Acetate cellulose film with bacteriophages for potential antimicrobial use in food packaging

Delaine Meireles Gouvêa a, *, Regina Cáelía Santos Mendonça a, Maryoris Lopez Soto a, Renato Souza Cruz b

a Federal University of Viçosa, Avenida P.H. Rolfs s/n, 36570-000, Viçosa, Minas Gerais, Brazil
b State University of Feira de Santana, Av Transnordestina, s/n, Novo Horizonte, 44036900, Feira de Santana, Bahia, Brazil

1. Introduction

The advent of new technologies aimed at ensuring the safety and quality of food has intensified, as has concern for consumer health, leading to the adoption of measures to reduce food-related risks. The purpose of the study was to evaluate the efficiency of active biodegradable films incorporated with bacteriophage for future application in packaging materials. Cellulose acetate films incorporated with solution of bacteriophages showed antimicrobial activity against Salmonella Typhimurium ATCC 14028 displayed the formation of inhibition zones in Muller-Hinton agar, and a growth curve, using the diffusion method in liquid medium. There was an increase in the lag phase and slower growth of microorganisms in the environment containing bacteriophages with the films, compared to control. The mechanical and physical properties of films, such as thickness, elongation and puncture resistance showed no significant effects. However, tensile resistance was different between control and treatments. The addition of bacteriophage altered the film surface, as observed by atomic force microscopy. There was a higher porosity of the films containing the bacteriophage solution compared to control. The acetate films may be incorporated with bacteriophages, since the physical and mechanical properties of the films were not changed drastically and there was an effect of the antimicrobial film.
In recent years, researchers have dedicated themselves to studies of packaging that not only acts passively, but also interacts with food. These studies have named the subject ‘active packaging’ (Karry, O’grady, & Hogan, 2006). Active packaging is widely used to ensure food quality, increase shelf life and ensure the hygiene of perishable products, especially those susceptible to oxidation and microbiological effects. For that, antimicrobial compounds such as essential oils and/or films covering the base of proteins, lipids and polysaccharides are used in the preparation of packaging for food (Ahmad, Benjakul, Prodpran, & Agustini, 2012).

Antimicrobial active packaging can reduce the rate of microbial growth, to increase the lag phase and/or inactivate microorganisms present in the target food or on the package itself (Appendini & Hotchkiss, 2002). In some of these systems for packaging food, contact with packaging is required so that migration of the antimicrobial takes place (Cooksey, 2001). In recent years, several studies have been carried out with the aim of developing new packaging films with antimicrobial properties (Hauser & Wunderlich, 2011). It has been noted that the effect of the antimicrobial will depend on its spectrum, the target microorganism and its growth rate, and the physico-chemical characteristics of the food in question, among other factors (Appendini & Hotchkiss, 2002).

An important material for antimicrobial packaging, cellulose acetate is a biodegradable compound formed from the acetylation of cellulose. The different degrees of acetylation affect solubility and biodegradability of the compound (Edgar et al. 2001). The polymer is amorphous, odorless, non-toxic, water vapor permeable, stable and soluble in acetone. It can form transparent and rigid film, but with some flexibility that supports high tension at room temperature (Cerqueira, Filho, Carvalho, & Valente, 2010). Various cellulose acetate-based films are already being used in foods. When in contact with food the polymer is able to release antimicrobials effectively for food preservation (Cooksey, 2005). The use of cellulose-based films is shown in quite efficient active packaging technology. Many positive results have been obtained featuring an enclosure capable of inhibiting the growth of pathogenic microorganisms (Karry et al., 2006). Complete inhibition of L. monocytogenes on ham, turkey breast and beef was achieved using pediocin or nisin in an acetate film, containing heat resistant Pediococcus-derived bacteriocins in synergistic combination with a chelating agent to inhibit or kill L. monocytogenes on contact with food (Katz, 1999).

