Antifungal edible coatings containing Argentinian propolis extract and their application in raspberries

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A B S T R A C T

Edible coatings based on gelatin containing an ethanolic extract of Propolis (PEE) collected of hives from Monte region in Argentina were developed and applied to raspberries (*Rubus idaeus* L.) with the aim of prolonging their shelf life. Initially, the antifungal activity of PEE was evaluated against the major fungal pathogens of fruit and vegetables. Then, two different strategies were followed to incorporate the PEE into gelatin-based films: i) by directly incorporating the PEE into the protein matrix and ii) by previously encapsulating the PEE within zein nanocapsules with the aim of controlling the release of the PPE and extending their efficiency with time. Dry films were evaluated in terms of their optical, morphological, mechanical and water barrier properties. Likewise, the antifungal activity of the films against *P. digitatum, P. expansum, P. italicum, A. alternata, A. carbonarius* and *B. cinerea* was also assessed. Finally, film-forming dispersions (FFD) were applied to cold-stored raspberries and the fungal decay was determined throughout cold storage at 5 ºC.

The incorporation of PEE affected the mechanical response of the films, giving rise to more flexible and deformable edible films, but also more colored films with lower transparency. The active films revealed a notable antifungal activity against the tested fungus, showing a greater inhibitory effect on *P. digitatum* and *B. cinerea.* Adding PEE enhanced the antifungal activity during cold storage in raspberries. Furthermore, this proof-of-concept study proved the efficiency of the encapsulation process to reduce the infection incidence in raspberries stored at refrigeration temperatures for a longer period of time.

Keywords: Propolis, gelatin, edible coatings, film-forming dispersions, antifungal activity.

1. Introduction

One of the key problems affecting postharvest industries is the losses caused by fungal pathogens that infect the fruit before, during or after harvest, but develop disease after harvest. These postharvest fungal infections are mainly controlled using fungicides, including inorganic compounds and synthetic chemicals. However, due to the increasing consumer demand for safe and nutritional food, as well as the negative opinion towards the use of synthetic preservatives in food due concerns over the risk and stability of inorganic and synthetic compounds (Hyldgaard, Mygind, & Meyer, 2012), new alternatives should be explored.

The development of active edible coatings is a plausible alternative to control the growth of phytopathogens in fruits during postharvest shelf life. Edible coatings are defined as a thin layer based on proteins, polysaccharides and/or lipids formed on food surfaces that function as a protective layer (Grosso, Asensio, Grosso, & Nepote, 2019). Hydrocolloid matrices can serve as an excellent carrier of active agents like fungicides. Although there are several factors that may affect the stability and release kinetics of the antifungal natural compounds, including their inherent characteristics (molecular weight, concentration or polarity) (Hu, Chen, & Wang, 2012; Suppakul, Sonneveld, Bigger, & Miltz, 2011), the chemical composition of the coated food (Gherardi, Becerril, Nerin, & Bosetti, 2016), or the environmental conditions (Chalier, Ben Arfa, Guillard, & Gontard, 2009; Chen, Wang, Hu, & Wang, 2012; Kurek, Guinault, Voilley, Galic, & Debeaufort, 2014), the hydrocolloids used as biopolymeric matrices play an important role in the design of antifungal edible coatings. Specifically, among the film-forming proteins, gelatin, obtained by hydrolysis of collagen, has effective barrier properties against oxygen and carbon dioxide (Jiang, Liu, Du, & Wang, 2010) and it has been reported as an excellent carrier of active compounds either as edible coatings (Tongdeesoontorn & Rawdkuen, 2019) or as encapsulating matrices (Gómez-Mascaraque et al., 2018). The application of natural extracts to food preservation has received increasing attention in the design of active edible coatings, as a suitable alternative to control fungal pathogens. Many naturally

occurring products, such as essential oils from edible and medicinal plants, herbs, spices and isolated compounds have been used for thousands of years for many purposes, such as food preservatives due to their well-known antimicrobial properties (Hamer et al., 1999; Tiwari et al., 2009).

In fact, the development of proper edible antifungal coatings to be used in postharvest is one of the major challenges for food technologist in the next few years. Natural extracts, such as the propolis extracts, have attracted interest from researchers, as several works have been conducted (Cosa, Vlietinck, Berghe, & Maes, 2006, Gargouri, Osés, Fernández-Muiño, Sancho, Kechaou, 2019; Tugba Degirmencioglu et al., 2019). Propolis is a complex mixture of resinous, gummous and balsamic substances collected by honeybees from exudates and plant sprouts, to which bees add saliva, wax, and pollen to elaborate the final product (Ghisalberti, 1979). Propolis is used in Argentina as a food supplement and alternative medicine (Salas, Mercado, Zampini, Ponessa, & Isla, 2016a). Moreover, it is included in the Argentine food code as an ingredient of sweets, honey, ethanolic extracts and as dietary supplements (Argentine Food Code, 2018). Although the active constituents of propolis differ according to the geographical source, its biological activity depends on compounds from the polyphenolic fractions, mainly flavonoids, followed by aromatic acids, phenolic acid esters and lignans or terpenoid components (Silici & Kutluca, 2005). For instance, due to the great diversity of ecoregions in Argentina, different chemical compounds were also identified in propolis depending on the area where the bees produced it (Isla et al., 2009; Agüero et al., 2011; Kumazawa et al. , 2010; Chaillou et al., 2009). In the case of the propolis used in this work, the botanical origin (*Zuccagnia punctata*) was determined by histological and chromatographic techniques. These medicinal plant as well as *Zuccagnia*-type propolis extracts are typical and exclusive of Argentina. In fact, in previous research works, two main chemical compounds (2´, 4´-dihydroxy-3´-metoxychalcone and 2´, 4´-dihydroxychalcone) were identified in this propolis and in *Zuccagnia punctata* extracts but they were not found in propolis from other regions of Argentina. The extracts and isolated compounds have antimicrobial activity on pathogenic bacteria and yeast (Salas et al., 2016a, b, 2018). However, this is

