. (2017), with some minor modifications. Briefly, gelatine films loaded with bacteriophages were cut into circles of 2 cm diameter, and their dry weight was obtained by drying them at 80 ºC in an oven for 24 h.

Other intact film circles were immersed in a buffered solution of Trizma 0.1 M pH 7.0 at room temperature and, after 24 h, the undissolved film remains were recovered by

vacuum filtration using Whatman nº 1 paper that had been weighed previously. Finally, the paper, together with the remains, was dried at 80 °C for 24 h and weighed. The following equation was used to calculate the percentage of undissolved film:

$$S(\%) = (m1 - m2/m1) \times 100 \quad (6)$$

Where *S(%)* is the percentage of solubilised film, *m1* is the initial dry weight of the film and *m2* is the dry weight of the non-solubilised film remains.

2.2.4. Scanning electron microscopy (SEM)

Micrographs were taken with a JSM-6610LV (JEOL, USA) scanning electron microscope with the aim of studying the microstructure of the transverse section of the gelatine films loaded with bacteriophages. For that purpose, film samples were cut into square pieces of 1 x 1 cm using a surgical blade. These films were attached perpendicularly around stubs using double-sided carbon-based tape as adhesive and then the films were gold-sputter-coated for 5 min in an argon atmosphere. The magnification used to observe the transverse section of the films was × 900, and the voltage was set at 20 kV.

2.3. Antimicrobial activity of films and coatings

2.3.1. In vitro antimicrobial activity of films

In order to determine the inhibitory capacity of phiIPLA-RODI in the films after the drying step, a test was performed in TSB liquid medium. For that purpose, a 0.4 g piece of each of the films prepared with the different bacteriophage concentrations described in section 2.1. was immersed in 100 mL of TSB medium with an initial concentration of 10^6 CFU/mL of *S. aureus* IPLA1. Therefore, once the pieces of film were dissolved in TSB, the concentrations of bacteriophages in the liquid medium were 5.25×10^6 PFU/mL (GF1-TSB), 3.48×10^6 PFU/mL (GF2-TSB) and 1.90×10^6 PFU/mL (GF3-TSB). Furthermore, a film made exclusively from gelatine was tested in the same conditions (G), and another control sample with just TSB infected with *S. aureus* was

also assessed (WB). These samples were incubated for 17 h at 37 ºC, under orbital stirring at 250 rpm. Afterwards, the liquid media were diluted with 0.7% NaCl and seeded in Baird-Parker agar medium enriched with egg yolk tellurite emulsion (Sigma-Aldrich, USA), a *Staphylococcus*-selective agar medium. After 48 h of incubation at 37 ºC, the colonies were counted, and the results expressed as $\log_{10}$ CFU/mL.

2.3.2. Antimicrobial properties of films and coatings on cheese pieces contaminated with *S. aureus*

To investigate the antimicrobial activity of the films and coatings, 100 g of fresh cheese was purchased in a local market and divided into several cylindrical pieces of 1 g using a hollow punch. Afterwards, every piece of cheese was infected with 100 µL of 10^5 CFU/mL of *S. aureus* in 0.7% NaCl. These contaminated pieces of cheese were then each submitted to one of the following treatments:

a) The bacteriophage stock solution was diluted in TSB to the same concentration as GF1, GF2 and GF3 for the film-forming solutions described in section 2.1. (1.75×10^8 PFU/mL (GF1), 1.16×10^8 PFU/mL (GF2) and 6.35×10^7 PFU/mL (GF3)) and, for every concentration prepared, three contaminated pieces of cheese were tested. For that purpose, the pieces of cheese were immersed in one of these solutions, stirred gently by hand for 3 min, recovered and saved in tightly closed polypropylene tubes under refrigeration (4 ºC). A control sample with contaminated cheese and immersed in TSB but without bacteriophages was also tested.

b) Contaminated pieces of cheese were immersed in gelatine film-forming solutions with bacteriophages at the GF1, GF2 and GF3 concentrations (as described in section 2.1.). After 3 min, the pieces were recovered, dried at room temperature for 10 minutes, and saved in polypropylene tubes at 4 ºC. A control sample with contaminated cheese immersed in gelatine with no bacteriophages was also assessed.

c) Gelatine films loaded with different proportions of bacteriophages were prepared as was described in section 2.1. These films were used to wrap the contaminated pieces of cheese, thermosealed, and stored at 4 ºC.