The use of bacteriophages to control pathogens is promising and is becoming a reality. Although the practice of primary bacteriophage therapy has been performed with a view to the treatment of bacterial infections in humans, the concept of removing undesirable bacterial populations using bacteriophages can be extended to animals, plants, foodstuffs and other domains (Gill & Young, 2011). However its incorporation in packaging is still unknown and it is necessary to better understand and evaluate its limitations. Thus, the aim of this study was to evaluate the efficiency of active biodegradable films incorporating bacteriophages for later use in packaging for chilled ready-to-use foods ready.

2. Materials and methods

2.1. Microorganisms used

Bacteriophages used were BFSE16, BFSE18, PaDTA1, PaDTA9, PaDTA10 and PaDTA11. Bacteriophages were isolated from chicken feces, poultry exudates and swine feces and characterized in the laboratory. The bacterial strain used as the host target was Salmonella enterica subsp. enterica serovar Typhimurium ATCC 14028. Studies with other strains of Salmonella were performed, but data were not shown in this study. We chose the strain above as pattern for the sampled data.

2.2. Elaboration of acetate films

The films were prepared by the cast method by Soares and Hotchkiss (1997) with adaptations. The flakes of cellulose acetate (Rhodia, Brazil) were added to acetone (Himedia, India) in a proportion of 1:10 (10% w/v) and left to stand for about 12–18 h. After the break the films were made at 1, 3 and 5% (v/v) (treatment T1, T3 and T5, respectively) of a mix of phase suspension buffer composed of SM (50 mmol l⁻¹ Tris–HCl — Sigma–Aldrich, USA; pH 7.5), 0.1 mol l⁻¹ NaCl — Vetec, Brazil, 8 mmol l⁻¹ MgSO₄·7H₂O — Chemco, Brazil, 0.01% gelatine — Merck, USA), at a concentration of 10⁹ PFU mL⁻¹ (Plaque Forming Units — PFU·ml⁻¹) (each bacteriophage previously propagated, purified, titrated and identified). The mixture was homogenized and spread on glass plates, previously cleaned with acetone. The films were removed from the plates after evaporation of the solvent under ambient conditions, and were stored in packages of polyethylene (PE) for further analysis. As a control, films with no suspension of bacteriophages were produced.

2.3. In vitro activity of acetate films

2.3.1. Evaluation of the bacteriophage lytic activity on film

For active films, the disk-agar diffusion method was used, as suggested by the National Health Surveillance Agency (ANVISA, 2009) for rapid in vitro susceptibility testing of aerobic bacteria and antimicrobial agents. The adapted method is similar to that used by Imran, El-Fahmy, Revol-Junelles, & Desobry, 2010. The analysis was performed in three replicates and duplicate. The plates containing Mueller-Hinton agar (Himedia, India) were inoculated with the bacterial suspension of Salmonella Typhimurium using the method of smear swab. Disks of 1 cm² acetate film were arranged in the plates, including a control (film without addition of bacteriophage), and then incubated at 35 ± 2 °C for 24 h. The zone of inhibition was measured by its diameter, including the disc. For the method of diffusion in liquid medium, a cut of 1 cm² per 10 ml of medium was immersed in 60 ml of TSB nutrient broth (Triptic Soy Broth — Himedia, India) in an Erlenmeyer flask. The medium was inoculated with the bacterial suspension of Salmonella Typhimurium at a concentration of 10⁶ CFU ml⁻¹ and the tubes were placed in a shaker (Bio Braun Biotech International, Germany) at 150 rpm at 35 ± 2 °C. Microbial growth was analyzed for 10 h at 30 min intervals; and the same process was done for the control. The optical density was measured at 600 nm (Spectrophotometer Model SP-22, Biospectro, Brazil) and evaluated for the same rate of growth of the microorganism. The entire experiment was performed in triplicate (Tankiwale & Bajpai, 2012).