the first report of antimicrobial activity of *Zuccagnia*-type propolis against a collection of different fungal postharvest pathogens. The application of natural compounds for food preservation has received increasing attention worldwide to control postharvest fungal pathogens and, thus, these bioactive propolis extracts could contribute as an alternative to synthetic antimicrobials in postharvest applications.

In this work, a previously characterized Propolis ethanolic extract (PEE) from the "Monte" region in Argentina, defined as "*Zuccagnia*- type Propolis" according to its botanical origin was used. This propolis has demonstrated interesting biological properties such as antibacterial, anticandida, antiinflammatory (Salas et al., 2016a, b; Salas, Zampini, Maldonado & Isla., 2018) which have been mainly attributed to their high content in chalcones, flavanones and phenolic acids, among other compounds (Salas et al., 2016a, b, 2018).

Although most of the works dealing with active edible coatings have been carried out by directly mixing the active compounds with the biopolymer matrices, their efficiency is somewhat compromised during the coating-forming process because volatile compounds can be lost during the coating-drying process (Sánchez-González, Cháfer, González-Martínez, Chiralt, & Desobry, 2011; Sanches-Silva et al., 2014). In an effort to reduce the losses in antifungal properties during film or coating development, the encapsulation of PEE is being here proposed as a feasible route to stabilize the active agent into biopolymer matrices and, thus to promote a sustained release of the active compounds. Concretely, zein is suggested as an encapsulating matrix to protect the PEE by using the antisolvent methodology (Patel, Hu, Tiwari, & Velikov, 2010).

This proof-of-concept study should provide a new route to be further explored for the development of novel antifungal active coatings with improved efficiency.

Considering these aspects, this work was designed to: (i) assess the antifungal efficiency of a Propolis extract from Argentina against fungal pathogens like *Penicillium digitatum*, *Penicillium expansum*, *Penicillium italicum*, *Botrytis cinerea*, *Alternaria alternata* and *Aspergillus carbonarius;* (ii)

investigate the physicochemical properties of the active edible coatings based on gelatin and PEE, (iii) compare the effect of PEE when it was directly incorporated into the gelatin matrix *vs.* it was previously encapsulated within zein nanocapsules (iv) evaluate the antifungal efficacy of the developed coatings when applied to raspberries at refrigerated temperature.

2. Materials and methods

2.1 Reagents

Gelatin from porcine skin, with reported gel strength of 180 g Bloom, was supplied by Gelita AG (Germany). Zein from corn (grade Z3625, 22-24 kDa) was purchased from Sigma-Aldrich (Madrid, Spain). Glycerol was purchased from Panreac- Aplichem. Potato dextrose broth (PDB) and potato dextrose agar (PDA) was purchased from Scharlau Chemie and Difco-BD Diagnostics (Sparks, MD, USA), respectively.

2.2. Propolis sample and extract preparation

Propolis sample was provided by INTA-PROAPI, Argentina, from hives located in the Agrotechnical School, Monte Region, Tucumán (26°35'S,65°55'W), Argentina. The sample was collected in March 2015 and is representative of the collection time of the raw material for phytotherapeutical purposes. The sample was weighed and frozen at –20 ºC until further processing.

Crude Propolis (20 g) was extracted with 250 mL 80% ethanol at room temperature for 7 days. The extract was taken to dryness under reduced pressure and lyophilized to obtain the Propolis ethanolic extract (denoted as PEE). Extract was stored at –20 ºC in the dark until analysis.

2.3 Preparation and characterization of film forming dispersions (FFD)

147 Two different groups of film-forming dispersions (FFD) (pH ~5.10 ± 0.03) were prepared. First,
148 gelatin films with and without extract were prepared. Likewise, FFD with gelatin and zein capsules
149 were prepared with and without PEE.
150 For gelatin-based FFD, 2.75 g of gelatin was dissolved in 90 mL of distilled water at 40 ºC. Propolis
151 extract dissolved in 10 mL 80% ethanol was added to gelatin solution to reach a final concentration of
152 0.5 mg PEE/mL and 0.5 g glycerol was added as plasticizer. Then, the mixture was magnetically stirred
153 for 30 min at ambient temperature. Control samples were prepared similarly without propolis extract.
154 In order to better protect the extract and to check if the antifungal activity could be prolonged with
155 time, PEE was previously encapsulated in zein nanocapsules. Based on a previous screening test, 0.25
156 g zein dissolved in 10 mL 80% ethanol (with and without propolis extract) were drop-wise added in
157 50 mL distilled water, following 15 min more under stirring at 60 rpm. The particle size of preformed
158 zein nanocapsules with and without PEE, measured by dynamic light scattering (Zetasizer Nano ZS,
159 Malvern Instruments, UK), was ~120 nm and 130 nm, respectively. PEE was incorporated into the
160 zein hydroalcoholic solution at a concentration of 2 mg PEE/g zein to reach a final concentration of
161 0.5 mg PEE/mL in the overall FFD, since the encapsulation efficiency was estimated to be ~ 100%,
162 according to the UV-Vis spectroscopy protocol from Moreno et al. (2019). Furthermore, 2.5 g of
163 gelatin were suspended in 40 mL of distilled water, under magnetic stirring, during 2 h at 40 ºC to
164 guarantee that it was completely dissolved. Then, 0.5 g glycerol was added to the solution and stirred
165 at room temperature during 2 h before being mixed with the as-prepared zein capsules solution
166 containing or not the extract (PEE). Physicochemical properties of FFD are given in Table S1 of
167 Supplementary material.
168 Edible films' nomenclature was: Gel; Gel/PEE; Gel/zein; Gel/zein/PEE.
169

