On the use of carrageenan matrices for the development of antiviral edible coatings of interest in berries

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ABSTRACT

Different film-forming dispersions (FFD) based on κ -, ι - and λ - carrageenans and green tea extract (GTE) have been developed as an innovative strategy to guarantee the food safety of blueberries and raspberries. First, the FFD were characterized (surface tension and viscosity) and the physicochemical properties (water vapour permeability-WVP-, water sorption, contact angle, mechanical properties) of the stand-alone films were evaluated. Then, the FFD were applied to refrigerated-stored raspberries and blueberries, and the antiviral activity against murine norovirus (MNV), a cultivable norovirus surrogate, and hepatitis A virus (HAV) of coated fruits was determined at refrigerated (10 °C) and ambient conditions (25 °C). The type of carrageenan used significantly affected the WVP and the mechanical properties of the stand-alone films, being κ -carrageenan films more rigid and less permeable. The incorporation of GTE resulted in less ductile and deformable films and slightly more permeable than their counterparts prepared without the extract. All the coatings were effective in extending the shelf life of raspberries and blueberries under refrigeration, preserving their firmness to a greater extent and promoting better appearance. In general, FFD with similar viscosity (κ - and λ - carrageenans) showed higher antiviral activity as the gelling capacity of the carrageenan increased (κ - carrageenan) because of the formation of a more cohesive polymer matrix and the higher solid surface density (SSD) deposited onto the berry surfaces. Adding GTE enhanced the carrageenan antiviral activity at both refrigerated and ambient temperatures in blueberries and raspberries, being slightly more effective in the case of MNV.

KEYWORDS: carrageenan, antiviral, active coatings, food-borne pathogens, berries

1. INTRODUCTION

Over the last decade, the inclusion of fresh produce in the human diet has been steadily increasing due to heightened consumer awareness of the associated health benefits. Unfortunately, this increase in fresh produce consumption has been associated with a simultaneous increase in the incidence of foodborne illnesses. Despite accounting for the major causes of foodborne outbreaks in high-income countries, human enteric viruses have received comparatively less attention than other foodborne pathogens. In US, viruses are the most common etiologic agents identified in produce associated outbreaks (54%), frequently linked with food-handling errors (Bennett et al., 2018). Among them, berries have been identified as important vehicles for the transmission of foodborne viruses, such as human noroviruses and hepatitis A virus (HAV) (Lynch, Tauxe, & Hedberg, 2009). For example, since January 2018, of the 53 alert notifications involving viruses reported in the European Union's Rapid Alert System for Food and Feed (RASFF) database, 11 were associated with berries, namely strawberries, raspberries, blueberries, blackberries, currants and cherries. Berries are usually picked for fresh consumption and are packed without washing because they are highly perishable, thus potential virus contamination coming from contaminated irrigation water or improper hygiene practices is unlikely to be removed (Butot et al., 2008; Maunula et al., 2013).

Given the lack of effective, realistic, and validated strategies to eliminate or reduce viral contamination in berries without significantly modifying the fruits' physicochemical and sensorial properties (Sánchez, 2015), the development of edible antiviral coatings has recently been postulated as an innovative strategy with the potential to guarantee the food safety of these products (Fabra et al., 2018; Falcó et al., 2019; Randazzo et al., 2018).

Research interest in edible coatings based on natural polymers and food-grade additives has been increasing in recent years (Fang et al., 2018; Poverenov et al., 2018; Saricaoglu et al., 2018). The natural polymers appropriate for this purpose include a variety of proteins, polysaccharides, and lipids that can be combined to extend the shelf life and improve the safety of food products. These polymers can be customized to actively inhibit the growth of fungi, pathogenic bacteria, and, most recently, human enteric viruses (Randazzo et al., 2018). Food-grade additives generally recognized as safe (GRAS), including natural extracts, for example, green tea extract (GTE) and grape seed extract (GSE), essential oils, and the groups of chemical compounds (i.e. polyphenols) are excellent candidates for the development of active biopolymer-based coatings (D'Souza, 2014; Fabra et al., 2018; Li et al., 2013; Randazzo et al., 2018; Ryu et al., 2015).

Many studies have been carried out to investigate the potential of marine polysaccharides extracted from algae (e.g., carrageenan, agar, and alginate) or crustaceans (e.g., chitin and chitosan) to be utilized as biopolymer matrices for the development of edible films and coatings (Fabra et al., 2018; Fang et al., 2018; Hajji et al., 2018; Poverenov et al., 2018; Ramu et al., 2018; Sapper et al., 2018). Marine polysaccharides, especially those from seaweeds, are also attracting increasing attention in medicine and pharmaceuticals because they have been found to possess unique structures that exert virucidal effects by interfering with different stages of the viral infection process (Bouhlal et al., 2011; Chen et al., 2018; Shi et al., 2017; Wang et al., 2012). An interesting group of marine polysaccharides are carrageenans, a family of linear sulfated polysaccharides that are extracted from red seaweeds and account for 30–75% of the algae's dry weight (Zhang et al., 2010). Carrageenans, sulfated D-galactans with high molecular weights, are composed of repeating disaccharide units with 3-linked- β -D-galactopyranose, 4-linked- β -galactopyranose, or 3,6-anhydro- β -

galactopyranose (Funami et al., 2007; Vera et al., 2011). The number and position of the sulfate groups on the repeating disaccharide units classify carrageenans into three major types: λ , ι , and κ .

A number of studies have found evidence for the antiviral activity of carrageenans, through a mechanism of neutralizing the positive charges present on cell surfaces with the negative charges from the sulfate groups, thus interfering with viruses' adsorption onto cells (Gomaa et al., 2016; Klimyte et al., 2016). However, although carrageenans have been widely used as polymer matrices (Fabra et al., 2009; Farhan et al., 2017; Hambleton et al., 2009; Nur Fatin Nazurah et al., 2017; Ramu et al., 2018; Sanchis et al., 2018; Thakur et al., 2016), their potential to assure the food safety by themselves has not yet been explored.

