Antiviral activity of alginate-oleic acid based coatings incorporating green tea extract on strawberries and raspberries

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ABSTRACT

Foodborne illnesses caused by the consumption of berries contaminated with human enteric viruses, namely human noroviruses (NoVs) and the hepatitis A virus (HAV), remain a significant food safety concern. The objective of this research was to investigate a food-grade edible coating composed of alginate/oleic and containing green tea extract (GTE) as an antiviral agent for the preservation of fresh strawberries and raspberries. Berries were stored at ambient (25 °C) temperature and refrigerated (10 °C) conditions. Initially, the effect of the pH of the film-forming dispersions (FFD) on their antioxidant and antiviral activity was analysed. Then, the physicochemical properties of edible alginate-oleic acid coatings containing GTE were studied, and finally, their antiviral efficacy when applied onto strawberries and raspberries at 10 and 25 °C was evaluated. The results showed that the antioxidant properties of the films were not pH-dependent, but the antiviral activity was higher at a pH 5.5. The infectivity of the murine norovirus (MNV), a human norovirus surrogate, and HAV in fresh strawberries after the coating treatments was reduced by approximately 1.5-2 log during the 4-days storage period at 10 °C as compared to the controls and a complete inactivation of both viruses was observed after overnight storage at 25 °C. However, the efficacy of the GTE-coatings was slightly reduced on the raspberries, probably due to the difference in the amount of coating that adhered to their surface (4.64 ± 0.23 g and 1.54 ± 0.15 g·cm⁻² for strawberries or raspberries, respectively). Therefore, this paper reports, for the first time, the potential of antiviral edible coatings to improve the safety of berries against foodborne pathogens.

KEYWORDS:

Edible coatings, foodborne viruses, green tea extract, alginate

1. INTRODUCTION

The global edible films and coatings market has recently become more interested in food preservation, and it is expected to grow in the future owing to the increased use of green strategies to minimize the use of chemical preservatives (Galus and Kadzińska, 2015, Hassan et al., 2018). Edible films and coatings are based on natural polymers such as proteins, polysaccharides and/or lipids, which can improve food quality and safety by providing selective barriers to oxygen, moisture and aromas or by avoiding lipid oxidation (Hassan et al., 2018, Rezaei and Shahbazi, 2018, Umaraw and Verma, 2017). Furthermore, the natural antimicrobial and antioxidant agents incorporated into the edible coatings could also be suitable for active packaging development (Kaya et al., 2018, Musso et al., 2017, Wang et al., 2017, Yuan et al., 2016), free from synthetic additives. These facts have boosted research in this area.

Active agents used for the formulation of edible films and coatings need to be included in the approved list of food-grade additives or compounds generally recognized as safe (GRAS) by the pertinent regulations. However, although the bactericide, fungicide and antioxidant properties of different GRAS compounds have been broadly investigated, little information is available about how biopolymers could act as carriers of antiviral compounds (Fabra et al., 2018, Randazzo et al., 2018), and to the best of our knowledge, there is no information about the application of antiviral edible coatings to foods.

Natural compounds such as polyphenols or essential oils having antiviral properties can be used for the development of edible films and coatings (Randazzo et al., 2018, Fabra et al., 2016). In particular, green tea extract (GTE) has demonstrated strong antiviral activity against foodborne pathogens such as hepatitis A virus (HAV) and murine norovirus (MNV), a human norovirus surrogate (Randazzo et al., 2017). GTE is a

polyphenol-rich extract obtained from the cultivated evergreen tea plant (*Camellia sinensis* L.) of the family *Theaceae*. Catechins are the main polyphenols present in GTE and they include (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), and (–)-epigallocatechin-3-gallate (EGCG) (Chan et al., 2011, Pekal et al., 2012, Seeram et al., 2006). The main beneficial properties of GTE are usually ascribed to EGCG, which is the main catechin in green tea (~50%, Cabrera et al., 2006), with proven antioxidant, anticarcinogenic, anti-inflammatory and antimicrobial (bactericidal and virucidal) properties against a wide range of foodborne pathogens (Perumalla, 2011). However, catechins are rather unstable at certain pH conditions and different tea-derived compounds can be formed as a consequence of degradation and epimerization reactions. In fact, Falcó et al. (2018) reported that the antiviral efficiency of GTE was pH-dependent, and that higher reductions against MNV and HAV were recorded for GTE at a pH of 7.2 probably due to the change in composition and, more specifically, in the amount of catechin derivatives. They found that the presence of catechin dimers such as theasinensins A and B, in combination with kaempferol 3-O-xylosyl-glucoside, seemed to contribute to the antiviral activity of the GTE at neutral pH.