2.3.2. Evaluation of the dissemination of the bacteriophage and its viability in the film

The film, cut into small pieces, was added to test tubes containing 10 ml of SM buffer (50 mmol l⁻¹ Tris–HCl — Sigma–Aldrich, USA; pH 7.5), 0.1 mol l⁻¹ NaCl — Vetec, Brazil, 8 mmol l⁻¹ MgSO₄·7H₂O — Chemco, Brazil, 0.01% gelatine — Merck, USA). One of the tubes was vortexed (Kingstic, China) for 2 min and the other part was stored for approximately 5 min to check whether the diffusion process was facilitated by agitation. After this time, serial dilutions were made from the tubes. To the agar overlay (Triptic Soy
Broth + 0.6% agar–agar (Himedia, India)) were added 100 µL of dilution and 100 µL of the previously active bacteria and this mixture was poured into Petri dishes containing agar base (Triptic Soy Broth + 1.2% agar–agar (Himedia, India)) (Carrillo et al., 2005). The plates were incubated (Fanem 002 CB, Brazil) at 35 ± 2 °C for 6–18 h. After incubation, the plates formed from lysis were counted and the results expressed as colony forming plates (Plaque Forming Units – PFU·mL⁻¹). This test was done weekly from the first day of manufacture of the film. A film without addition of phage was used as a control. Tests were performed in triplicate.

3. Results and discussion

3.1. Development and in vitro activity of acetate films incorporated with bacteriophages

The control films produced by casting had become uniform without forming aggregates and without visible color (transparent). When the solution was added the bacteriophage films presented a whitish appearance, as can be seen in Fig. 1.

The rapid sensitivity test obtained by the agar diffusion method showed the following results (Table 1).

There was a significant difference (p < 0.05) between the films that had the solution of bacteriophages added and the control. The T1 and T3 treatments differed statistically and the T5 treatment did not differ from the T1 and T3 films. Therefore, with additions of 1% solution the same effect on inhibition of bacteria was observed, since the concentration of the bacteriophage added was the same in all three films (10⁶ PFU·mL⁻¹). Only the amount added to solution (1, 3 and 5%) changed.

The formation of an inhibition zone is related to the absence of growth of the microorganism and spread of antimicrobial factor in solid medium after 24 h of evaluation. For the addition of bacteriophages in acetate film, it was noted that the amount of solution did not influence its action spectrum. We know that the higher the concentration of phage (high titer), the smaller the volume that needs to be added to obtain the desired effect (Sillankorva, Neubauer, & Azeredo, 2008).

With the addition of bacteriophage to the film it was observed that its diffusion into the medium containing the bacteria was evident through the contact of the film with the medium in which it was inoculated. The diffusion of the antimicrobial factor through the medium can be influenced by several factors, such as the growth rate of the microorganism, the composition of the agar and the interactions that occur between the antimicrobial and the film (Almeida et al., 2013).

The bacteriophage, being an organic antimicrobial agent, shows different behavior from a chemical antimicrobial. Being a virus, it needs the host to replicate, and therefore it must initially find the host in the environment, then infecting it and causing bacterial lysis (host death). Thus the time of action of the bacteriophage is more dynamic. The chemical antimicrobial agent acts on the host immediately, eliminating it, but if there is a new infection, the antimicrobial chemical would not continue to act. In the case of bacteriophages, which are more dynamic, the process is better because it finds the host, infects it, replicates itself, causes lysis and releases new phage particles into the medium, restarting a new cycle. Within 24 h the multiplication process is still occurring because new virus particles are still being released to infect the host. Where the host is present, the bacteriophage is also able to restart a new cycle of replication and cell lysis. The diffusion

2.4. Physical and mechanical properties of the films

2.4.1. Storage of films

The films were stored at room temperature controlled at 23 ± 2 °C and relative humidity 50 ± 10% for 48 h, according to D618-08 standards of the American Society for Testing and Materials (ASTM, 2008), using a desiccator-saturated solution of calcium nitrate (Prolab, Brazil) (Mesquita, Andrade, & Corrêa, 2001). They were evaluated for thickness, puncture resistance and mechanical resistance.

2.4.2. Thickness

The thickness of the films was determined using a micrometer (Mitutoyo, USA) with a precision of 0.01 mm. Ten readings were taken at random points in the film, using the mean as result. Each film was produced in three replications.

2.4.3. Mechanical properties

The mechanical properties of films, such as tensile resistance, modulus of elasticity and elongation of the film, were evaluated with the aid of a texturometer (model TA.XT plus, Stable Micro Systems, United Kingdom) according to ASTM D882-09 (ASTM, 2009), with initial separation of 50 mm and test speed of 1 mm·min⁻¹.