To the best of our knowledge, there is no existing literature on the formulation and characterization of carrageenan-based antiviral coatings. Furthermore, no data are present in the literature regarding the antiviral activity of κ -, ι -, and λ - carrageenans against human enteric viruses. Therefore, the main goals of this study were first to develop and characterize edible active antiviral coatings based on κ -, ι -, or λ - carrageenans and then to assess their antiviral efficacy when applied onto the surfaces of blueberries and raspberries at environment and refrigeration temperatures. The synergic antiviral effect of these polysaccharide matrices with GTE, a natural compound recently reported to possess antiviral properties (Falcó et al., 2018; Fabra et al., 2018), was also evaluated. In fact, several chemical constituents from the GTE have been previously identified as having antiviral capacity (Falcó et al., 2018, 2019). Furthermore, the morphology and physicochemical (mechanical, water vapour barrier, water sorption, contact angle) properties of the edible carrageenan–GTE film were analyzed to evaluate the applicability of these edible coatings.

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120 **2. MATERIALS AND METHODS**

121 **2.1 Materials**

122 κ - ι - (specifically, sodium iota carrageenan) and λ - carrageenan were kindly provided by
123 Ceamsa (Pontevedra, España). GTE with high oxygen radical absorbance capacity (ORAC)
124 (> 70% catechins content and > 50% EGCG content, measured by HPLC) was kindly donated
125 by Naturex, S.A. (France). Blueberries (*Vaccinium corymbosum*) and raspberries (*Rubus*
126 *idaeus L.*) were obtained from a local market, then immediately transported to the laboratory
127 where berries were selected for uniformity of size and freedom from pathological and
128 physiological defects for use in the experiments.

129 **2.2 Preparation of film forming dispersions (FFD)**

130 Six different film-forming dispersions (FFD) based on κ -, ι -, λ - carrageenan were prepared.
131 FFD containing GTE were prepared as follows: each carrageenan (1 % w/w) was directly
132 dissolved in water at 40 °C and, once the carrageenan was completely dissolved, 0.7 % (w/w)
133 GTE was added to the aqueous dispersion and stirred until it was solubilized in the
134 carrageenan-based matrices. Control κ -, ι - and λ - carrageenan films without GTE were also
135 prepared for comparative purposes.

136 **2.3 Preparation and characterization of stand-alone films**

137 Stand-alone coatings (films) were obtained by casting. FFD were poured onto levelled Teflon
138 casting plates of 15 cm in diameter and were dried at 30 °C and 45% relative humidity (RH)
139 for 24h. Films were prepared by pouring the amount of FFD that would provide 1 g of total
140 solids, so as to keep the total solids content constant in the dry films (56 g/m²). Dried films
141 were peeled off from the casting surface and preconditioned in desiccators at ~54% RH and

23 $\pm$ 2 °C, using an oversaturated salt solution of magnesium nitrate. Film thickness was measured in quintuplicate using a hand-held digital micrometer (Palmer-Comecta, Spain, $\pm$ 0.001 mm) and the average value was used in mechanical properties and water vapour permeability (WVP) calculations.

2.3.1 Scanning electron microscopy (SEM)

The microstructure of the films was visualized in a Hitachi SEM microscope (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance of 8-10 mm. The films (three samples per formulation) were frozen in liquid nitrogen and fractured immediately. Then, they were mounted on M4 Aluminium Specimen Mount and fixed on the support using double-side adhesive tape and, finally, a thin coating of palladium-gold was sprayed on their surface to explore the cross-section of the samples.

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 to determine tensile strength (TS), elastic modulus (E) and elongation (EAB) properties, according to ASTM standard method D882-09 18 (ASTM, 2010a). E, TS and EAB properties were obtained from the stress-strain curves estimated from force-deformation data. After drying, three samples of each obtained film were selected for the tensile property measurements and they were equilibrated for four days at 54% relative humidity (RH) in a cabinet using magnesium nitrate saturated solution at 23 $\pm$ 2 °C. Prior to the test, the thickness of the samples was randomly measured at four points. At least eight replicates of each film formulation were tested. Equilibrated specimens were mounted in the film extension grips and stretched at 50 mm min⁻¹ until breaking. 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 magnesium nitrate saturated solution (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.

2.4. Challenge tests

2.4.1. Surface solid density (SSD)

Selected blueberries (*Vaccinium corymbosum*) and raspberries (*Rubus idaeus L*) were dipped in the FFD for 2 min and air-dried for 1 h at room temperature. The mean value of the coating was calculated in ten samples by quantifying the SSD, as described in Falcó et al., 2019 (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 blueberry and raspberry geometries as sphere and cone, respectively 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.

2.4.2. Water vapour resistance (WVR), firmness and colour properties.

Coated and non-coated samples were stored on PET trays at 10 °C, where the pieces did not come into direct contact with each other. Physicochemical analysis of non-coated (control) and coated samples were performed during 7 and 14 days for raspberries and blueberries, respectively. The water vapour resistance (WVR) of raspberries and blueberries was determined in eight samples per FFD and in eight non-coated samples that were kept in desiccators containing an oversaturated magnesium nitrate solution to generate 54% RH. The desiccators were kept in an incubator Hot-Cold (Selecta, Barcelona, Spain) at 10° C. WVR was calculated using a modified equation of Fick's First Law (Eq. 2), as described by Avena-Bustillos et al., 1994.

$$WVR = \frac{a_w - (\%HR/100) \times P_{wv}}{R \times T} \times \frac{A_s}{J} \quad (\text{Eq. 2})$$

where J is the slope of the weight loss curve in stationary conditions, A_s is the average sample surface area, a_w water activity of samples ($a_w \sim 0.99$), P_{wv} saturated water vapour pressure, T absolute temperature and R the universal constant of gases.