The development of antiviral edible coatings could be of great interest, for instance, in relation to raw and minimally processed fruits and vegetables, which have been identified as important vehicles for foodborne virus transmission (Lynch, Tauxe, & Hedberg, 2009, Randazzo et al., 2018). In fact, there is increasing awareness of the significance of foodborne diseases caused by the consumption of berries contaminated with human enteric viruses (Scavia et al., 2017; Severi et al., 2015). For example, since January 2018, of the 48 alert notifications reported in the EU RASFF database that involved “viruses”, 9 were associated with berries, namely strawberries, raspberries,

blueberries and blackberries. Berries are usually eaten raw and are not subjected to any industrial processes which facilitate virus inactivation or removal (Butot et al., 2008). Thus, the incorporation of antimicrobial agents into edible coatings can be used to control viral contamination.

To the best of our knowledge, there is no published work related to the antiviral properties of edible coatings applied to berries. Considering all these aspects, this work was designed to: (i) assess the effect of pH on the antiviral and antioxidant activity of films containing GTE; (ii) investigate the physicochemical properties of edible alginate-oleic acid films containing GTE; and (iii) evaluate the antiviral efficacy of the developed coatings when applied to strawberries and raspberries at 10 and 25 °C.

2. MATERIALS AND METHODS

2.1 Materials

Alginic acid sodium salt from brown Algae (medium viscosity), oleic acid (technical grade 90%) and Tween 80 were purchased from Sigma-Aldrich (Steinheim, Germany). GTE was kindly donated by Naturex, S.A. (France). Strawberries (*Fragaria x ananassa*) and raspberries (*Rubus idaeus L.*) were obtained from a local supermarket, then immediately transported to the laboratory where fruits were selected for uniformity of size and freedom from pathological and physiological defects for use in the experiments.

2.2 Preparation of film forming dispersions

Four different film-forming dispersions (FFD) based on alginic acid sodium salt were prepared: two with GTE at two different pHs (5.5. and 7.0) and two without the extract. FFD containing GTE were prepared as follows: alginic acid (A) sodium salt (1 % w/w)

was directly dissolved in water at room temperature (RT). Once it was completely dissolved, a controlled amount of GTE was added to obtain 1:0.7 alginate:extract ratio. At this point, for one of the formulations, the pH of the alginate-GTE solution was modified until 7.0 by means of NaOH 1 M. The pH of the other alginate-GTE solution was not modified, being ~5.5. Then, 0.5 % (w/w) oleic acid and 0.2 % (w/w) Tween 80 were mixed, under magnetic stirring with the alginate-GTE solution at RT. Oleic acid and Tween 80 were used as a hydrophobic compound to improve the applicability of these coatings and as a surfactant to improve the stability of FFD, respectively. Finally, FFD were prepared by means of a rotor-stator homogenizer (D9, MICCRA GmbH, Müllheim, Deutschland) for 2 min at 13,500 rpm at RT. After homogenization, FFD were degasified at RT with a vacuum pump.

Control films without GTE were also prepared for comparative purposes.

Edible films' nomenclature was 'A' for control alginate film (without lipid), 'A-OA' for those containing oleic acid, 'A-OA-GTE x' for those containing GTE where 'x' refers to the pH.

2.3 Preparation and characterization of the stand-alone coatings

Stand-alone coatings (films) were prepared by weighing the amount of the FFD that would provide 1 g of total solids on Teflon casting plates of 15 cm in diameter, so as to keep the total solids content constant in the dry films (56 g/m²). The films were dried for approximately 24 h at 45% relative humidity (RH) and 30 °C on a levelled surface and, subsequently, they were peeled intact from the casting surface and conditioned in desiccators with an oversaturated salt solution of magnesium nitrate (~ 54% RH) at 23 ± 2 °C.

Film thickness was measured with a Palmer digital micrometer to the nearest 0.0025 mm at five random positions around the film used for WVP measurements and at four positions along the strips used for the mechanical properties. Average values of film thickness were used in all WVP and tensile property determinations which ranged between 94 and 115 μm .

2.3.1 Scanning electron microscopy (SEM)

The microstructure of the films was observed by means of a SEM (Hitachi S-4800). The films (three samples per formulation) were cryofractured by immersion in liquid nitrogen, and then mounted on M4 Aluminium Specimen Mount and fixed on the support using double-side adhesive tape to explore the cross-section of the samples. After gold–palladium coating, the images were captured using an accelerating voltage of 10 kV and a working distance of 10 mm.

2.3.2 Mechanical properties

A Mecmesin MultiTest universal test machine (Landes Poli Ibérica, S.L., Barcelona, Spain) equipped with a 100-N static load cell was used for the tensile testing, according to ASTM standard method D882-09 18 (ASTM, 2010a). The mechanical parameters: elastic modulus (E), tensile strength at break (TS) and elongation percentage at break (EAB) were obtained from the stress vs. strain curves. Equilibrated specimens were mounted in the film extension grips and stretched at 50 mm min⁻¹ until breaking. Eight replicates of each formulation were tested. The experiments were carried out 54% RH and 24 °C.