170 **2.4 Preparation and characterization of stand-alone coatings**

171 Films were prepared by pouring the amount of FFD that would provide 1 g of total solids, so as to
172 keep the total solids content constant in the dry films (56 g/m^2). FFD were poured onto levelled Teflon
173 casting plates (diameter = 15 cm), resting on a level surface. Films were dried during 24 h at 30 °C
174 and approximately 40% relative humidity (RH). Dry films were peeled off the casting surface and pre-
175 conditioned in desiccators at 20 ºC and 53% RH, by using oversaturated solutions of magnesium
176 nitrate-6-hydrate (Panreac Química, SA, Castellar del Vallés, Spain) for one week before the analysis.
177 A hand-held digital micrometer (Palmer-Comecta, Spain, ± 0.001 mm) was used to measure film
178 thickness at five different points of the same sample and, the average value of six repetitions was used
179 in mechanical and water vapor permeability calculations.
180
181 *2.4.1. Microstructural analysis*
182 The cross-section of the stand-alone films were observed in a Hitachi scanning electronic
183 microscope (SEM) (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance
184 of 10 mm. Films were previously cryo-fractured in liquid nitrogen, mounted on an 'Aluminium
185 Specimen Mount', fixed on the support using double-side adhesive tape and coated with a thin
186 layer of gold-palladium sprayed on their surface.
187
188 *2.4.2. Optical properties*
189 The transparency of the films was determined through the surface reflectance spectra in a
190 spectrocolorimeter CM-3600d (Minolta Co., Tokyo, Japan) with a 10 mm illuminated sample area.
191 Measurements were taken from three samples in each film using both a white and a black background.
192 The transparency was determined by applying the Kubelka–Munk theory for multiple scattering to the
193 reflection spectra. Transparency (Ti) was calculated, as indicated by Hutchings (1999), from the
194 reflectance of the sample layer on a white background of known reflectance and on an ideal black
195 background.

196

197 *2.4.3. Mechanical properties*

198 Mechanical properties were measured using a Mecmesin MultiTest universal test machine (Landes
199 Poli Ibérica, S.L., Barcelona, Spain) with a 100-N static load cell, equipped with tensile grips. Sample
200 films, previously equilibrated at 53 % RH and 20 ºC, were cut into 1 mm wide and 8 mm long strips,
201 according to the ASTM D882-09 18 standard method (ASTM, 2010a). Grip separation was set at 50
202 mm and cross-head speed was 50 mm · min^{-1}. Elastic modulus (E), tensile strength (TS) and percentage
203 of elongation (EAB) at break were evaluated in six samples from each type of film.

204

205 *2.4.4. Water vapor permeability (WVP)*

206 WVP was measured in dry film discs (3.5 cm diameter) which were equilibrated at 53% RH and 20
207 ºC according to the "water method" of the ASTM-E96-95 (ASTM2010b), using Payne permeability
208 cups (Elcometer, SPRL, Hermelle/s Arganteau, Belgium). De-ionized water was used inside the testing
209 cup to achieve 100% RH on one side of the film, while an oversaturated magnesium nitrate solution
210 was used to control the RH on the other side of the film. During WVP testing, the side of the film in
211 contact with the teflon plate was placed in contact with that part of the test cup having the highest RH.
212 This situation tries to simulate the case of a film applied on the wet surface of a fresh fruit. Water vapor
213 transmission rate measurements (WVTR) were performed at 20 ºC. To calculate WVTR, the slopes of
214 the steady state period of the curves of weight loss as a function of time were determined by linear
215 regression. The WVP was determined form WVTR values, as previously described by Fabra, Talens,
216 Gavara, & Chiralt (2012).

217

218 **2.5 *In vitro* antifungal activity of propolis extract and active coatings**