The firmness of berries was measured by using a TA-XTplus Texture Analyser (Stable Micro Systems, Surrey, UK), with a 50 N load cell, using a 2 mm diameter cylindrical probe. Berries were cut longitudinally and 80% compressed at a 0.2 mm/s deformation rate. Force and

distance at the failure point were used as mechanical parameters. Measurements were carried out in four fruit (eight halves) per coating formulation and in four non-coated strawberries (eight halves).

The colour parameters of berries were determined using a CM-3600d spectrophotometer (Minolta Co, Tokyo, Japan) with a 10 mm diameter window. The measurements were taken in four samples per FFD before and after coating and at each time of storage. CIE-L*a*b* coordinates, hue (h^*_{ab}) and chroma (C^*_{ab}) (CIE, 1986) were obtained from the reflection spectra of the samples using D65 illuminant/10° observer.

2.4.3 Antiviral test on berries

Murine norovirus (MNV-1) (kindly provided by Prof. H. W. Virgin, Washinton University School of Medicine, USA) and hepatitis A virus (HAV) strain HM-175/18f (purchased from ATCC VR-1402) were propagated and quantified in the cell line RAW 264.7 (also provided by Prof. H. W. Virgin) and in FRhK-4 (kindly provided by Prof. A. Bosch, University of Barcelona, Spain), respectively. Viruses were harvested and enumerated as described by Falco et al., (2017)

Blueberries and raspberries were exposed to UV for 15 min on a sterile plate in a laminar flow hood to reduce the microbial load. Then 50 µL of MNV and HAV suspensions (about ca. 5 log TCID₅₀/mL) were inoculated on the berry surfaces and let dry for 1 h at room temperature under a laminar flow hood. Then each berry was immersed for 2 min into carrageenan-based FFD, after drying, samples were incubated ON at 25 and 10 °C. Samples incubated at 10 °C were also stored for 4 days. On each sampling day, individual berry samples were placed in a tube containing 5 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS and shaken for 2 min at 180 rpm to release viral particles from the surface. Berries were removed from the DMEM suspension, and then

viruses were recovered and titrated. Serial ten-fold dilutions were performed from the resultant virus suspension and confluent cell lines in 96-well plates were used to evaluate the antiviral effect of the coatings. Each treatment was performed in triplicate. Positive controls were uncoated berries and coated berries without GTE in its formulation 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 carrageenan coatings and N_x is the infectious virus titer for carrageenan-GTE coatings (Falcó et al., 2018).

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. Furthermore, data were analyzed using a Principal Component Analysis (PCA) to explain the total variance resulted in challenge tests. To this end, physicochemical data (SSD, WVR, F/D, and optical proprieties) were normalized and subjected to PCA. Only factors resulted to have Eigen-values higher than 1.00 were selected according to the Kaiser criterion (Jolliffe, 2011). Statistical data processing and graphical elaborations were achieved by using STATISTICA software version 7 (StatSoft Inc., Tulsa, OK, USA).

3. RESULTS AND DISCUSSION

3.1. Properties of film-forming dispersions (FFD)

Table 1 summarizes the results of the surface tension test and the rheological analysis of the FFD under study. Surface tension is an important property for food coating solutions since it significantly affects both the suspension spreadability (the ability of the FFD to spread over

a solid surface and adhere to it) and the integrity of the coating layer after drying (Mostafavi, Kadkhodae, Emadzadeh, & Koocheki, 2016). The presence of carrageenan reduced the surface tension of water (71 mN/m at 35 °C, Walstra, 2003) to 44-64mN/m, being κ - and λ -carrageenan those with lower surface tension values probably due to the higher molecular weight of these carrageenans as compared to ι -carrageenan based FFD. Furthermore, the incorporation of GTE extract, with marked polar character, provoked a slight reduction of the surface tension of the carrageenan dispersions ($p<0.05$), in agreement with a partial substitution of the polysaccharide by polyphenolic molecules at the air-water interface which could contribute to reducing the surface tension. Similarly, Katsouli et al., 2010 also reported a decrease in the net air/water surface tension of aqueous solutions by the presence polyphenols.

Table 1. Surface tension and rheological parameters of the FFD: Ostwald de Waale model parameters and apparent viscosity (η_{ap}) at 100 s⁻¹, at 35°C. Mean values (standard deviation).

FFD	μ (Pa×s)	n	κ (Pa×s)	η_{ap} (Pa×s) (g =100 ⁻¹)	Surface tension (mN/m)
κ -	-	0.584 (0.080) ^a	1.067 (0.613) ^a	0.146 (0.035) ^a	46.17 (2.92) ^a
κ - GTE	-	0.681 (0.015) ^a	0.533 (0.078) ^a	0.122 (0.010) ^a	42.57 (1.71) ^b
ι -	0.035 (0.002) ^a	-	-	-	63.70 (1.51) ^b
ι - GTE	0.036 (0.002) ^a	-	-	-	51.63(2.32) ^a
λ -	-	0.504 (0.007) ^a	1.436 (0.131) ^a	0.146 (0.008) ^a	48.87(1.08) ^a
λ - GTE	-	0.513 (0.002) ^a	1.284 (0.011) ^a	0.136 (0.013) ^a	41.67 (2.12) ^b

Different letters in superscripts (a-b) indicate significant differences among the samples. n: flow index, κ : consistency index, η_{ap} : apparent viscosity at shear rate of 100 s^{-1} .