2.3.3 Water vapour permeability (WVP)

WVP was determined gravimetrically at 23 ± 2 °C and 54-100% RH gradient, according to the ASTM E96/E96M-10 (ASTM 2010b) gravimetric method for hydrophilic films. Prior to the test, the thickness of the samples was randomly measured at five points. Payne permeability cups of 3.5 cm in diameter (Elcometer SPRL, Hermelle/s Argenteau, Belgium) were filled with 5 mL of distilled water (100% RH) and then, film samples (35 mm diameter) were secured with the outwards-facing side in contact with the air during drying. The cups were placed in pre-equilibrated cabinets at 54% RH using oversaturated solutions of $\text{Mg}(\text{NO}_3)_2$ (Panreac Quimica, SA, Barcelona, Spain) and they were weighted periodically (± 0.00001 g) until the steady state was reached. The free film surface during film formation (air side) was exposed to the lowest relative humidity to simulate the actual application of the films in high water activity products when stored at intermediate relative humidity. Cups with aluminium samples were used as control samples to estimate solvent loss through the sealing. WVP was calculated as previously described by Fabra et al., 2012. Four replicates per formulation were made.

2.3.4 Antioxidant activity

The Trolox Equivalent Antioxidant Capacity (TEAC) of the GTE and the films was determined using a modification of the original TEAC method (Re et al., 1999). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), was used as a standard of antioxidant capacity. Each film sample of 12 ± 2 mg was cut into small pieces and hydrated with 2 mL of distilled water for two hours, and then, 8 mL of methanol were added in order to favour the extraction of the natural extracts. The supernatant (film extract) obtained was analysed for $\text{ABTS}^{+\cdot}$ (2,20-azinobis(3-4ethylbenzothiazoline- 6-sulfonic acid) radical scavenging activity. To this end, 10 μL of the film extracts were added to 1 mL of the $\text{ABTS}^{+\cdot}$ solution, and absorbance at 734 nm was registered every

minute for 6 min. For calibration, Trolox standards of different concentrations were prepared, and the same procedure was followed. The TEAC of the film samples was determined by comparing the corresponding percentage of absorbance reduction at 6 min with the Trolox concentration–response curve. All the determinations were carried out at least six times using a spectrophotometer (Beckman Coulter DU 730, England) and methanol: water solutions as the blank.

2.3.5 Determination of antiviral activity

MNV-1 was propagated and quantified in the murine macrophage cell line RAW 264.7 (both kindly provided by Prof. H.W. Virgin, Washington University School of Medicine, USA), while HAV strain HM-175/18f (ATCC VR-1402) in the FRhk-4 cells (kindly provided by Prof. A. Bosch, University of Barcelona, Spain).

Pieces of each edible film (25 ± 5 mg) were sterilized with UV light in a safety cabinet under laminar flow for 15 min each side and then placed into test microtubes containing 500 μ L of MNV and HAV suspensions diluted in PBS pH 7.2 (*ca.* 5 logs TCID₅₀/mL). Samples were incubated overnight (ON) at 10, 25 or 37 °C and during 4 days at 10 °C in a shaker (180 rpm). Then, the effect of the active compound was neutralizing with complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fecal calf serum (FCS). Each treatment was performed in triplicate. Confluent RAW 264.7 and FRhk-4 monolayers in 96-well plates were used to evaluate the antiviral effect of the edible films. Positive controls were virus suspensions incubated with alginate films without GTE under the same experimental conditions. The decay of MNV and HAV titers was calculated as $\log_{10} (N_x/N_0)$, where N_0 is the infectious virus titer for alginate films and N_x is the infectious virus titer for alginate films containing GTE (Falcó et al., 2018).

2.3.6 HPLC analysis

GTE-containing films (5 mg film/mL) in PBS at pH 7.0 were subjected to HPLC-MS analysis. Samples were analyzed after ON incubation in the PBS. An Agilent 1290 HPLC system equipped with an Acquity UPLC BEH C18 column (Waters, 50 mm × 2.1 mm, 1.7 mm of particle size) was used, following the method described in Gómez-Mascaraque, Soler, and Lopez-Rubio (2016). Data were evaluated using the XIC manager in the PeakViewTM software (version 2.2). The compounds detected were tentatively identified with the aid of the Phenol Explorer and Chemspider databases (<http://phenol-explorer.eu> <http://www.chemspider.com>).