The puncture test was performed according to ASTM F1306-90 (98) (2008) using Heavy Duty Plataform/Film Support Rig (HDP/FSR) with P/5S spherical probe tip with penetration distance of 4 mm and test speed of 1 mm·min⁻¹.

2.4.4. Atomic force microscopy

The morphology and surface roughness of the films equilibrated at relative humidity of 53% was studied by atomic force microscopy. The experiment was conducted at the laboratory of the Department of Physics, Federal University of Viçosa, Viçosa, MG, Brazil.

The samples were fixed using double-sided tape, in support of the Atomic Force Microscope NT-MDT (Model NTegra PRIMA, Russian) using the intermittent contact mode with soft cantilever and force constant 0.02 N·m⁻¹.

The sharp support arm was positioned over the sample, and images (50 µm × 50 µm) were obtained, which were then turned into a three-dimensional image for viewing roughness (Shojaee-Aliabadi et al., 2013).

2.5. Statistical analysis

For the in vitro activity of acetate films CRD (completely randomized design) was used with three treatments (T1, T3 and T5) and a control, four treatments.

The data were analyzed at 5% probability by analysis of variance (ANOVA). Differences between means were analyzed by Tukey’s HDS test using the “MINITAB” 16 (Minitab Inc., USA) as a statistical resource.

Fig. 1. Samples of films produced from cellulose acetate: A – control, B – film with addition of 1% (T1) of the mix of bacteriophage; C – film with addition of 3% (T3) of the mix of bacteriophage; D – film with addition of 5% (T5) of mixed bacteriophages.
method in liquid medium held the growth curve of the bacterium *Salmonella Typhimurium* in TSB. There was a change in the growth rate ($\mu$ = slope of the line) of the microorganism using the points that make up the linear part of the curve, using as control the film without addition of bacteriophage. Fig. 2 evaluates the results.

The analysis shows the control effect of the film added the mix of bacteriophages on pathogen growth. The values of $\mu$ (calculated by statistical software “MINITAB” 16, USA) corresponding to the points that compose the exponential phase for the control film and treatments T1, T3 and T5 are 0.832 h$^{-1}$, 0.820 h$^{-1}$, 0.792 h$^{-1}$ and 0.824 h$^{-1}$, respectively, where $\mu$ is the specific growth rate of the microorganism under the conditions tested. This growth parameter corresponds to the slope of the resulting graph of the natural log of cell number versus time (logarithm neperian (ln) representation in Fig. 2). The smaller the value of $\mu$, the better the inhibition effect of the film.

Although there was no significant effect of time on the effect of films impregnated with bacteriophage in relation to growth, when ($p > 0.05$), in the graph we observed an increase in the lag phase for the films containing bacteriophages, and growth curves were below the control curve, demonstrating an inhibitory effect of the film on pathogen growth. Note that bacteriophages have an important part as regulators of microbial growth provided that they are present in high density environments (Miller & Martin, 2008), in favor conditions (Moisture, Water Activity, Nutrients, etc.) to the meeting with the bacteria and its subsequent infection because bacteriophages are non-motile. The efficiency of the bacteriophages can be affected by the nutritional and physiological state of the host bacterium and this infection may determine the parameters and their efficiency. When the film is diffusion in the liquid medium in TSB medium favors the growth of bacteria, affecting the latency period and the cell lysis time, since the medium is rich in nutrients that can directly affect the phage receptor, the ATP levels and cyclic AMP or even enhanced expression of genes characteristic of lysogenic replication (Miller & Martin, 2008). These details could explain the limited behavior of bacteriophage in the curve described in the figure.

### 3.2. Evaluation of the dissemination of the bacteriophage and its viability in acetate film

We can observe from Fig. 3 that after 14 days of evaluation the bacteriophage was no longer detected. However, we know that the shelf life of a refrigerated product is on average 3–10 days. On the first day after the preparation of the film, the concentration of bacteriophage is in the order of $10^8$ PFU·mL$^{-1}$, and the initial concentration was $10^{10}$ PFU·mL$^{-1}$. So initially the bacteriophage reduced two log cycles and after 14 days it dropped considerably, reaching non-detectable levels.