219 2.5.1 *Fungal strains and culture conditions*

220 *Penicillium digitatum* (Pers.:Fr.) Sacc., strain Pd1 (PDIP, deposited at the Spanish Type Culture
221 Collection with the accession code CECT20795) was isolated from an infected grapefruit (Marcet-
222 Houben et al., 2012).
223 *Penicillium expansum* Link, strain CMP-1 (CECT20906) was isolated from a decayed 'Golden' apple
224 and *Penicillium italicum* Wehmer, strain PHI-1 (CECT20909) was isolated from a mandarin stored at
225 4 ºC (Ballester et al., 2015). *Aspergillus carbonarius* ITEM 5010 was obtained from the Agri-Food
226 Toxigenic Fungi Culture Collection of the Institute of Sciences of Food Production, CNR, Bari, Italy.
227 *Botrytis cinerea* CECT2100 (*Botryotinia fuckeliana* (de Bary) Whetzel 1945) was obtained from the
228 Spanish Type Culture Collection, and *Alternaria alternata* pv. *citri* (AF2440-1).
229 All strains were grown on potato dextrose agar (PDA, Difco-BD Diagnostics, Sparks, MD, USA)
230 plates. Cultures were incubated at 24 ºC for 7-10 days. Conidia were scraped off the agar with a sterile
231 spatula, suspended in sterile distilled water, and filtered through a nylon mesh. Conidia concentration
232 was determined with a hemocytometer adjusting to 10^5 conidia/mL with sterile water.
233
234 *2.5.2 In-vitro antimicrobial assays*
235 Antimicrobial activity of PEE against a collection of different fungal postharvest pathogens was
236 conducted in sterile 96-well flat-bottom microtiter plates (Nunc, Thermo Scientific). Fungi were grown
237 at 24 °C for 7 days in a final volume of 100 µL of potato dextrose broth (PDB, Scharlau Chemie)
238 containing 10^5 conidia per well and different amounts of PEE, in two-fold serial dilutions ranging from
239 1 to 0.03 mg DW/mL. To prevent evaporation, plates were incubated inside a box on top of water-
240 saturated filter paper. Growth was followed by measuring the absorbance at 600 nm with a FLUO star
241 Omega microplate reader (BMG LABTECH GmbH, Ortenberg, Germany). To minimize the effect of
242 non-homogeneous fungal growth within the well, each A600 value was the average of nine
243 measurements regularly distributed within the well. Each treatment was conducted in triplicate within
244 the same plate. Values given represent the mean ± standard deviation of the mean, after background

245 subtraction, of these three replicates. IC_{50} (the concentration required to obtain 50% inhibition of
246 growth) values were determined at 4 days by adjustment of growth data to a four-parameter sigmoid
247 curve. The minimum inhibitory concentration (MIC) is defined as the minimum concentration at which
248 no growth was observed at the end of the incubation period.
249 Additionally, sterile 24-well flat-bottom microtiter plates (Nunc, Thermo Scientific) were used. Plates
250 were prepared by adding 800 μL of potato dextrose agar (PDA; Difco-BD Diagnostics, Sparks, MD,
251 USA) containing two different PEE concentrations (0.5 and 0.05 mg/mL). Control plates without PEE
252 were also included. Then, a drop of 2 μL of 10^5 conidia/mL of each fungus was added at the top of the
253 agar. Plates were incubated at 25 °C for 4 days.
254 In order to determine the *in vitro* antifungal activity of the active coatings, PDA plates were inoculated
255 with 100 μL of a 10^5 conidia/mL. Propolis active coatings containing gelatin or gelatin/zein, and
256 control coatings without propolis, as previously described, were cut with a 10 mm diameter cork borer
257 and placed on the top of the PDA plates previously inoculated with the conidia suspensions. Plates
258 were incubated at 24 ºC for 8 days and the absence or presence of fungal development was observed.
259

260 **2.6 Application of active coatings on fresh fruit**

261 Selected raspberries (*Rubus idaeus* L) obtained from a local supermarket were used. Each raspberry
262 was immersed in gelatin-based FFD for 1 min and, after drying, the samples were stored under
263 refrigerated conditions at 5 °C for 14 days (in the same commercial containers). Periodic reviews were
264 performed to check the fungal contamination of berries. Controls without extract (Gel and Gel/zein
265 coatings) and controls without FFD were included. A total of 30 raspberries were used for each
266 treatment. The test was carried out in duplicates.
267 The amount of coating adhered was calculated by means of the Surface Solid Density (SSD). To this
268 end, commercial raspberries were dipped in the FFD for 1 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, Randazzo, Sánchez, López-Rubio, & Fabra (2019) (Eq.1).

$$SSD = (MCA \cdot X_s)/A_s \quad (1)$$

Where MCA is the mass of coating solution adhered to the fruit surface, Xs is the mass fraction of solids present in the FFD, and A_s is the average sample surface area. A_s was estimated by considering raspberry geometry as a cone, 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 raspberry surfaces (MCA).

3. RESULTS AND DISCUSSION

3.1 Antifungal activity of propolis extract

Argentine propolis polyphenols were extracted by using 80% aqueous ethanol solution, and the extraction yield was 51% based on the dry weight of propolis. The extract was previously characterized in terms of antioxidant, anti-inflammatory, anthelmintic, antibacterial and anti-Candida activities (Salas et al., 2016a, b, 2018) and the chemical composition of the sample was reported by Salas et al. (2016).

In this work, the antifungal activity of PEE was assessed against different fungal pathogens: *P. digitatum*, *P. expansum*, *P. italicum*, *A. alternata*, *A. carbonarius*, and *B. cinerea*. Results of IC_{50} and MIC are presented in Table 1. All the tested fungal pathogens were sensitive towards the PEE. The IC_{50} suggested that *P. digitatum* and *B. cinerea* were the most sensitive to the PEE, with 0.04 and 0.05 mg/mL, respectively. The strongest effect was observed for *P. digitatum* and *B. cinerea*, with MIC values of 0.14 and 0.17 mg/mL, respectively. A moderate effect was observed for *A. alternata*, *P. italicum*, and *A. carbonarius*, with a MIC value around 0.40 mg/mL. The lowest sensitivity was exhibited by *P. expansum* (MIC 0.58 mg/mL). Pobiega et al. (2019) have also tested the antifungal