2.4. Challenge tests

Selected strawberries (*Fragaria x ananassa*) and raspberries (*Rubus idaeus L*) were dipped in the FFD for 2 min and air-dried for 1 h at RT. The mean value of the coating was calculated in ten samples by quantifying the surface solid density (SSD) (Eq. (1)).

$$SSD = (M_{CA} \cdot X_s) / A_s \quad (\text{Eq. 1})$$

Where M_{CA} is the mass of coating solution adhered to the fruit surface, X_s is the mass fraction of solids present in the FFD and A_s is the average sample surface area. The average sample surface area (A_s) was estimated by considering strawberry and raspberry geometries as cones with a known height (measured in triplicate using a digital micrometer) and volume (measured with a pycnometer, by using water as reference liquid). Samples were weighed before and after coating, to determine the mass of coating solution adhered to the strawberry or raspberry surfaces (M_{CA}). The non-coated sample was used as a control.

The viscosity of the FFD used as a coating (A-OA and A-OA-*GTE5.5*) was measured using a rotational viscosity meter Visco Basic Plus L (Alpha Series) from Fungilab S.A. (Spain).

In order to evaluate the appearance of the coating, coated and uncoated berries were observed by means of Eclipse 90i Nikon microscope (Nikon corporation, Japan) equipped with 5-megapixels cooled digital color microphotography camera Nikon Digital Sight DS-5Mc. Strawberries were observed by illuminating either with visible or UV light, and acquiring the images along the complete visible spectrum without any filter or with a long pass emission UV-2A fluorescent filter block (Nikon corporation, Japan), respectively. Raspberries were observed by means of a band pass emission G-2E/C filter for red fluorescence (Nikon Corporation, Japan). Acquired images were analyzed and processed by using Nis-Elements Br 3.2 Software (Nikon Corporation, Japan).

2.4.1 Antiviral test on berries

Berries were exposed to UV for 15 min on a sterile plate in a laminar flow hood. Thereafter, berries were inoculated by spotting 50 μ L of MNV or HAV suspension and dried under continuously circulating laminar flow for 1 h at RT before application of the coating treatments. Each sample was dipped in the coating solution for 2 min. Berry samples were dried under the laminar flow hood and then stored for 24 h at 10 and 25°C. Samples incubated at 10°C were also stored during 4 days. On each sampling day, individual untreated and treated berry samples were placed in a tube containing 5 mL of DMEM supplemented with 10% FCS and shaken for 2 min at 180 rpm. Finally, berries were removed from the tube and serial dilutions were performed from the resultant virus suspension. Each treatment was performed in triplicate. Positive controls were uncoated

berries and coated berries without GTE in its formulation under the same experimental conditions. Infectious viruses and effectiveness of the treatments were calculated as described above.

2.5 Statistical analysis

Data of each test were statistically analysed. The statistical analysis was carried out by means of IBM SPSS Statistics software (v.23) (IBM Corp., USA) through the analysis of variance (ANOVA). Comparison of the means was done employing the Tukey's Honestly Significant Difference (HSD) at the 95% confidence level. All data are presented as mean $\pm$ standard deviation.

3. RESULTS AND DISCUSSION

3.1. Effect of pH on the antioxidant and antiviral properties of films

In the first part of this work, the antioxidant and antiviral activity of the A-OA-GTE films were evaluated depending on the pH of the film-forming solution to explore if the effect reported in the pure extract by Falcó et al., 2018 was also reproduced in the films. As clearly observed in Table 1, the antioxidant activity of the films expressed, as mmol Trolox per g antioxidant, was not pH-dependent which means that the pH did not alter the antioxidant properties of the extract when incorporated within the films. Thus, although epimerization and degradation reactions can be produced at neutral pH (Falcó et al., 2018), tea-derived compounds also have antioxidant properties (Gómez-Mascaraque et al., 2015). Furthermore, the antioxidant activity of the GTE was significantly higher (20.1 ± 0.41 mmol Trolox per g antioxidant, Fabra et al., 2018) than those reported in the literature for tea extracts (Magcwebeba et al., 2016, Majchzak et al., 2004) and those obtained for the A-OA-GTE edible films. The lower antioxidant

activity of the edible films as compared to the pure extract can be ascribed to the existing interactions between the polyphenols with compounds present in the films, preventing a complete release of the GTE from the film structure (Fabra et al., 2018). In fact, interactions between GTE and lipids have been previously reported (Fabra et al., 2018, Rashidinejad et al., 2016, 2017, Tamba et al., 2007).