In all cases, samples were taken at time 0 and after 3 and 6 days, a characteristic sampling time for this type of food product (Amatiste, et al., 2014). The pieces of cheese to be sampled were placed in sterilised plastic bags with 10 mL of 0.7% NaCl and triturated using a Stomacher (IUL Instruments, Barcelona, Spain) for 120 s. Finally, the liquid sample was diluted and seeded in Baird-Parker medium with 2% agar. After 48 h of incubation at 37 ºC the colonies were counted, and the results expressed as $\log_{10}$ CFU/mL.

2.4. Statistical analysis

Analysis of variance (ANOVA) was applied. Least significant differences (LSD) were calculated by Fisher's test to determine significant differences between the tested samples. These analyses were performed using Statgraphics® V.15.2.06 statistical software.

3. RESULTS AND DISCUSSION

3.1. Physical properties of gelatine films loaded with increasing concentrations of bacteriophages

3.1.1. Visual aspect of the films, light transmission and transparency

The prepared films were easily peeled from the Petri dishes in one piece and were homogeneous and easy-to-handle in every case tested; moreover, they were found to be neither brittle nor sticky. Their visual appearance is shown in Figure 1, and it was noticeable that all of them were completely transparent, with no visual difference

between the control and the samples loaded with bacteriophages, even at the highest bacteriophage concentration tested.

The transmittance values for the films at 280 nm were lower than at the other wavelengths tested (Table 1), which could be explained by the presence of aromatic amino acids in the gelatine's composition. However, in comparison with other edible protein-based films, such as those prepared using delipidated egg yolk proteins (Marcet, et al., 2017), these light transmission values at 280 nm could be considered high. This is because, although these aromatic amino acids are present, their contribution to the primary structure of the gelatine peptides is low in comparison to that found in the components of other protein-based films. To be more specific, pork skin gelatine protein contains just 3 amino acid residues of tyrosine, 14 of phenylalanine, and no tryptophan per 1000 amino acids. (Zhou, Mulvaney, & Regenstein, 2006). In fact, a slight difference can be observed between the light transmission properties of the films tested; the film with the highest concentration of bacteriophages in its composition showing the lowest values at every wavelength tested. These differences can also be observed in the transparency value, for which the higher the concentration of bacteriophages, the lower was the transparency value. However, although these differences were measurable, they were not great enough to be appreciated by visual inspection.

3.1.2. Mechanical properties

The mechanical properties of the gelatine films loaded with bacteriophages are shown in Table 2. In this case, no statistical difference was detected between any of the films tested. They all showed a statistically similar thickness, PS and PD value. The bacteriophage phiIPLA-RODI is a *Myoviridae*, which belongs to the *Spounavirinae*

subfamily (Gutiérrez, et al., 2015); the members of this family possess heads of 87-94 nm diameter and tails that are 140-219 nm long (Lavigne, et al., 2009). So, taking into consideration the thickness of the gelatine film, the dimensions of one bacteriophage are small enough not to produce any disruption in the protein packaging film; however, a high bacteriophage concentration may lead to structural problems in the film matrix, since the protein chain packaging involves forces such as disulphide bonds, hydrophobic interaction, electrostatic forces and hydrogen bonds (Wihodo & Moraru, 2013). These compacting forces can be weakened if the bacteriophages introduce sufficient heterogeneity into the film matrix to hinder the physical approach of the protein chains. In this study, a stock with a bacteriophage concentration of $7x10^8$ PFU/mL was used, but this number cannot be related to a particular number of bacteriophage particles, and in any case, the results obtained indicate that the phage concentrations used in these films were too low to produce any effect on either the strength or the flexibility of the gelatine films prepared, even for the films with the highest concentration of bacteriophages. Similar findings were reported by other authors, and in this regard, Gouvêa et al. (2015) did not find any statistical difference in the values of the PS parameter between control films prepared using acetate cellulose and those prepared with acetate cellulose and bacteriophages.