activity of ethanol extracts of propolis from Polish against different fungal pathogens such as *Alternaria solani*, *Aspergillus niger*, and *P. expansum*. Results showed that the MIC values ranked between 4 and 32 mg/mL. Differences in the botanical origin and the chemical composition of the propolis may be the reason of the different MIC values observed for *P. expasum* by Pobiega et al. (2019) and the Argentine propolis used in present work. In a second study, no antimicrobial activity of Portuguese propolis extracts has been observed against *Aspergillus fumigatus* in the experimental conditions, with an IC_{50} higher than 0.064 mg/mL (Falcão et al., 2014). A reduced growth of *P. expansum* and *P. italicum* was observed when fungal pathogens were inoculated at the surface of solid medium containing 0.50 mg/mL of Argentine propolis extract. However, no growth was observed for the other three pathogens tested. Growth and sporulation of all fungal pathogens tested were affected by using 0.05 mg/mL of PEE (**Supplementary Figure 1**). Inhibition of mycelial growth of *A. alternata*, *B. cinerea* and *P. expasum* was also observed when the fungi was grown in a medium containing a PEE with high concentration of total polyphenols and flavonoids (Curifuta et al., 2012). Comparing our results with literature data, the chemical composition of propolis extracts is different throughout the world, according to its botanical origin, and the antifungal properties of this extract are ascribed to a synergetic effect from the various chemical compounds present in the extract rather than to one single chemical substance (Boisard et al., 2015, Serra Bonvehi, Ventura Coll, & Escola Jorda, 1994, Kujumgiev et al., 1999). Thus, the antifungal activity of PPE from Argentine, is associated with different combinations of esters of phenolic acids and, more specifically, caffeates and ferulates as well as chalcones and flavonoids which have been previously identified as antifungal principles of Argentine propolis (Salas et al., 2016 a, b, 2018).

Table 1. IC_{50} and MIC (mg/mL) of the Argentine propolis ethanolic extract against different fungi.

	IC_{50}	**MIC**
Penicillium digitatum	0.04	0.14

Penicillium expansum	0.14	0.58
Penicillium italicum	0.17	0.41
Alternaria alternata	0.15	0.40
Aspergillus carbonarius	0.13	0.42
Botrytis cinerea	0.05	0.17

317

318 **3.2 Characterization of the developed stand-alone films**

319 Film components can be arranged in different form in the dried film, depending on their interactions
320 in the FFD and during film drying. The microstructure of the different films was qualitatively studied
321 using Scanning Electron Microscopy (SEM). Figure 1 displays representative SEM micrographs
322 obtained from the cross-section of the gelatin and gelatin/zein based films, with and without PEE. As
323 observed, gelatin-based films exhibited a homogeneous structure. However, in films containing zein
324 capsules, small white spots and irregularities were observed throughout the cross-section, which were
325 more accentuated in the bottom phase than in the upper, indicating that zein capsules were
326 preferentially located in this lower part of the film.

327 Generally, when PEE was incorporated to FFD, some stretch marks was observed being more
328 pronounced in the gelatin-based films than in gelatin/zein, and no phase separation was observed. This
329 suggests that interactions between proteins and polyphenol as chalcones, phenolic acids and flavonoids
330 could take place and might favor the integration of the PEE in the biopolymer matrix. In fact,
331 polyphenols and proteins could combine to form complexes by non-covalent interactions (Jöbstl,
332 O'Connell, Fairclough, & Williamson, 2004). These interactions and the internal appearance of the
333 cross-section could also explain the higher extensibility observed in PEE-containing films, as it will
334 be detailed below.