Tables 2 and 3 show the effect of edible films against MNV and HAV, respectively, at different temperatures and exposure times. Overall, A-OA-GTE5.5 films exhibited the most effective antiviral activity at 37 °C. In line with these results, higher reductions were reported for MNV after ON incubation at 37 °C, decreasing titers by 3.42 log TCID₅₀ /mL and 5.76 log TCID₅₀/mL for A-OA-GTE 7.0 and 5.5, respectively. Statistically significant reductions ($p < 0.05$) on MNV infectivity were observed for both films at 25°C, while no effects ($p > 0.05$) were reported at 10°C (Table 2) since viruses usually persist better at lower temperatures than at higher temperatures. For HAV significant differences were reported for A-OA-GTE5.5 films after ON incubation at 25 and 37 °C (Table 3). In contrast, A-OA-GTE7.0 films had almost no effect against HAV after ON incubation at 10, 25 and 37 °C. These results differ from those reported by, Falcó et al., 2018 for pure GTE. They found that freshly prepared pure GTE was very effective in inactivating either MNV or HAV at neutral pH but was ineffective at pH 5.5 because of the change in GTE composition and, more specifically, in the amount of catechin derivatives (caused by degradation and epimerization reactions) that took place in aqueous solutions at pH 7.0. Thus, the differences in the antiviral efficiency between pure extract and GTE-containing films may be mainly attributed to the methodology followed for determining the antiviral activity. In the previous work, pure GTE extract was incubated in PBS under the same experimental conditions (pH in the original sample) (Falcó et al., 2018) whereas, in the present work, GTE-containing films were

incubated with PBS at 7.0 after having been prepared at 5.5 or 7.0. Thus, one would hypothesize that during the ON incubation of A-OA-GTE 5.5 films, part of the GTE was released into the PBS (pH 7.0) becoming degraded as a consequence of the pH (as previously reported). In contrast, in the case of A-OA-GTE 7.0, the GTE was already degraded in the original FFD and the antiviral activity of the catechin derivative compounds formed during film-formation, decreased during storage, as demonstrated by Falcó et al., 2018 for pure GTE. To confirm this hypothesis, HPLC analysis of the developed active films at both pH conditions and after overnight incubation in PBS at pH 7.0 were carried out. Table S1 of the Supplementary Material summarizes the main phenolic compounds found in both films. These compounds were tentatively identified based on the m/z values obtained from the mass spectra. Figure S1 of the Supplementary Material depicts the profiles of phenolic compounds found in both types of films. In general, a decrease in the amount and concentration of polyphenolic compounds was observed in the films prepared at pH 7.0, probably due to the degradation induced by the long-time exposure at this specific pH, as previously reported (Falcó et al., 2018). Unexpectedly, both theasinensin A and B, which were previously described as potentially responsible of the increased virucidal activity in the pure extract (Falcó et al., 2018), did not appear neither in the A-OA-GTE 5.5 nor in A-OA-GTE 7.0 films after ON incubation at pH 7.0. This could be due to existing interactions between lipids and GTE as it has been previously reported in several works (Fabra et al., 2018, Tamba et al., 2007).

Interestingly, these results showed that alginate films containing GTE were more effective in reducing the titers of MNV and HAV than previous studies (Fabra et al., 2018) where films containing 0.75 g GTE/g alginate prepared at pH ~ 5.5 reduced MNV and HAV infectivity by 1.97 and 1.25 log after ON incubation at 37 °C. These

differences can be ascribed to the different composition of the lipid fraction of the films, which seem to play a crucial role in the availability of the GTE to exert the antiviral activity.

Overall, edible films containing GTE at pH 5.5 showed higher antiviral efficiency than their counterparts prepared at pH 7.0, with higher activity on MNV than HAV.

Therefore, taking into account that the antioxidant properties were not pH-dependent and the antiviral activity was higher at pH 5.5, a deeper physicochemical characterization of the A-OA-GTE films prepared at pH 5.5 and the antiviral efficiency on raspberries and strawberries coated with A-OA-GTE_{5.5} were carried out.

3.2. Characterization of the developed edible films

The microstructure was characterized by SEM to examine the arrangement of the different compounds in the alginate matrix. Figure 1 shows the cross-section SEM micrographs of the films where notable differences can be observed. Pure alginate films (Figure 1A) exhibited a smooth appearance whereas the presence of OA (Figure 1B) introduced discontinuities in the polysaccharide matrix. Films containing GTE (Figure 1C) appeared similar to their counterparts prepared with OA but with a rougher aspect.

Water vapor is one of the main permeants studied in the application of edible films and coatings, because it may permeate from the internal or external environment through the coating, resulting in possible negative changes in product quality and shelf-life. Table 4 summarizes the measured WVP and water uptake values for the neat alginate films and those containing OA or OA-GTE. As previously reported by other authors (Ma et al., 2016, Mohammad Amini et al., 2016), both WVP and water sorption were reduced by the addition of OA although a very limited reduction in the former was observed with respect to the pure alginate film due to the well-known plasticizing effect of this