3.1.3. Water vapour permeability (WVP) and solubility

The WVP of biopolymer-based films depends on several factors, such as the kind and concentration of biopolymer used, the kind of plasticiser, and the amount and nature of the additives included in the film matrix to extend their functional properties. Furthermore, there are two models to explain the water barrier properties of a protein-based film, one of which refers to the formation of voids in the internal structure of the film matrix during the drying step (Ukai, Ishibashi, Tsutsumi, & Marakami, 1976), while the other involves the formation of water micropathways, which are a result of the

hydrophilic nature of the polymer matrix itself (Krochta, 1990). Therefore, it is a film property that is closely related to the microstructure of a protein-based film. In these experiments, as is shown in Table 3, the incorporation of bacteriophages in the film matrix did not produce any effect on the WVP of the gelatine films tested, which suggests that there was no alteration of the film microstructure caused by the presence of bacteriophages at the concentrations tested. Similar results were obtained by Alves et al. (2020), who found no statistically significant differences in this parameter between sodium alginate films with bacteriophages and those prepared without bacteriophages used as control.

The solubility values of the films are also shown in Table 3. In this case as well, the incorporation of the bacteriophage did not produce any statistically significant change in the parameter assessed, which supports the previous suggestion that the addition of bacteriophages to the film-forming solution did not change the overall hydrophilicity of the films produced.

3.1.4. Scanning electron microscopy (SEM)

Micrographs of the gelatine films loaded with bacteriophages are shown in Figure 2. Their microstructure was seen to be similar for every sample tested, showing a smooth, compact, continuous film matrix, similar to other gelatine film micrographs found in the literature (Ge, Wang, Shi, & Yin, 2012). As was suggested in the tests performed before, the incorporation of bacteriophages did not produce any noticeable change in the film microstructure.

3.2. Antimicrobial activity of films and coatings

3.2.1. In vitro antimicrobial activity of films

S. aureus IPLA1 growing free in a TSB liquid medium reached a mean concentration of 3.85×10^9 CFU/mL after 17 h of incubation at 37 °C (Figure 3, sample WB), while a similar value to that was obtained for the contaminated TSB liquid medium treated with gelatine film without bacteriophages (G), confirming that gelatine proteins do not have any growth-inhibiting effect on *S. aureus* IPLA1. On the other hand, every microbial assay performed with gelatine films loaded with phiIPLA-RODI at any of the concentrations tested showed a decrease in the microbial load at the end of the test. In this case, samples GF2-TSB and GF3-TSB exerted a similar effect on the microbial population, producing a reduction of five log units in the microbial load. The best result was achieved with the GF1-TSB sample, which contained the highest concentration of bacteriophages tested and consequently, reduced the viable counts to 70 CFU/mL. These results show that the antimicrobial activity of the bacteriophage phiIPLA-RODI remains after the film-forming solution drying step and therefore, that the incorporation of bacteriophages in films allows them to conserve their infective capacity. It is also shown that the activity is dependent on the concentration of bacteriophages included in the film matrix. Similar antimicrobial properties and results were also observed by other authors employing other bacteriophages in different types of films, such as whey-protein (Vonasek, et al., 2014), sodium alginate (Alves, et al., 2019) and acetate cellulose (Gouvêa, et al., 2015).

3.2.2. Antimicrobial properties of films and coatings on cheese pieces contaminated with *S. aureus*.

The films analysed in the previous section were used to wrap contaminated pieces of cheese and then thermosealed. However, it is also possible to prepare an edible coating with the same film-forming solution by immersing the foodstuff pieces in it, and recovering them in such a way that a thin layer of edible film is formed on their surface (Lacroix & Vu, 2014). Therefore, to test the inhibitory effect of the films on a real food model and to investigate the repercussions of the way these gelatine-based packaging

363 materials are applied, three different tests were carried out, using previously
364 contaminated pieces of cheese.