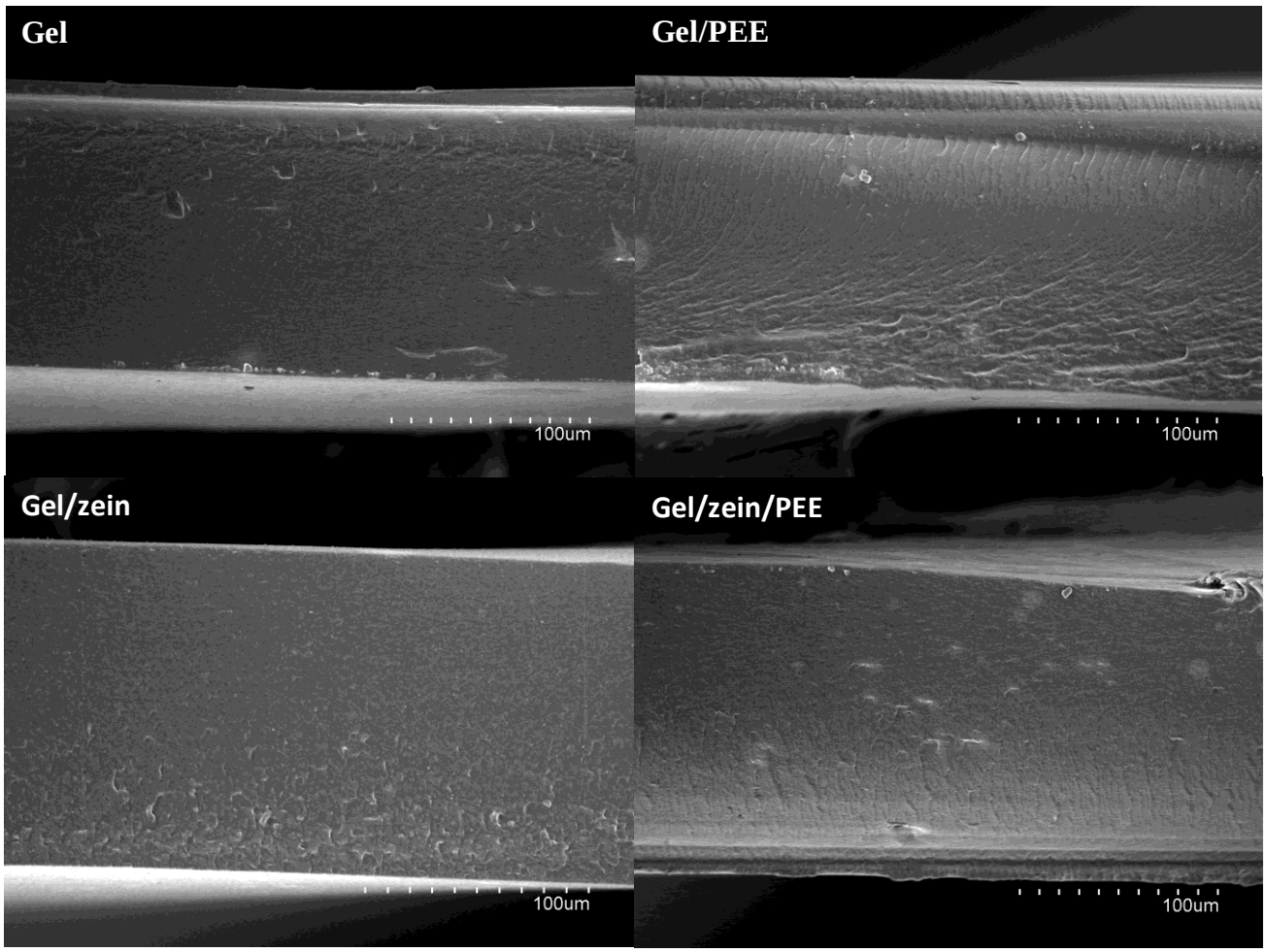


Figure 1. Cross-section images of the developed active films.

Optical properties (transparency and color parameters) are key parameters in the development of edible coatings because they play an important role in consumer acceptance. Spectral distribution curves of internal transmittance (Ti) are plotted in Figure 2 where high Ti values are related with a greater film homogeneity. These curves showed that both Gel and Gel/zein films presented similar spectra, which indicates that the addition of zein capsules did not notably increase the opacity of the films, although gelatin/zein films were slightly yellow due to the greater light absorption (lower Ti values) in the blue wavelength range. In contrast, PEE-containing films modified film spectra to a greater extent, promoting a selective decrease in the Ti of films in the same wavelength range (between 400 and 550 nm). This can be explained by both the selective absorption of the red-brown components of the

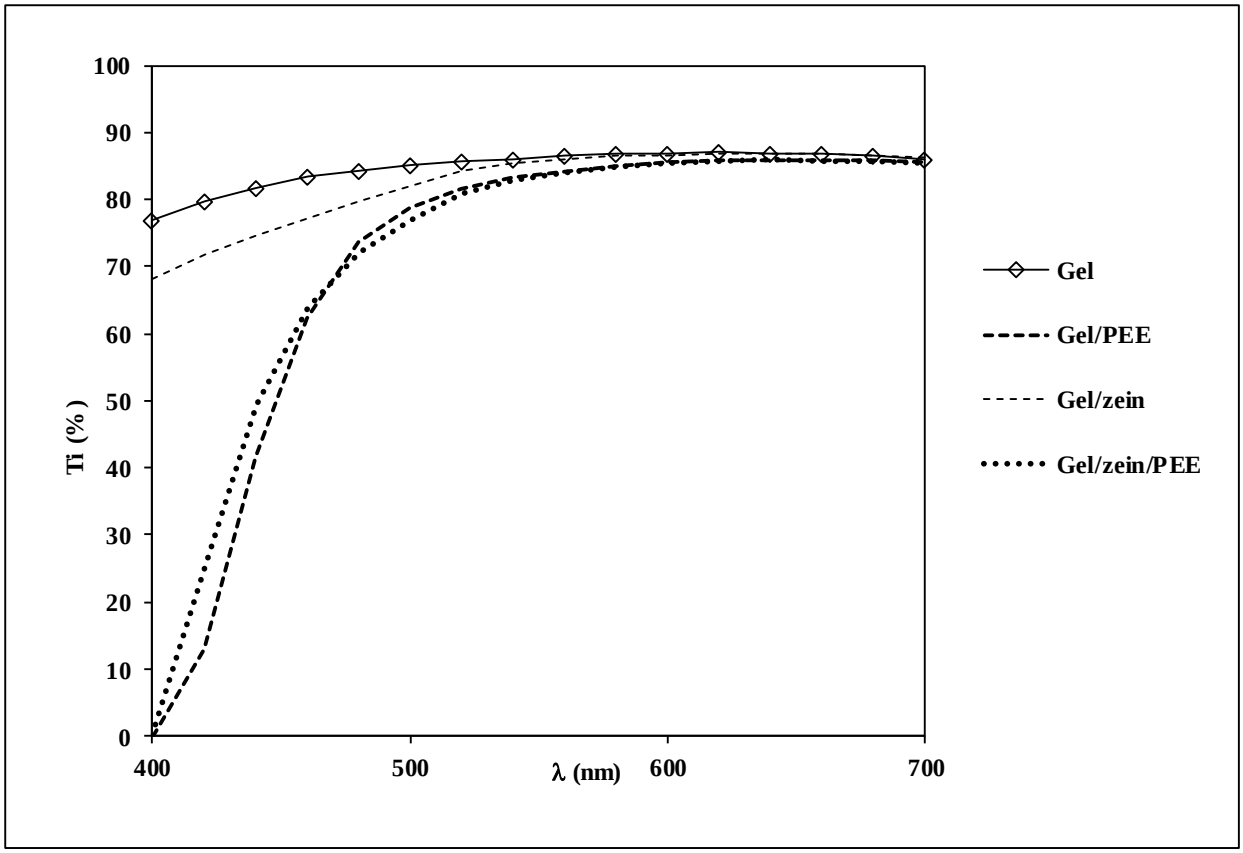
polyphenolic extract and the differences in the refractive indexes between PEE components and the biopolymer matrix, which provoked light dispersion. However, although the addition of PEE imparts yellowish color to the films, all of them preserved food contact transparency (see Figure 3). Similar effects have been previously reported either in gelatin or polysaccharide-based films incorporating natural polyphenolic compounds (Halim, Kamari, & Phillip, 2018; Wu, Sun, Guo, Ge, & Zhang, 2017; Moreno et al., 2020; Li, Miao, Wu, Chen, & Zhang, 2014).