The average liquid film thickness (and the amount of liquid coating adhered to the surface on coated products) is directly related to viscosity and draining time of the FFD (Cisneros-Zevallos and Krochta, 2003). Complete flow curves presented in Figure 1 show that ι -carrageenan dispersions behaved as Newtonian liquids with a linear relationship between the shear-stress (σ) and the shear-rate ($\dot{\gamma}$). However, a shear thinning behavior (pseudoplastic) was observed for κ - and λ -carrageenan dispersions, which from the viscosity values and effect on the surface tension of the solutions, seem to be higher molecular weight carbohydrates. The rheological data of pseudoplastic FFD were fitted to the Ostwald de Waale model. Table 1 gathers the viscosity values for the FFD with Newtonian behavior and, the flow (n) and consistency indexes (κ), together with the apparent viscosity (η_{ap}) values at a shear rate of 100 s^{-1} for the pseudoplastic FFD. The values of the correlation coefficient of the Ostwald de Waale model were in all cases around 0.99. The first clear observation was that κ - and λ - carrageenan made the FFD more viscous and more shear thinning ($n < 1$). When GTE was incorporated into the carrageenan-based solutions, the rheological behavior was very similar to that obtained for their counterparts prepared with pure κ -, ι - or λ -carrageenan.

Figure 1. Experimental (symbols) and predicted (lines) flow curves obtained by Ostwald de Waale model for the different FFD.

3.2. Properties of the stand-alone films

Physicochemical and functional properties of edible films, such as mechanical, water vapour/oxygen barrier and optical properties are directly related to their microstructure and affected by the interactions between film components and drying conditions (Fabra et al., 2009b). Figure 2 displays representative images of the cross-sections of the films. On the microstructural level, the first thing to highlight is that κ -carrageenan films with and without GTE showed fragility probably due to the formation of a more cohesive structure than their counterparts ι - or λ -carrageenan and ascribed to the higher gelling capacity of κ -carrageenan. Therefore, despite of having a similar behavior in terms of surface tension and viscosity than the λ -carrageenan, the different sulphate content is known to affect the hydrogel network formed after drying (Campo et al., 2009) and, thus, to the final properties of the stand-alone films.

Generally, carrageenan and GTE are highly compatible as deduced from the SEM micrographs since no phase separation was observed. Therefore, interactions between carrageenan and GTE might favor the integration of the polyphenolic compounds in the polysaccharide matrices. In fact, polyphenols contain hydrophobic aromatic rings, which can interact with hydrophobic substrates (Fabra et al., 2018, Tamba et al., 2007) and, hydrophilic hydroxyl groups, which can interact with the carrageenan. These potential interactions between carrageenan and polyphenolic extracts via hydrogen bonding could be the responsible of the formation of a more fragile structure in GTE containing films as shown below in the analysis of the mechanical properties.

Figure 2. SEM micrographs of the cryo-fractured section of the developed stand-alone coatings (scale marker 100 µm).

Optical properties (transparency and color parameters) are of great importance because they directly influence consumer acceptability. Figure 3 shows the spectral distribution curves of the internal transmittance (T_i) of the developed films. Over the wavelength range considered,

a similar pattern was observed for neat carrageenan films (without GTE), although λ -carrageenan films were less transparent. The incorporation of GTE, promoted a selective decrease in the T_i of the films between 400 and 550nm due to the selective absorption of the red-brown components of GTE as well as the some additional light scattering brought about by the polyphenol extracts (with a different refractive index). Similar effects have been previously observed in chitosan-GTE (Siripatrawan and Harte, 2010), agar/carrageenan/GSE (Kanmani and Rhim, 2014 ab) and alginate/lipid/GTE films (Fabra et al., 2018). Table 2 gathers the values of the color coordinates (L^* , lightness; C_{ab}^* , chrome; h_{ab}^* , hue) of the different films. Due to the typical color of GTE, films with GTE were darker (lower L^*), with a more saturated reddish color (higher C^* and lower h^*).

Table 2. Color parameters of the developed films. Mean values (standard deviation).

Films	L^*	C_{ab}^*	h_{ab}^*
κ -	75.2 (0.5)	13.1 (0.9)	82 (2)
κ - GTE	50.9 (1.2)	27.5 (1.0)	55 (2)
ι -	76.4 (0.4)	13.7 (0.5)	85 (1)
ι - GTE	41.9 (1.5)	20.1 (0.9)	57 (2)
λ -	58.0 (2.0)	20.4 (1.9)	69 (3)
λ - GTE	47.7 (1.6)	19.9 (2.0)	60 (3)

Different superscripts within a column indicate significant differences among formulations ($p < 0.05$).
 L^* lightness, C_{ab}^* Chroma, h_{ab}^* hue

Figure 3. Spectral distribution of internal transmittance (Ti) of the developed edible films.

Table 3 shows the tensile parameters of the developed films: Young's modulus (E, MPa), tensile strength (TS, MPa) and elongation at break (EAB, %), which are closely related to the film microstructure. As it is well known, E represents the stiffness of the films, TS is the resistance to break and EAB refers to the stretching capacity. The analysis of tensile properties is interesting since edible films have to preserve their integrity during food manipulation until consumption. It is noticeable that κ -carrageenan films were much more rigid than ι - and λ -carrageenan, with a Young's modulus increased by more than twice. This different behavior can be mainly ascribed to a higher interaction between κ -carrageenan polymer chains (due to the lower sulfate content), which conferred them the gelling capacity and promoted different structural properties once they were dried. The tensile behavior of the films was also strongly affected by the incorporation of GTE, leading to stiffer films (higher E values) but less resistance to break (higher TS values) and stretchable (lower EAB values) than their counterparts prepared without GTE. This increase in stiffness and decrease in extensibility could be attributed to the carrageenan-polyphenols interactions, as it will be