365 In the first experiment, contaminated pieces of cheese were directly immersed in TSB
366 liquid medium with the same bacteriophage concentrations as the film-forming
367 solutions prepared according to section 2.1 (Figure 4A). In the case of the GF1 and
368 GF2 bacteriophage concentrations, on day 3 a slight decrease in the concentration of
369 *S. aureus* was observed, and the populations remained almost constant until day 6.
370 The final concentration of *S. aureus* for the cheese pieces immersed in the liquid with
371 the highest concentration of bacteriophage (GF1) was 407 CFU/g, which was similar to
372 the value for sample GF2 (524 CFU/g). The lowest bacteriophage concentration tested
373 (GF3) resulted in a microbial load at the end of the test similar to that for the control
374 sample.

375 In the second experiment, the cheese pieces were coated with a gelatine solution
376 containing different concentrations of bacteriophages (GF1, GF2 and GF3) (Figure 4B).
377 The gelatine coating formed around the cheese pieces is distributed evenly over the
378 entire surface with a similar appearance in all cases (Figure 5A). As regards the
379 inhibitory effect, a decrease in microbial load was observed in all cases, but it was
380 slightly more pronounced for GF1, with a final value of 60 CFU/g.

381 In the last experiment, the cheese pieces were coated with previously dried films
382 containing the three concentrations of bacteriophages (GF1, GF2 and GF3) (Figure
383 4C). The appearance obtained was similar in every case tested. There were no breaks
384 and the films were in contact with the entire surface of the cheeses (Figure 5B). In this
385 case, a great reduction in the number of *S. aureus* was observed for the samples
386 wrapped using the film with the highest concentration of bacteriophages (GF1), with a
387 mean final value of 44 CFU/g. Cheese samples wrapped with GF2 and GF3 showed a
388 decrease in the microbial load until day 3, but then the microorganism proliferated
389 again, reaching a microbial concentration similar to that for the control sample. Both in

Figure 4B and 4C, there is a noticeable decrease in the number of viable bacteria in the control sample, which suggests a slight inhibitory effect produced only by the gelatine material covering the cheese samples. This decrease in the number of viable bacteria may have occurred due to the physical presence of the gelatine surrounding the piece of cheese, which may affect the growth of the bacteria, possibly by hindering their nutrient intake or limiting their growing space. Other authors, studying chitosan films loaded with phages and *Escherichia coli*, another anaerobic facultative bacteria, noted the same inhibitory effect produced by the unloaded films covering infected pieces of meat (Cui, et al., 2017).

When analysing the results of these experiments, it should be borne in mind that it is easier for bacteriophages to infect bacteria in a liquid medium (Gutiérrez, et al., 2015) or in an environment with a high level of humidity (Götz, 2002). In this case, the poorest antimicrobial results were obtained for those pieces of contaminated cheese that were directly immersed in a liquid medium with no gelatine. This may be explained because over time, the surface of the cheese dried faster than when a gelatine coating or film was used, since these protein-based packaging materials have the ability to preserve the water near the food surface for a longer period of time (Lin & Zhao, 2007). In addition, during the experimental time span, the cheese begins to ripen, with a high loss of moisture (Everett & Auty, 2008), making it more difficult for the phage to infect *S. aureus.* This may explain why the highest antimicrobial effect was observed during the first three days. Furthermore, the coatings formed by the immersion of the pieces of cheese in film-forming solutions GF2 and GF3 showed better antimicrobial performance after six days of storage than the films that were made with the same film-forming solutions and then used to wrap the cheese. These results suggest that the coatings were better at retaining the moisture on the surface of the cheese, but further investigation is required to corroborate this assumption. However, this difference was diminished at the highest bacteriophage concentration tested (GF1), so increasing the

phiIPLA-RODI concentration in coatings and films could lead to a reduction in the relative importance of the manner of application of these materials on the foodstuff.