To better understand optical properties of films, color coordinates (L*, lightness; C_{ab}*, chrome; h_{ab}*, hue) were analyzed and color differences (ΔE) due to zein or PEE addition were determined. Table 2 gathers the values for these optical parameters for all tested films. All films showed high luminosity, being the values greater for gelatin films, as expected from the reflection and Ti spectra. Addition of zein slightly decreased luminosity, being more accentuated by the addition of PEE. The higher chroma values reflect the greater orange-brownish color of PEE containing films as compared with Gel and Gel/zein films. These differences were clearly evidenced by the increase ΔE values in PEE containing films.

Table 2. Color parameters of the developed stand-alone films.

Films	L*	C^*_{ab}	h^*_{ab}	ΔE
Gel	81.3 (0.7)^a	15.9 (0.5)^a	90.0 (0.5)^a	-
Gel/PEE	71.2 (0.9)^b	47.8 (0.5)^b	90.2 (1.0)^a	33.5
Gel/zein	75.2 (0.4)^c	29.4 (0.3)^c	86.0 (1.0)^b	14.9
Gel/zein/PEE	70.6 (0.3)^b	44.9 (0.2)^d	87.2 (1.5)^b	30.9

L* lightness, C^*_{ab} Chroma, h^*_{ab} hue, ΔE color difference in comparison with the neat gelatin film. Different superscripts within a column indicate significant differences among formulations ($p < 0.05$). Mean values (standard deviation).



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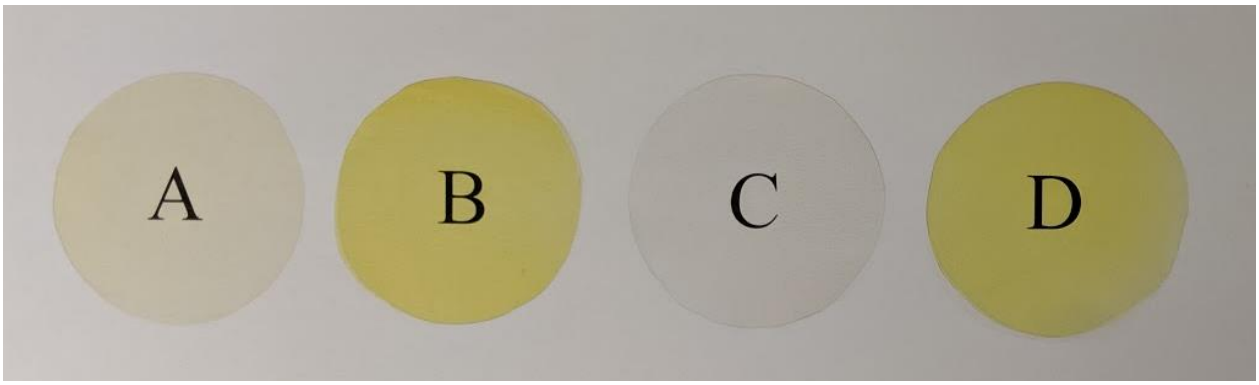
367 **Figure 2**. Spectral distribution of internal transmittance (Ti) of the developed films.



368

369 **Figure 3.** Films: **A:** Gel/zein; **B:** Gel/zein/PEE; **C:** Gel; **D:** Gel/PEE.

370

371 The mechanical properties of the tested films are given in Table 3. TS and EAB represent the film's
372 resistance to elongation and its stretching capacity, respectively, whereas E is a measure of the stiffness
373 of films. The TS and EAB values of gelatin-based film was in the same range of those found in
374 literature (Benbettaïeb et al., 2016, Nilsuwan, Guerrero, de la Caba, Benjakul, & Prodpran, 2020; Wu
375 et al., 2017). However, Cano, Andres, Chiralt, & González-Martinez (2020) reported higher E and TS

values than the ones found in this work for pure gelatin films. This agrees with the absence of glycerol in the films prepared by Cano et al. (2020) since the plasticizer weakens the protein intermolecular forces and improves the molecular chain mobility. Although EAB values were not substantially modified when the zein nanocapsules were incorporated, TS values decreased, thus increasing the film flexibility. This effect can be attributed to the development of discontinuities in the protein network induced by the zein capsules addition.