detailed below. Unexpectedly, κ -carrageenan/GTE films showed lower TS values than the corresponding κ -carrageenan prepared without GTE, although they were more rigid and less stretchable than neat κ -carrageenan films (without GTE), in agreement to that previously observed when ι - and λ -carrageenan films with and without GTE were compared. Sivarooban et al. (2008) have reported similar results for soy protein films containing GSE who also attributed this behavior (increased E and TS values and decreased EAB values) to the existing polyphenol-protein interactions. In fact, several works have demonstrated protein-polyphenol interactions (Gómez-Mascaraque et al., 2018, Jin et al., 2018, Liu et al., 2018, Ramos et al., 2018). However, there is a controversy concerning the behavior of TS, based on the type of hydrocolloid and the concentration of polyphenolic extract. Giménez et al., 2013 pointed out that the decrease in TS due to the incorporation of polyphenolic compounds into agar-based films could be attributed to a decrease in the molecular interactions between agar molecules. In contrast, other studies have reported an increase in TS in different polymer matrices such as agar-gelatin, chitosan or soy protein that incorporate GTE or GSE in concentrations up to 50% in the FFD. They attributed this effect to the established interactions between polyphenolic compounds and polymer matrices (Hong et al., 2009, Siripatrawan and Harte, 2010, Sivarooban et al., 2008). On the other hand, when the amount of extract was higher, it might not be interacting with the matrix, inducing the development of a heterogeneous structure and causing a decrease in TS (Bravin et al., 2004). Infrared spectroscopy was used to investigate potential interactions between GTE and carrageenan based matrices. ATR-FTIR spectra related to carrageenan-based films containing or not GTE are gathered in Figure 4. In general, all the carrageenans considered presented a broad absorption band in the 1210-1260 cm^{-1} spectral region which is ascribed

to the S=O stretching vibration of the sulfated groups and it was correlated to the sulfate content of the samples: the intensity of the band decreased from the λ -carrageenan (higher intensity) to κ -carrageenan (lower intensity). The presence of the sulfate groups gave rise to characteristic bands with the frequency being dependent on the position of the sulfate ring within galactose and 3, 6 -anhydrogalactose units (Pereira et al., 2009). For instance, κ - and λ -carrageenan showed an absorption band at approximately 845-850 cm^{-1} which is assigned to the sulfate group at the C₄ position in the D-galactose ring (C₄-O-S stretching vibrations), and the band at around 805 cm^{-1} resulting from the sulfate group at the C₂ position in the 3,6, anhydro-D-galactose ring (C₂-O-S bending vibrations) appeared in ι -carrageenan.

The broad absorption band in the region of 2900 and 3800 cm^{-1} corresponds to hydroxyl group vibrational stretching and it is associated to OH bond in water or hydroxyl groups of the carrageenan. If the hydroxyl groups of the biopolymer matrices are largely in interaction with water, some water adsorption occurred under environmental conditions used. This is undoubtedly evidenced by the H-OH bending vibration at 1640 cm^{-1} , characteristic of physisorbed water.

When incorporating GTE, the contribution of the -OH with a stretching vibration band at approximately 3400 cm^{-1} slightly shifted to lower wavenumbers, mainly in ι - and λ -carrageenan probably due to interactions with the hydroxyl groups of the GTE. Similarly, shifts in the bands associated with the physisorbed water region, mainly observed in ι - and λ -carrageenan-incorporating GTE can be ascribed to interactions that occur between GTE and carrageenan. These changes could also explain the lower water sorption found in GTE-containing films instead of their higher WVP values when they were compared to their counterparts without the extract. Furthermore, when GTE was incorporated in the

carrageenan matrices, the band in the sulfate groups region of 1210 and 1260 cm^{-1} slightly shifted to lower wavenumbers. Similarly, a characteristic band at 1618 cm^{-1} , attributed to the aromatic ring quadrupole and the band at 1097 cm^{-1} owed to the aromatic rings stretch of GTE (spectrum showed elsewhere, Robb et al., 2002) were slightly displaced in the GTE-containing films with respect to free GTE, being centered at 1690 cm^{-1} and 1113 cm^{-1} , respectively in the GTE-containing films.

Thus, the ATR-FTIR characterization allowed identifying the groups involved in GTE-carrageenan interactions, and confirming the assumptions from the physicochemical properties.

Figure 4. ATR-FT-IR spectra of representative samples.

Barrier properties of edible films are usually described by their permeability values. Table 2 summarizes the measured water vapour permeability (WVP) for the developed films. The WVP values of the neat carrageenan-based films were lower than those previously reported

for carrageenan cast films (Shojaee-Aliabadi et al., 2014, Rhim and Wang, 2014) since the films prepared in the present work did not incorporate plasticizers. It should be noted that the type of carrageenan affected the WVP values, observing a statistically significant increase ($p < 0.05$) as the number of sulfate groups increased in the carrageenan, that is, the WVP was lower in films formulated with κ – carrageenan than that obtained for ι –carrageenan based film and, they were also less permeable than films formulated with λ –carrageenan. This could be related to the gelling capacity of the carrageenan (it is well-known that weaker gels are obtained when the number of sulfate groups in the carrageenan molecules increases). Accordingly, the water sorption of κ – carrageenan films (Table 3) was lower than their counterparts ι – and λ – carrageenan films, suggesting that the diffusion of water molecules throughout the carrageenan-based matrices is dependent on the gel cohesion, being lower for κ – carrageenan -based films which had a greater gelling capacity than ι – and λ – carrageenan. It should be noted that, in film format (or dehydrated gel), the apparent diffusivity of water in carrageenan can be 100 times lower (Rondeau-Mouro et al., 2004). This is due to the increase in stiffness (hardening) of the polymer network and the subsequent decrease in mobility. In fact, the hydration process involves interactions with the hydroxyl (-OH) and sulfate ($-\text{SO}_3^-$) groups of the carrageenan, which can decrease the diffusion of water through the polymer matrix. In addition, the diffusion coefficient may vary with time. More probably, thickness could increase during the time of the experiment with the hydration shell and this could be a determining factor in the WVP.

Table 3. Mechanical properties, water vapour permeability, water vapour sorption and contact angle of the stand-alone coatings.