4. CONCLUSIONS

The bacteriophage phiIPLA-RODI was introduced successfully into gelatine-based films, and the physical and antimicrobial properties of these films were assessed. It was found that the prepared films were not physically affected by the bacteriophages in the film-forming solution, even at the highest bacteriophage concentration assessed. The antimicrobial properties of the prepared packaging materials were tested using pieces of cheese previously contaminated with *S. aureus*, and except at the highest concentration of bacteriophages tested, the best results were obtained when the cheese was immersed in the film-forming solution mixed with bacteriophages and the coating was directly formed on the surface of the cheese. Taking all this into consideration, a liquid solution of gelatine, glycerol and bacteriophages could be sprayed on the surface of foodstuffs that are commonly contaminated with *S. aureus,* such as fresh cheese, fruits and vegetables, to protect the consumers from this pathogenic bacteria, although further investigation into the performance of this coating on fruits and vegetables has to be conducted, as well as studies into the optimization of the concentrations of bacteriophages, protein and glycerol in order to maximize the antimicrobial properties of the coatings.

Declarations of interest

None.

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Table 1. Light transmission and transparency of gelatine films prepared with film-forming solutions with bacteriophage concentrations of 1.75×10^8 PFU/mL (GF1), 1.16×10^8 PFU/mL (GF2) and 6.35×10^7 PFU/mL (GF3). Control is a phage-free gelatine film.

Film	**Light Transmission (%)**						**Transparency**
	280nm	300nm	350nm	400nm	500nm	600nm	
Control	6.58	61.16	82.92	89.25	91.70	92.30	0.39
GF1	6.52	39.59	63.54	76.23	82.98	85.33	1.04
GF2	6.69	44.36	67.99	79.55	85.87	88.21	0.70
GF3	10.33	55.14	75.97	85.49	89.46	90.89	0.61

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571 **Table 2.** Thickness, puncture strength (PS) and puncture deformation (PD) of gelatine
572 films prepared with film-forming solutions with bacteriophage concentrations of
573 1.75×10^{8} PFU/mL (GF1), 1.16×10^{8} PFU/mL (GF2) and 6.35×10^{7} PFU/mL (GF3).
574 Control is a phage-free gelatine film.

Film	Thickness (mm)	PS (N/mm)	PD (%)
Control	0.070 ± 0.003 [a]	823.01 ± 46.54 [a]	17.96 ± 1.51 [a]
GF1	0.075 ± 0.007 [a]	803.37 ± 86.21 [a]	20.85 ± 2.41 [a]
GF2	0.073 ± 0.01 [a]	725.45 ± 77.61 [a]	24.12 ± 8.28 [a]
GF3	0.076 ± 0.005 [a]	754.83 ± 51.90 [a]	16.56 ± 4.76 [a]

575 Different letters in the same column indicate significant differences ($P < 0.05$).

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Table 3. Water vapour permeability (WVP) and solubility of gelatine films prepared with film-forming solutions with bacteriophage concentrations of 1.75×10^8 PFU/mL (GF1), 1.16×10^8 PFU/mL (GF2), and 6.35×10^7 PFU/mL (GF3). Control is a phage-free gelatine film.

Film	WVP (g*mm/m^2*h*kPa)	Solubility (%)
Control	1.61 ± 0.17 [a]	21.33 ± 3.32[a]
GF1	1.41 ± 0.38 [a]	27.79 ± 4.95[a]
GF2	1.49 ± 0.21 [a]	28.50 ± 6.24[a]
GF3	1.83 ± 0.11 [a]	24.22 ± 0.31[a]

Different letters in the same column indicate significant differences ($P < 0.05$).

Figure 1. Visual aspect of the films. A) Gelatine film without bacteriophages. B) GF1: Gelatine film prepared with a film-forming solution with a bacteriophage concentration of 1.75×10^8 (GF1), C) 1.16×10^8 PFU/mL (GF2), D) 6.35×10^7 PFU/mL (GF3).

Figure 2. Micrographs of the gelatine films loaded with bacteriophages. A) Gelatine film without bacteriophages. B) Gelatine films prepared with film-forming solutions with bacteriophage concentrations of 1.75×10^8 PFU/mL (GF1), C) 1.16×10^8 PFU/mL (GF2), D) 6.35×10^7 PFU/mL (GF3).