compound (Fabra et al., 2010, Kowalczyk et al., 2016). Water sorption reduction can be ascribed to the fact that lipids correspond to a fraction of solids with small water uptake capacity (Fabra et al., 2010) and to the interactions between the polar groups of OA and the hydrophilic sites of the alginate matrix by means of hydrogen bonds, which could substitute the polymer-water interactions. Interestingly, GTE also contributed to reduce the water uptake capacity of A-OA films although its effect on the WVP was negligible. Thus, it seems that the polyphenolic compounds, which are mainly hydrophilic, when incorporated within the A-OA matrix may be actually confined and more restricted in terms of their interaction with water. In fact, this could also contribute to the formation of more rigid films as compared to the A-OA. GTE-lipid interactions have been previously described (Fabra et al., 2018) and could help to explain the reduction in the water uptake capacity without altering the WVP despite of the hydrophilic character of this extract. Similarly, interactions between GTE and milk fat globules surfaces (Rashidinejad et al., 2016, 2017) and tea catechin (-)-epigallocatechin gallate with lipid membranes (Tamba et al., 2007) have been previously reported.

Table 4 summarizes the mechanical properties of the developed films. This analysis is interesting since an edible film or coating should be resistant in order to withstand manipulation and keep its integrity. As shown in Table 4, the addition of oleic acid induced a significant decrease in the elastic modulus and tensile strength at break, consistent with other proteins and polysaccharide-based films containing lipids (Fabra et al., 2010, Monedero et al., 2010). This fact can be ascribed to the presence of discontinuities in the polysaccharide matrix, which reduced the cohesion forces of the alginate network, thus implying a loss of mechanical resistance. In the case of GTE, a significant increase in the cohesive strength of the films occurred, thus increasing the hardness as compared to its counterpart prepared with OA. This seems to indicate that

polyphenol extract could interact with the polysaccharide matrix or even with the OA, producing harder films and could also explain the above-mentioned lower antioxidant activity obtained in GTE-containing films as compared to the pure extract.

Both OA and GTE reduced the film stretchability, indicating that these components could interact to a certain extent with alginate, thus modifying the chain aggregation pattern in the biopolymer matrix. Both hydrophobic interactions and hydrogen bonds can occur between alginate and OA or GTE. Polar groups of OA and phenolic compounds present in the GTE will interact through hydrogen bond formation with the polar regions (-OH) of alginate chains. Similarly, hydrophobic interactions can occur between the hydrocarbon regions of the OA and the non-polar groups of GTE, as it has been demonstrated in a recent work (Fabra et al., 2018).

3.3. Challenge tests: application of edible coatings in strawberries and raspberries

Challenge tests on coated strawberries and raspberries at two different temperatures (10 and 25 °C) were carried out to ascertain the virucidal effectiveness of A-OA-GTE5.5 edible coatings containing 0.7 % wt. GTE on real food samples. Firstly, the surface solid density (SSD) which can be used as an estimation of coating thickness was calculated, being significantly higher in strawberries ($4.64 \pm 0.43 \text{ g}\cdot\text{cm}^{-2}$) than in raspberries ($1.54 \pm 0.15 \text{ g}\cdot\text{cm}^{-2}$). No significant effect of the apparent viscosity of the formulations with and without GTE were observed on the amount of FFD adhered to the samples, as reported by other authors (Villalobos et al., 2009), since strawberries and raspberries coated with GTE-containing films did not show significantly different SSD values with respect to those coated with A-OA solutions (see Table 5).

The overall appearance of the coatings on the fruit surface was qualitatively evaluated by means of optical microscopy and representative images of the samples are presented

in Figures 2 and 3. It should be mentioned that images were captured in different conditions for strawberry and raspberry due to the amount of the total solids adhered on the surface, which was nearly three times greater in the case of strawberries. This means that the microscope parameters established to get the relevant information of each sample were completely different, as it has been described in section 2.4. Specifically, the overall appearance of the strawberry's coating was clearly observed by coating only half of the strawberry and taking the rest as non-coated control. A detail of the coating is given in Figure 2C where a continuous coating can be easily distinguished (see white arrows). However, it should be noted that a greater amount of coating was deposited in the seed hall due to the morphology (furrow-like structure) attained in these specific places of the strawberry surfaces, as it is shown in Figures 2A and 2B.

Due to the lower amount of total solids adhered on the surface, differences between coated and uncoated raspberries were detected under fluorescence illumination using the red filter. As shown in Figure 3, reduced fluorescence was observed in the uncoated samples (Figures 3A and 3C) and, the fluorescence intensity detected on the coated raspberries (Figure 3B and 3D) was higher at the surface of the cross-section images (figure 3D), indicating that the coating was mainly located on the surface.

Although it is outside the scope of this paper, it should be mentioned that the appearance of the coated strawberries and raspberries was completely different after 14 days of storage (supplementary material Figure S1), indicating that the presence polyphenols might also favour a delay in the growth of fungus in the berries.