Films	E (MPa)	TS (MPa)	EAB (%)	WVP (Kg- /Pa-s-m ²) 10 ¹⁴	w _e (100%) (g water/100g sample)	θ (°)
κ-	7532 (678) ^a	84.4 (17.6) ^a	2.0 (0.4) ^a	7.2 (0.5) ^a	88.4 (2.7) ^a	67.5 (5.5) ^a
κ- GTE	13370 (291) ^b	23.8 (6.9) ^b	0.4 (0.2) ^b	12.8 (1.5) _c	64.7 (2.8) ^b	29.5 (1.5) ^b
ι-	3160 (144) ^c	41.8 (10.8) ^b	2.9 (0.6) ^b	9.3 (0.3) _{ab}	127.8 (0.2) _c	78.9(5.6) ^c
ι- GTE	7884 (330) ^a	83.9 (10.8) ^{cd}	2.0 (0.7) ^c	15.5 (4.0) _{cd}	84.5 (5.3) ^d	27.0 (6.0) ^b
λ-	2772 (267) ^c	18.9 (9.0) ^c	1.2 (0.5) ^d	12.6(0.4) _{bc}	131.2 (3.0) _c	109.5 (7.6) _d
λ- GTE	6649 (703) ^d	37.4 (4.0) ^{ad}	1.2 (0.3) ^d	17.3 (1.1) _d	106.6 (1.6) _e	37.0 (7.0) ^e

WVP: water vapour permeability. E: Elastic modulus. TS: Tensile strength. EAB: elongation at break. w_e: water vapour sorption. θ: contact angle values. Mean values (standard deviation).

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

All the carrageenan films incorporating GTE had significantly (p<0.05) higher WVP, with a maximum permeability increase of 43%. Similar effects were previously observed in *Gelidium corneum*/nano-clay composite film containing GSE or thymol (Lim et al., 2010) and in rapeseed protein–gelatin film containing GSE (Jang et al., 2011). This increase in permeability was ascribed to a loss of cohesiveness of polymer matrices prepared with GSE or GTE contents up to 0.5 (w/v). Interestingly, the incorporation of GTE into the carrageenan matrices significantly reduced (p<0.05) the water sorption of the films (Table 3). This effect can be ascribed, on the one hand, to the confinement of the polyphenolic compounds into the carrageenan matrices, interacting with the polymer chains through hydrogen bonds and limiting their interaction with water. In fact, this could also contribute to the formation of more rigid and less deformable films, as it has been described above. On the other hand, the addition of GTE into the carrageenan matrices decreased the gelling capacity of the carrageenan, giving rise to less cohesive networks and, thus, the sorbed water could easily diffuse through the biopolymer matrices to a greater extent than their counterparts prepared without GTE, promoting an increase in the WVP. Similar effects have been previously

observed in tuna-fish gelatin with antioxidant extracts of two different murta ecotypes leaves (Gómez-Guillén et al. 2007) and chitosan/GTE films (Peng et al., 2013).

The water contact angle values are normally used to estimate the degree of hydrophobicity of the material. Therefore, the wettability properties of the neat carrageenan films and those containing GTE were also determined by direct measurement of contact angles of a water drop deposited on the upper surface of samples and the results are listed in Table 3. The contact angle values (θ) are reported as a function of carrageenan type and GTE addition. As observed, the contact angle increased as the SO_3^- increased, being higher for λ -carrageenan and lower for κ -carrageenan. The θ values were in the range of those found in the literature for κ -carrageenan) and ι -carrageenan, being 66 ° and 88 °, respectively)(Pinhero et al., 2012. Karbowiak et al., 2016, Rhim. 2012. Rhim & Wang. 2013). From Table 3, GTE addition provoked a significant decrease ($p<0.05$) of the contact angle values, suggesting the more hydrophilic character of these films. This is explained by the increase of polar groups from the polyphenols, leading to an increase of the hydrophilic character of GTE-containing films. These results are in line with those reported by Moradi et al. (2012), who also noted a significant reduction in the contact angle values for chitosan-GTE films.

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

Challenge test on coated blueberries and raspberries at refrigerated temperatures were carried out under conditions of *in vivo* storage, mimicking realistic scenarios of fresh fruit handling. The physicochemical quality and the antiviral effectiveness (for safety assurance of the fruits) of carrageenan-based edible coatings on berries under refrigeration were evaluated.

3.3.1. Surface solid density (SSD) and water vapour resistance (WVR)

SSD values and WVR of non-coated and coated blueberries and raspberries are shown in Table 4. SSD, which can be used as an estimation of coating thickness was significantly affected ($p<0.05$) by the type of berry, type of carrageenan as well as the presence of GTE. The first clear observation is that the SSD was more than three-fold higher for raspberries than blueberries, which can be explained by the different roughness of the skin (reaching higher values for rougher skins such as raspberries).

The trend was broadly the same for raspberries and blueberries when comparing the different film forming dispersions (FFD). Regarding the FFD without GTE, it was observed that SSD significantly decreased ($p<0.05$) when the amount of sulphate groups increased (lower gelling capacity) and the contact angle values of the corresponding stand-alone films increased (more hydrophobic films), being lower in the case of ι - and λ -carrageenan and higher in κ - carrageenan coatings. It should be noted that the incorporation of GTE to the FFD, significantly increased ($p<0.05$) the amount of SSD as compared to their counterparts prepared without GTE. This effect can be ascribed to the lower contact angle and surface tension of these FFDs, which could favor a greater adhesion and spreadability of the coating to the solid surface. Therefore, the SSD is closely related to the hydrophilic character of the stand-alone films, the surface tension of the FFD and to the gelling capacity of each carrageenan. Furthermore, FFD with similar viscosity values were greater deposited onto the surface of raspberries and blueberries as the gelling capacity increased (κ - carrageenan) because of the formation of a more cohesive polymer matrix.