Figure 3. Bacteriophage inhibitory capacity of films in TSB medium previously contaminated with *S. aureus* IPLA1 (10^6 CFU/mL). Bacteriophage concentration of the films was GF1-TSB: 5.25×10^6 PFU/mL. GF2-TSB: 3.48×10^6 PFU/mL. GF3-TSB 1.90×10^6 PFU/mL. WB: pure culture of *S. aureus* IPLA1 without phages or films. G: assay with a gelatine film.

Figure 4. Evolution of *S. aureus* growth in cheeses treated with: (A) bacteriophages in TSB liquid medium at three different concentrations (GF1, GF2 and GF3); (B) bacteriophages dissolved in the film-forming solution at three different concentrations (GF1, GF2 and GF3); (C) gelatine films prepared with film-forming solutions containing bacteriophages at three different concentrations (GF1, GF2 and GF3). In every case, the bacteriophage concentrations used were either 1.75×10^8 PFU/mL (GF1), 1.16×10^8 PFU/mL (GF2) or 6.35×10^7 PFU/mL (GF3).

Figure 5. Visual appearance of coated cheeses. Gelatine-coated cheeses (A) and cheeses wrapped with films (B). 1.- Control (gelatine coating or film); 2.- Gelatine coating or film prepared with a film-forming solution with a bacteriophage concentration of 1.75×10^8 PFU/mL (GF1); 3.-1.16×10^8 PFU/mL (GF2); 4.- 6.35×10^7 PFU/mL (GF3).

Figure 3.