In relation to loss of viability of the bacteriophage in the film, this probably occurred because the film does not come into contact with food and, consequently, the bacteriophage is not in contact with the bacteria. The in vitro test was performed without the film being in contact with food to represent the same conditions. The release to the environment occurred only during the analysis process, when the film was stored during this time (14 days). As previous studies have shown that encapsulation of phages in various biopolymer matrices lead to significant loss in activity. The dried microspheres with encapsulated phages were combined with trehalose, a common stabilizing agent for viruses, to increase the phage stability after drying (Bieganski, Fowler, Morgan, & Toner, 1998). Similar encapsulation of phages in synthetic PVA (polyvinyl alcohol) polymer using the electrospinning process has shown a significant loss in number of viable phages (~1e 6% viable phages) immediately after electrospinning (Salalha, Kuhn, Dror, & Zussman, 2006).

### Table 2

Mechanical properties of tensile resistance and elasticity module of the films added to the mixture of bacteriophages and control film.

<table>
<thead>
<tr>
<th>Acetate film</th>
<th>Maximum tensile resistance (MPa)</th>
<th>Module of elasticity (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>$17.7 \pm 0.48^{a}$</td>
<td>$289.67 \pm 10.07^{b}$</td>
</tr>
<tr>
<td>T1</td>
<td>$5.76 \pm 0.36^{bc}$</td>
<td>$205.67 \pm 17.90^{b}$</td>
</tr>
<tr>
<td>T3</td>
<td>$5.78 \pm 0.54^{b}$</td>
<td>$211.33 \pm 28.54^{b}$</td>
</tr>
<tr>
<td>T5</td>
<td>$4.15 \pm 0.67^{c}$</td>
<td>$166.67 \pm 8.02^{bc}$</td>
</tr>
</tbody>
</table>

* a, b, c Means followed by different letters in the same column differ statistically by the Tukey test ($p < 0.05$). C — control film; T1 — film with addition of 1% of the mixture of bacteriophages, T3 — film with addition of 3% of the mixture of bacteriophages, and T5 — film with addition of 5% of the mixture of bacteriophages.
Bacteriophages cannot survive for long without the presence of the host cell because they are obligate intracellular parasites and host-specific, lacking their own metabolism (Húngaro, Mendonça, Gouveia, Vanetti, & Pinto, 2013).

3.3. Physical and mechanical properties of elongation, tensile resistance, modulus of elasticity and puncture of acetate films

The thickness of the films did not differ (p > 0.05), with the treatments having a mean thickness of 0.068 ± 0.006 mm. The standard deviation was low, indicating good fit in the manual production of the film.

The presence the solution of bacteriophages in acetate films did not affect (p > 0.05) the percentage of elongation of the films. For tensile resistance and modulus of elasticity, there was a significant difference (p < 0.05) between the films with bacteriophages and the film control. T1, T3 and T5 showed a reduction in the maximum values for tension and elasticity in relation to the control film. The T5 treatment showed a significant difference (p < 0.05) between T1 and T3 for maximum tensile resistance. Mean values of tensile strength and modulus of elasticity are shown in Table 2.

One of the key challenges in developing phage based antimicrobial packaging materials is the stability of bacteriophages in material formulations. Most of the current understanding of phage stability is based on the analysis of phages in an aqueous environment while there is very limited understanding of the stability of phages in material formulations (Jepson & March, 2004; Schaper, Duran, & Jofre, 2002).

Assessing the mechanical properties, we found that the work of Almeida et al. (2013), who prepared films based on cellulose with added nanocomposites and clay, reduced tensile strength compared to the control, due to the fact that the structure of the biopolymer was not exfoliated, and there was low interaction between the clay and the polymer and the formation of aggregates of silver nanoparticles. Similar results were obtained by Ahmad et al. (2012) on basis of gelatin film with added essential oil, which showed reductions in the tensile resistance and elongation as compared to control at concentrations above 10%.