Coatings did not significantly improve the WVR of samples, in agreement with the high permeability values of carrageenan-based films, especially at high RH (100%) when the films are highly plasticized and barrier properties are greatly reduced (Fabra et al., 2009a).

Table 4. Surface solid density (SSD) and water vapour resistance (WVR) of non-coated and coated raspberries and blueberries. Mean value (standard deviation).

FFD	Raspberries		Blueberries	
	WVR (s/cm)	SDD (g/m ²)	WVR (s/cm)	SDD (g/m ²)
C	1.19 (0.36) ^a	-	1.96 (0.23) ^a	-
κ-	1.27 (0.19) ^a	7.08 (0.25) ^a	1.96 (0.23) ^a	1.85 (0.35) ^{ab}
κ- GTE	0.94 (0.15) ^a	10.05 (1.50) ^b	1.51 (0.30) ^a	4.41 (0.80) ^c
ι-	0.76 (0.26) ^a	4.56 (0.72) ^c	1.91 (0.28) ^a	1.08 (0.50) ^b
ι- GTE	1.32 (0.40) ^a	9.92 (0.81) ^d	1.73 (0.21) ^a	2.26 (0.65) ^d
λ-	0.86 (0.28) ^a	4.31 (0.70) ^c	1.73 (0.35) ^a	1.11 (0.81) ^b
λ- GTE	1.36 (0.33) ^a	9.72 (0.89) ^d	2.25 (0.43) ^a	2.42 (0.48) ^d

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

3.3.2. Physicochemical properties

The physicochemical quality of the coated and uncoated samples was evaluated in terms of firmness and color appearance. Firstly, the ratio force-deformation at the break point (F/D) is shown in Figure 5. The first clear observation is that, this parameter, which is related to the resistance to fracture of the product, did not change ($p < 0.05$) by the presence of the coating solutions, except in those coated with κ-carrageenan-GTE which showed even a two-fold increase for blueberries, in line with the greater rigidity of the corresponding stand-alone films (see Table 3). For raspberries, the F/D significantly decreased after one-week storage, except in the case of those coated with κ-carrageenan-GTE, indicating that this latter coating preserved the firmness of the raspberries to a greater extent. It should be noted that the decrease in the F/D parameter was significantly higher in non-coated raspberries than in coated ones. For blueberries, the F/D significantly decreased ($p < 0.05$) during cold storage in non-coated samples but these changes were not significant ($p > 0.05$) in the coated samples, indicating that, in general, coatings had an important role in keeping the firmness of blueberries. However, the mechanical response of blueberries coated with κ-carrageenan-

GTE also changed ($p<0.05$) during cold storage, reaching values in the range of those obtained in non-coated and coated fresh samples ($t=0$ days). These changes in the mechanical response of the samples can be attributed to changes in the structure of the cellular tissue during ripening and senescence (Chiralt et al., 2001).

Figure 5. Mechanical response (F/D) of non-coated and coated samples throughout at refrigerating conditions: (A) raspberries, (B) blueberries

Figure 6 displays color parameters: luminosity (L^*), chroma (C^*_{ab}) and hue (h^*_{ab}) of blueberries and raspberries before and after coating applications. In general, non-significant differences were observed in raspberries although they became more red (lower h^*_{ab} values) during storage. Coated blueberries had a slightly more bluish hue (lower h^*_{ab} values) and a more vivid color (higher C^*_{ab} values) than the non-coated samples (Supplementary material S1), being this effect more pronounced in those coated with κ-carrageenan probably due to the higher SSD. During refrigerated storage, luminosity did not vary significantly ($p>0.05$) neither in non-coated blueberries nor in coated ones. However, the C^*_{ab} values decreased in non-coated blueberries and this decrease was less marked in blueberries coated with the different FFD. The sample hue remained practically constant throughout the storage time and

only significantly decreased in non-coated samples (blueberries became more bluish) between 11 and 14 storage days, reaching values in the range of coated samples. Furthermore, a PCA was also carried out to evaluate the variance related to the physicochemical characteristics of coatings applied on blueberries and raspberries. Results of PCA analysis are represented as scatterplots showing the relationship between factors and samples (score plot, A) and variables (loading plot, B) for raspberries (Supplementary Material Fig. S2) and blueberries (Supplementary Material Fig. S3). For both coated food matrices, the first two Factors gained eigenvalues higher than 1, representing up to 82.3 and 81.0% of the total variance for raspberry' and blueberry 'coatings, respectively. In raspberries (Supplementary Material Fig. S2), Factor1 was negatively correlated with all variables, except for L^* , while Factor2 was positively correlated with SSD and WVR and negatively with F/D , C^*_{ab} and h^*_{ab} . As a result, ι^- and λ^- carrageenans were correlated with L^* . On the contrary, in blueberries 'coatings, ι^- and λ^- carrageenans were positively correlated with variable h^*_{ab} , resulting plotted together in the lower-right quarter. The coatings with GTE resulted widely spread on the score plot, showing any discrimination clearly correlated with variables (Supplementary Material Fig. S3A). In summary, the discrimination of samples based on the scatterplots highlighted differences among the samples that resulted in widely spaced points (Supplementary Material Fig. S2A and S3A). The PCA indicated a correlation among optical proprieties (L^* and h^*_{ab}) and ι^- and λ^- carrageenans, while a discrimination of samples based on the incorporation of GTE was not observed. In conclusion, throughout storage, raspberries samples preserved their appearance although they became slightly redder and the coating did not have a notable effect on this development.

Coated blueberries under refrigeration were slightly lighter and less bluish at the end of storage.