GTE and control coatings were used to treat fresh strawberries and raspberries inoculated with MNV and HAV and stored at 10 °C (ON and 4 days) and 25 °C (ON). The infectivity of MNV and HAV in fresh strawberries after coating treatments was reduced by approximately 1.5 logs after ON storage at 10 °C. Additionally, more than

2.5 log reduction of MNV titers was observed after 4-days storage at 10 °C compared to the controls (Fig 4). Complete inactivation for both viruses was observed after ON storage at 25 °C. The efficacy of GTE-coatings was slightly reduced on raspberries since MNV titers were reduced by 1.25 and 1.54 log at 10 °C, ON and 4 days storage, and by 1.96 after ON incubation at 25 °C (Fig. 5). These differences in the antiviral activity of the A-OA-GTE 5.5 coating can be ascribed to the amount of coating adhered (and thus, the amount of GTE) onto the surface of the berries, which was significantly lower in the case of raspberries (~ 1.47 or 0.49 g GTE·cm⁻² for strawberries and raspberries, respectively).

4. CONCLUSIONS

In conclusion, GTE was successfully incorporated into alginate-oleic acid film-forming dispersions at two different pH conditions (7.0 and 5.5). Results showed that the active antioxidant properties of the films were not pH-dependent and the antiviral activity of the films was reduced at basic pH (7.0). Active coating solution prepared at pH 5.5 was effective in controlling the infectivity of MNV and HAV at ambient and refrigerated conditions (25 and 10 °C) although the efficacy of GTE-coatings was greater in strawberries than in raspberries. This indicates that the amount of coating adhered onto the surface of the berries, and, more concretely, the amount of GTE played an important role. Thus, the active edible coatings prepared in this work exhibited potential antiviral properties to improve food safety of fresh strawberries and raspberries.

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Table 1. Antioxidant capacity (TEAC: trolox equivalent antioxidant capacity) of the developed active films at pH 5.5 and 7.0

FFD/coating	TEAC (mM Trolox/g antioxidant in the film)
A-OA	-
A-OA-GTE 7.0	14.1 (0.6) ^a
A-OA-GTE 5.5	12.5 (1.1) ^a

Mean value (standard deviation). Different letters denote significant differences between samples. A-OA alginate film containing oleic acid; A-OA-GTE 7.0: alginate film containing oleic acid and GTE at pH 7.0; A-OA-GTE 5.5: alginate film containing oleic acid and GTE at pH 5.5

Table 2. The effect of edible films on the infectivity of murine norovirus (MNV)

FFD/coating	Temperature							
	37°C		25°C		10°C			
	Overnight		Overnight		Overnight		4 days	
	log TCID ₅₀ /mL	Reduction	log TCID ₅₀ /mL	Reduction	log TCID ₅₀ /mL	Reduction	log TCID ₅₀ /mL	Reduction
A-OA	6.91 (0.31) ^a		6.24 (0.26) ^a		7.45 (0.25) ^a		7.32 (0.54) ^a	
A-OA-GTE 7.0	3.49 (0.14) ^b	3.42	4.49 (0.47) ^b	1.75	6.99 (0.26) ^a	0.46	6.62 (0.14) ^a	0.71
A-OA-GTE 5.5	1.15 (0.00) ^c	5.76	4.43 (0.19) ^b	1.71	7.53 (0.40) ^a	-0.08	6.53 (0.52) ^a	0.79

Mean value (standard deviation). Within each column for each temperature and time, different letters denote significant differences between treatments. A-OA alginate film containing oleic acid; A-OA-GTE 7.0: alginate film containing oleic acid and GTE at pH 7.0; A-OA-GTE 5.5: alginate film containing oleic acid and GTE at pH 5.5

Table 3. The effect of edible films on the infectivity of hepatitis A virus (HAV).

FFD/coating	Temperature							
	37°C		25°C		10°C			
	Overnight		Overnight		Overnight		4 days	
	log TCID ₅₀ /mL	Reduction	log TCID ₅₀ /mL	Reduction	log TCID ₅₀ /mL	Reduction	log TCID ₅₀ /mL	Reduction
A-OA	4.70 (0.45) ^a		4.53 (0.31) ^{ab}		6.07 (0.45) ^a		5.74 (0.72) ^a	
A-OA-GTE 7.0	4.82 (0.25) ^a	-0.13	4.95 (0.22) ^a	-0.42	6.07 (0.66) ^a	0.00	4.87 (0.36) ^b	0.87
A-OA-GTE 5.5	3.33 (0.07) ^b	1.67	3.32 (0.13) ^b	1.21	5.74 (0.14) ^a	0.33	4.78 (0.59) ^b	0.96

Mean values (standard deviation). Within each column for each temperature and time, different letters denote significant differences between treatments.