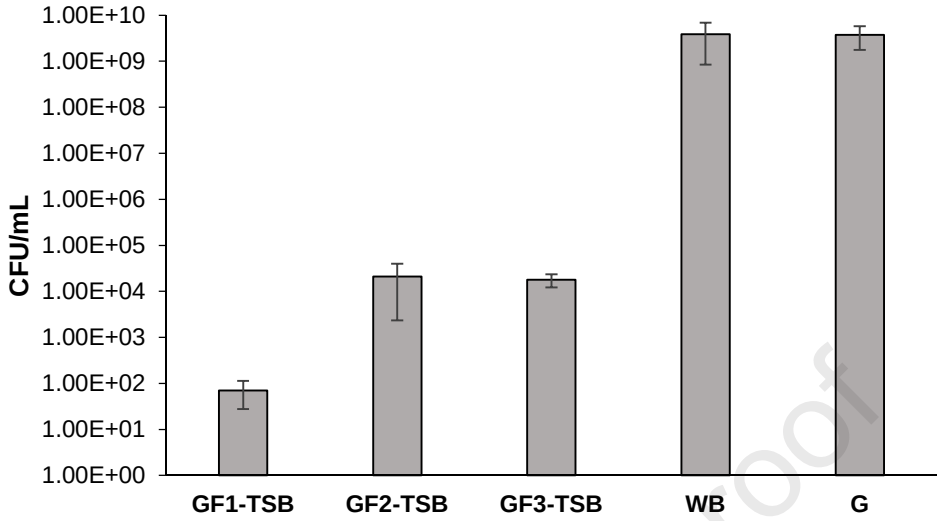
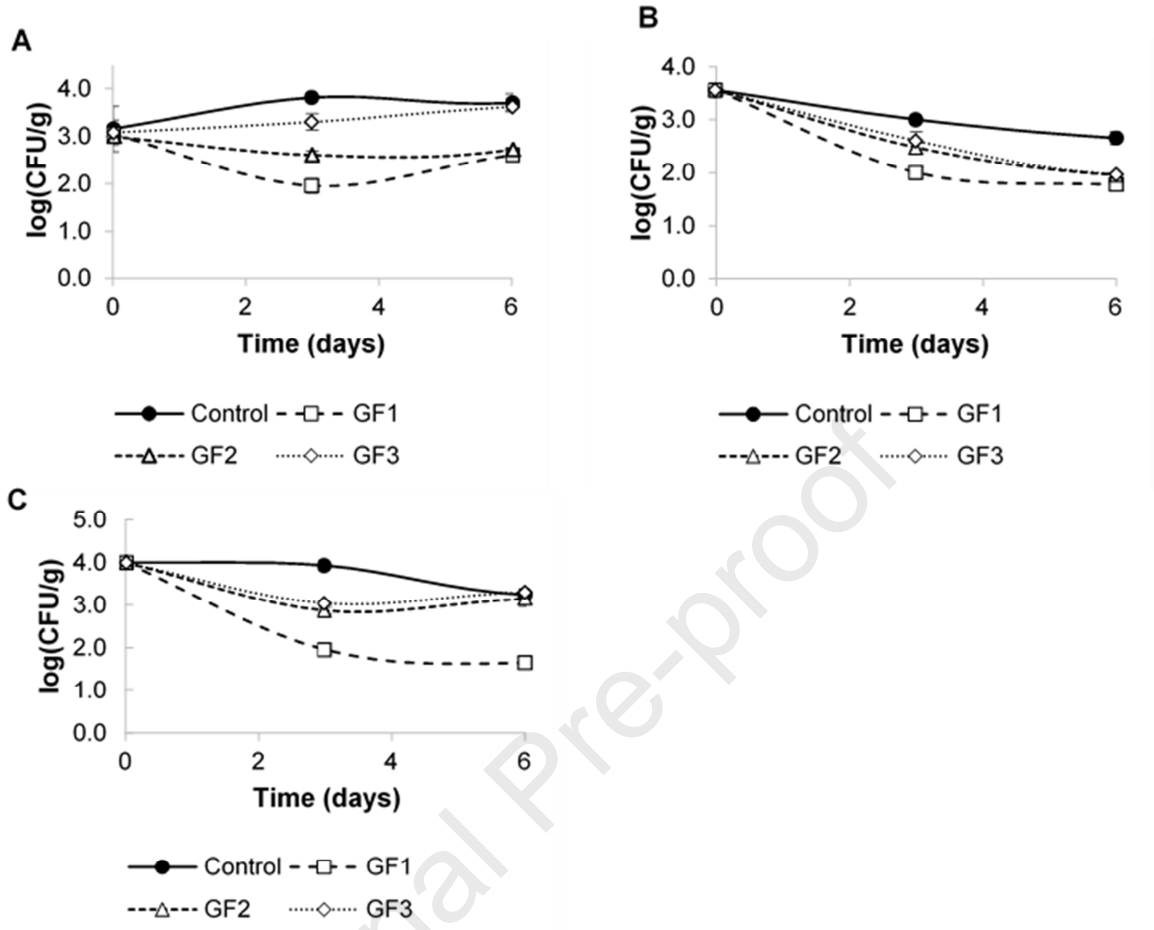
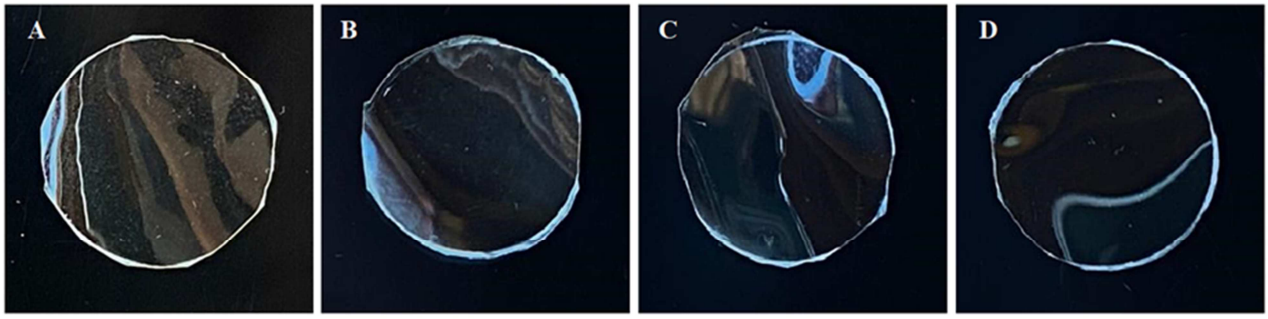
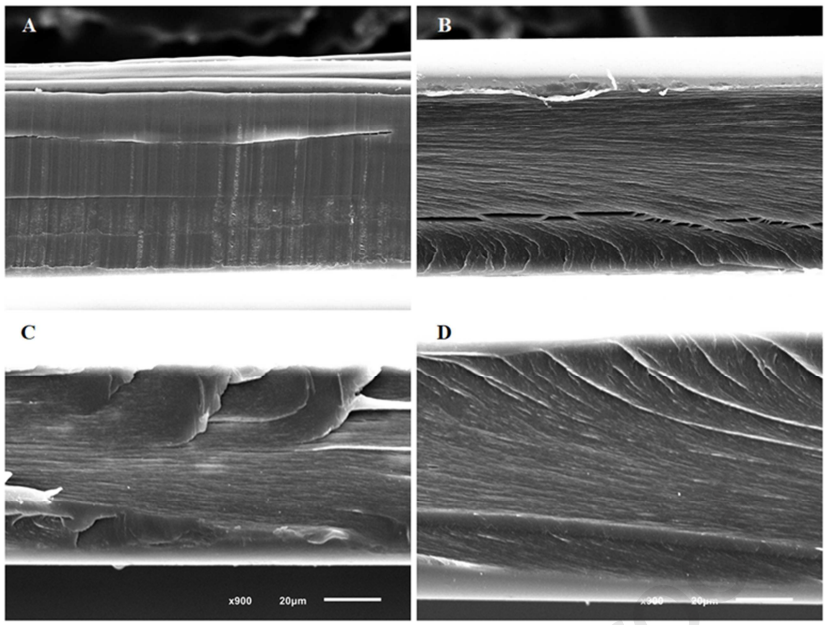
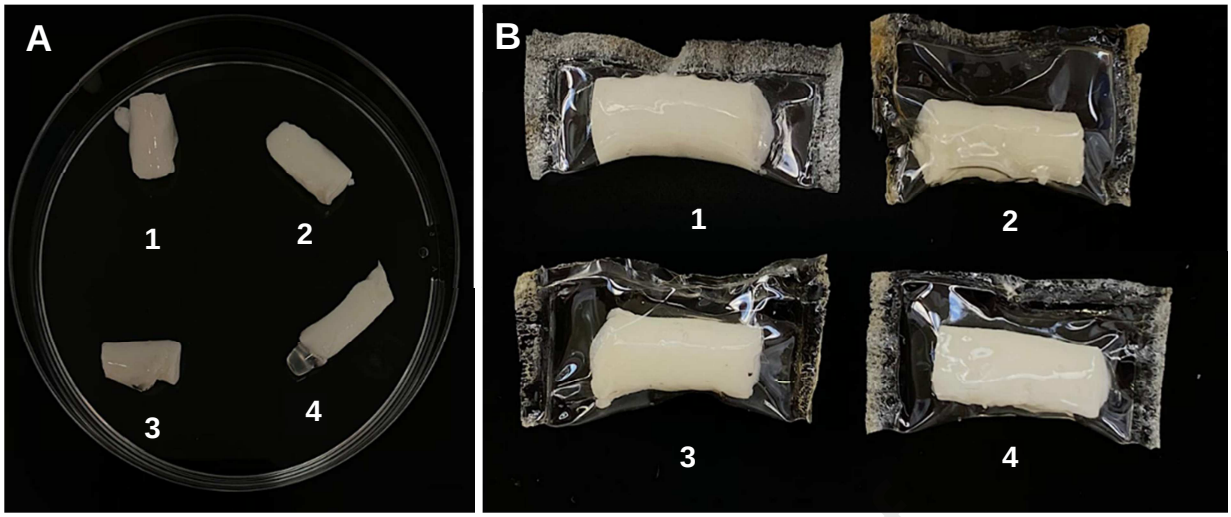


Figure 4









- Bacteriophage phiIPLA-RODI in gelatine films remained active against *S. aureus*.
- Films' physical properties were unaffected by incorporation of bacteriophages.
- The use of a gelatine-free solution of phages was the least effective treatment.
- Coatings showed better antimicrobial performance than previously dried films.

Declarations of interest

None