A good exfoliation and dispersion of the cellulose matrix components caused the mechanical properties of the films to be improved. This technique requires the film to be manufactured in a way that facilitates a greater dispersion of the components to be used and a greater homogeneity of the film.

### Table 3
Mechanical properties of resistance to puncture of the control films and T1, T3 and T5.

<table>
<thead>
<tr>
<th>Acetate film</th>
<th>Puncture resistance (strength in N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2.44 ± 0.57a</td>
</tr>
<tr>
<td>T1</td>
<td>2.24 ± 0.38a</td>
</tr>
<tr>
<td>T3</td>
<td>1.80 ± 0.29a</td>
</tr>
<tr>
<td>T5</td>
<td>1.33 ± 0.44a</td>
</tr>
</tbody>
</table>

* Means followed by the same letter in the same column do not differ statistically by the Tukey test (p > 0.05).

**Fig. 4.** Photomicrographs of 2D films obtained by AFM: A – Film with 1% of the mix of bacteriophages, B – Film with 5% mix of bacteriophages, C – Control film (50 μm × 50 μm).
The addition of extra-polymeric components in excessive amounts can result, in a heterogeneous film structure with discontinuities and irregularities. However, these components at appropriate levels can favor the polymeric matrix and increase the interaction between the polymer and the added component (Ahmad et al., 2012; Gemili, Yemenicioglu, & Altinkaya, 2009; Melo et al., 2012; Ramos, Jimenez, Peltzer, & Garrigós, 2012). Majdzadeh-Ardakani, Navarchian, and Sadeghi (2010) evaluated the method of 'casting' in starch-based biopolymer films with added clays and concluded that the mechanical properties of the films are strongly influenced by the type of clay and the stirring mode used in the process. Mechanical combined with ultrasonic stirring favored the dispersion of clays in the film and this led to an increase in the elasticity modulus of the nanocomposites.

With respect to the puncture test, the mean of treatments related to puncture strength showed no significant effect (p > 0.05) between control and treatments. The results are shown in Table 3.

3.4. Atomic force microscopy (AFM)

Photomicrographs obtained by atomic force microscopy allowed us to evaluate the microtopography of the surface of the films in two and three dimensions (Figs. 4 and 5).

From 3D figures we can see that the films containing the mix of bacteriophages have a more irregular and porous surface. In 2D figures it was observed that pore irregularity formed in the darker areas and aggregate roughness in the lightest part. These pores may have been formed due to a solution in which the bacteriophages are present.

With regard to the evidence provided by Atomic Force Microscopy (AFM), Almeida et al. (2013) reported that when there was the addition of silver nanoparticles to different films containing clay Nanofil there was a corresponding increase in the roughness of the films. This increase in roughness was probably caused by low interaction between silver/Nanofil/polymer, allowing the formation of aggregates and increased roughness. Compared to our work, the formation of pores may have been influenced by the solution in which the bacteriophage is stored.

4. Conclusion

Bacteriophages incorporated into acetate film caused visual changes in the film and changes in its tensile properties. The thickness, elongation and puncture resistance were not altered with the addition of bacteriophages.

The antimicrobial activity of the film was shown in both the diffusion method in liquid medium and in the method of solid medium diffusion evidenced by the growth curve and the inhibition formed, respectively, when incubated at 35°C.

Bacteriophages remained viable for 14 days of evaluation. After that time, they were no longer detected in the film. The Atomic Force Micrograph showed a change in the morphological structure

---

**Fig. 5.** Photomicrographs of 3D films obtained by AFM: A – Film with 1% of the mix of bacteriophages, B – Film with 5% mix of bacteriophages, C – Control film.
of the surface of the films with the addition of bacteriophages as compared to control.

The techniques of incorporation of bacteriophages into film need to be improved so that they remain viable for longer in the packaging and can be used in future refrigerated food storage and consumption.

Acknowledgments

The authors would like to express their sincere thanks to the National Council for Scientific and Technological Development (CNPq), Brazil, and we thank Federal University of Viçosa, MG, Brazil, for technical support for this study.

References


1 These five references bring principial ideas and relations with the results of our work. They are also references which we can compare our work and draw a conclusion, because it had not been done the way it was conducted.