A-OA: alginate film containing oleic acid; A-OA-GTE 7.0: alginate film containing oleic acid and GTE at pH 7.0; A-OA-GTE 5.5: alginate film containing oleic acid and GTE at pH 5.5

Table 4 Mechanical properties, water vapour permeability and water vapour sorption of the stand-alone coatings.

FFD/coating	E (MPa)	TS (MPa)	EAB (%)	We (g water/ 100 g sample) at 100% RH	WVP 10 ¹³ (Kg·Pa ⁻¹ s ⁻¹ m ⁻²)
A	4524 (364) ^a	59 (5) ^a	1.5 (0.1) ^a	93 (1) ^a	2.45 (0.23) ^a
A-OA	883 (64) ^b	7.44 (0.24) ^b	0.8 (0.1) ^b	63 (6) ^b	1.87 (0.10) ^b
A-OA-GTE 5.5	1607 (76) ^c	11 (3) ^b	0.8 (0.2) ^{ab}	43 (3) ^c	2.00 (0.14) ^{ab}

WVP: water vapour permeability, E: Elastic modulus, TS: Tensile strength, EAB: elongation at break, We: water vapour sorption. Mean values (standard deviation).

Different superscripts within a column indicate significant differences among FFD/coating ($p < 0.05$).

Table 5. Surface solid density (SSD) in coated strawberries and raspberries and apparent viscosity values at 100 s⁻¹ of FFD.

FFD/coating	SSD /g cm ⁻²		η_{ap} (Pa·s)
	Strawberries	Raspberries	
A-OA	4.13 (0.35) ^{a1}	1.70 (0.20) ^{a2}	281 (6) ^a
A-OA-GTE 5.5	4.64 (0.23) ^{a1}	1.54 (0.15) ^{a2}	230 (10) ^b

Mean value (standard deviation). SSD: Surface Solid Density,

Different superscripts within a column indicate significant differences among FFD/coating ($p < 0.05$).

Different superscripts within a file indicate significant differences among the strawberries and raspberries ($p < 0.05$).

Figure Captions

Figure 1. SEM micrographs of the cryo-fractured section of the A (A), A-OA (B) and A-OA-GTE 5.5 (C) stand-alone coatings.

Figure 2. Optical images of uncoated (A) and coated (B) strawberries. A detailed of the coating is also given (C).

Figure 3. Optical images of uncoated (A, C) and coated (B, D) raspberries: surface (A, B) and cross-section (C, D) images.

Figure 4. Reduction of murine norovirus (MNV) (A) and hepatitis A virus (HAV) (B) titers (log TCID₅₀/mL) on strawberry surfaces after treatment with GTE-coatings at two different storage temperatures.

Each bar represents the average of triplicates. Within each column, different letters denote significant differences between treatments.

Black bars: Control; grey bars: overnight storage period; white bars: 4-day storage period; dashed line depicts the detection limit.

Figure 5. Reduction of murine norovirus (MNV) (A) and hepatitis A virus (HAV) (B) titers (log TCID₅₀/mL) on raspberry surfaces after treatment with GTE-coatings at two different storage temperatures

Each bar represents the average of triplicates. Within each column, different letters denote significant differences between treatments.

Black bars: Control; grey bars: overnight storage periods; white bars: 4-day storage periods; dashed line depicts the detection limit.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

SUPPLEMENTARY MATERIAL

Table S1. Green tea derived phenolic compounds identified in the HPLC/MS spectra of GTE.

RT (min)	m/z	Tentative identification
5.46	305 [M-H] ⁻	Epigallocatechin/gallocatechin
5.79/6.01	289 [M-H] ⁻	Epicatechin/catechin
0.91	169 [M-H] ⁻	Gallic acid
5.46/6.19	457 [M-H] ⁻	Epigallocatechin gallate
5.46	457 [M-H] ⁻ /479 [^] M + Na-2H]; 915 [2M-H] ⁻	Gallocatechin gallate
7.84	441 [M-H] ⁻	Epicatechin gallate/catechin gallate
8.50/8.81	463 [M-H] ⁻	Quercetin galactoside
9.50	593 [M-H] ⁻	Kaempferol rutinoside
8.3/8.5	447 [M-H] ⁻	Kaempferol galactoside/ Kaempferol glucoside
7.90	479 [M-H] ⁻	Myricetin gallactoside
8.47	463 [M-H] ⁻	Quercetin
9.8/10.05	563 [M-H] ⁻	Theaflavin
8.80	609 [M-H] ⁻	Quercetin rutinoside

RT= retention time (min)

Figure S1. Detailed images of uncoated (A, C) and coated A-OA-GTE 5.5 (B, D) berries after two-week storage at refrigerated conditions.

Figure S2. Polyphenolic profile of A-OA-GTE 5.5 o A-OA-GTE 7.0 films.