Figure 6. Optical properties (L^* : luminosity, C^*_{ab} : Chroma and h^*_{ab} : hue) of non-coated and coated samples throughout at refrigerating conditions: (A) raspberries, (B) blueberries

568

569 **3.3.3. Antiviral activity**

570 Carrageenan-based FFD, with and without GTE, were used to treat fresh raspberries and
571 blueberries artificially inoculated with MNV and HAV and stored at 10 °C (ON and 4 days)
572 and 25 °C (ON). In general, the effect of carrageenan type on the infectivity of MNV and
573 HAV in fresh raspberries and blueberries after coating treatments was higher at 25 °C
574 although a similar trend was observed at lower temperatures. As observed, FFD with similar
575 viscosity (κ - and λ - carrageenan) showed higher antiviral activity as the gelling capacity of
576 the carrageenan increased (κ - carrageenan) because of the formation of a more cohesive
577 polymer matrix and the higher DSS deposited onto the fruit surfaces. In fact, the infectivity
578 of MNV in fresh blueberries after coating treatments at 25°C for ON incubation was reduced
579 under the detection limit for κ - and ι - carrageenan films containing GTE and by
580 approximately 3.54 log for λ -carrageenan coatings containing GTE (Fig. 7A). Additionally,
581 under the same experimental conditions, κ - and ι - carrageenan coatings without GTE reduced
582 by more than 2.5 log MNV infectivity. Surprisingly, despite of having higher DSS, lower
583 reductions were reported in coated raspberries where MNV titers were significantly ($p<0.05$)
584 reduced by 2.25 and 2.79 log for ι - and λ -carrageenan coatings containing GTE, respectively.
585 This effect can be ascribed to the different roughness of the fruit surface, which can affect
586 the recovery of viruses during the assay. Furthermore, when blueberries were stored at 10°C,
587 great potential was showed obtaining reductions of 2.38 and 3.13 log after ON and 4 days
588 storage while MNV infectivity was below the detection limit for both storage periods for
589 raspberries coated with λ -carrageenan coatings containing GTE. Remarkable, levels of
590 infectious MNV in uncoated raspberries (Fig. 7B and C) were lower than in blueberries

associated to the lower pH of the raspberry surface. Thus, the effect of coated-raspberries was, in some instances, below the detection limit most likely due to a synergistic effect of pH and GTE. Overall carrageenan coatings containing GTE showed higher antiviral activity against MNV than recently described alginate-oleic acid based coatings incorporated with GTE (Falcó et al., 2019) due to the higher SSD deposited onto the fruit surface and the intrinsic antiviral properties of the carrageenan-based matrix, as compared to the alginate matrices.

In addition, there was noticeable less reduction of HAV titers in coated berries (Fig. 8). For instance, HAV titers in blueberries were reduced by 2.88, 2.92 and 1.83 log after ON incubation at 25°C for κ -, ι - and λ -carrageenan coatings containing GTE, respectively. However, only λ -carrageenan coatings prepared with GTE significantly reduced HAV infectivity by 1.37 log on coated raspberries. At refrigerated temperatures, higher efficacy of coatings was observed in raspberries than blueberries, with HAV titers by 1.79, 1.75 and 1.71 after an ON incubation for κ -, ι - and λ - carrageenan coatings containing GTE, respectively (Fig 8B).

Figure 7. Reduction of murine norovirus (MNV) titers (log TCID₅₀/mL) on blueberry and raspberry surfaces after treatment coatings at different temperatures and storage times.

*A: 25°C/ON; B: 10°C/ON; C: 10°C/4 days.

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

***White bars: control without coating; black bars: coating control κ; black-dashed bars: coating κ-GTE; dark grey bars: coating control ι; dark grey-dashed bars: coating ι-GTE; light grey bars: coating control λ; light grey-dashed bars: coating λ-GTE. Horizontal line depicts the detection limit.

Figure 8. Reduction of hepatitis A virus (HAV) titers (log TCID₅₀/mL) on blueberry and raspberry surfaces after treatment coatings at different temperatures and storage times.

*A: 25°C/ON; B: 10°C/ON; C: 10°C/4 days.

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

***White bars: Control without coating; black bars: coating control κ; black-dashed bars: coating κ-GTE; dark grey bars: coating control ι; dark grey-dashed bars: coating ι-GTE; light grey bars: coating control λ; light grey-dashed bars: coating λ-GTE. Horizontal line depicts the detection limit.

4. CONCLUSIONS

In this work, antiviral edible coatings based on κ-, ι- and λ- carrageenan and GTE have been successfully developed. Specifically, κ-, ι- and λ- carrageenan based coatings showed antiviral activity against MNV and HAV. A direct correlation between the surface tension of FFD, the contact angle of the corresponding stand-alone films, the gelling capacity of carrageenan, the SSD and the antiviral activity was observed. On the one hand, FFD with similar viscosity showed higher antiviral activity as the gelling capacity increased (κ-carrageenan) because of the formation of a more cohesive polymer matrix and the higher DSS deposited onto the raspberry or blueberry surfaces. On the other hand, when comparing FFD with and without GTE, lower contact angle and surface tension values (FFD containing GTE) favored a higher SSD onto the fruits, increasing the antiviral activity of the coatings, probably due to a synergistic effect of carrageenan and GTE. In general, carrageenan-based edible coatings did not promote significant changes in the physicochemical quality of

raspberries and blueberries throughout refrigerated storage, although they provided better appearance. Nevertheless, they provided a better preservation of the fruits in terms of antiviral infectivity at refrigerated and ambient conditions, being accentuated by the presence of GTE. Overall, the antiviral activity varied depending on the formulation of coating forming solution, the tested virus, the type of berries and the storage conditions, thus, specific studies to optimize the formulation of antiviral coatings are needed for each specific food matrix.

Acknowledgments

M.J. Fabra is recipient of a Ramon y Cajal (RYC-2014-158) contract from the Spanish Ministry of Economy, Industry and Competitiveness. W. Randazzo was supported by a postdoctoral fellowship from Generalitat Valenciana (APOSTD/2018/150). We would like to thank Tina Lindemann and Nicolás Gil for technical assistance.

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