The incorporation of PEE to Gel and Gel/zein films significantly affected the mechanical behavior, which was not dependent on the film´s composition. PEE addition pointed to the formation of a mechanically weaker network, turning the films more elastic ($p<0.05$) (lower E values) and more stretchable ($p<0.05$) (greater EAB values) than control Gel and Gel/zein films. The tensile strength (TS) of PEE-containing films was not significantly modified, thus suggesting that these interactions were not strong enough to enhance the film´s mechanical resistance.

Similarly, Bodini, Sobral, Favaro-Trindade, & Carvalho (2013) displayed that the E of gelatin films decreased by the addition of ethanol-propolis extract, suggesting that propolis acted as a plasticizing agent, increasing the mobility of the polymer matrix which, in turn, promoted reduced tensile strength and increased film elongation.

Table 3. Mechanical properties[1], and water vapor permeability of tested stand-alone films.

	E (MPa)	**TS (MPa)**	**EAB (%)**	**WVP (10^{-10} g/Pa·s·m^2)**
Gel	1375 (179)^{a,b}	32.9 (2.0)^{a}	4.9 (1.2)^{a}	6.87 (0.28)^{a}
Gel/PEE	1020 (126)^{a,b}	29.7 (3.5)^{a,b}	30.1 (5.2)^{b}	7.07 (0.01)^{a}
Gel/zein	1693 (166)^{b}	22.4 (2.6)^{b}	6.4 (1.9)^{a}	6.76 (0.18)^{a}
Gel/zein/PEE	972 (92)^{a}	29.4 (0.9)^{a,b}	26.2 (1.7)^{b}	7.23 (0.26)^{a}

[1] Elastic modulus (E), tensile strength (TS), and elongation at break (EAB) of films equilibrated at 53% relative humidity for one week. WVP: water vapor permeability. PEE: propolis ethanolic extract. The values are reported as averages of six repetitions. Different superscripts within a column indicate significant differences among formulations according to Tukey's test ($p < 0.05$).

Table 3 also displays the water vapor permeability (WVP) of gelatin-based films containing or not PEE. WVP values ranged between 6.8 and 7.3 g/Pa s m^2, which agree with those from previous studies in which gelatin-based films were obtained (Benbettaïeb, Tanner, Cayot, Karbowiaa, & Debeaufort, 2018). No significant ($p<0.05$) differences of WVP values was observed for all the films, regardless of the PEE addition. Similarly, Bodini et al. (2013) did not observed any change in the WVP when propolis extract produced with type 12 resin was incorporated into gelatin-based films. Inversely, other authors noticed a 16% decreased of the WVP values for gelatin films enriched with another polyphenolic extract such as green tee extract (Wu et al., 2013). This contradictory behavior observed on WVP of gelatin films could be related to the type of phenolic compounds (especially the number and position of OH groups) which could interact with the biopolymer matrices.

Antifungal activity of tested stand-alone films was evaluated against a battery of different fungal pathogens: *P. digitatum, P. expansum*, *P. italicum*, *A. carbonarius*, *A. alternata*, and *B. cinerea*. Four gelatin-based coatings were tested: Gel, Gel/PEE, Gel/zein and Gel/zein/PEE. Only the coatings containing the PEE were active reducing the growth and sporulation of the fungal pathogen tested (Figure 4). Similar effects have been previously reported by Pastor, Sánchez-González, Cháfer, Chiralt, & González-Martínez (2010) in hydroxypropylmethylcellulose-based films containing propolis from other geographic origen. The results suggest that the combination of zein and gelatin, or only gelatin, with the PEE from Argentina had *in vitro* antifungal effects against the major fungal pathogens of fruit and vegetables, which could be attributed to the presence of active components in the PEE, such as phenolic acids, including caffeic acid, chalcones and flavonoids (Salas et al., 2016). In fact, caffeic acid phenethyl ester (CAPE) has been previously identified as one of the main compounds in this PEE (Salas et al., 2016, 2018). CAPE has proved a large number of biological activities including antimicrobial capacity (Murtaza et al., 2014). This compound has been also found as one of the main components of Chinese (Kumazawa, Hamasaka, & Nakayama, 2004) and Indian propolis (Kasote et al., 2017), showing a remarkable antimicrobial activity against different

424 microorganisms. Caffeic acid, cinnamic acid and its derivatives, with well-known antifungal properties
425 (Korošec et al., 2013), were also found in the propolis extract used in the present work (Salas et al.,
426 2016a,b¸2018).
427 The major components of PEE from Monte region in Argentina are chalcones, precursors of
428 flavonoids, which have been proved as antimicrobial compounds. Their antimicrobial activity and
429 particularly the antifungal action has been largely attributed to the reactive enone moiety. As a Michael
430 reaction acceptor the enone unit binds thiol groups of certain proteins (Lahtchev et al., 2008).
431 Although the antimicrobial mechanism of chalcones and flavonoids has been previously described
432 (Lahtchev et al., 2008, Xiao, Zhu, & Zhang, 2014), several authors state that the antifungal activity of
433 this type of extracts cannot be ascribed to a single chemical compound or a specific group of
434 compounds, being the effect derived from the combination of various flavonoids (Boisard et al., 2015,
435 Kujumgiev et al., 1999, Pobiega et al., 2019).
436
437

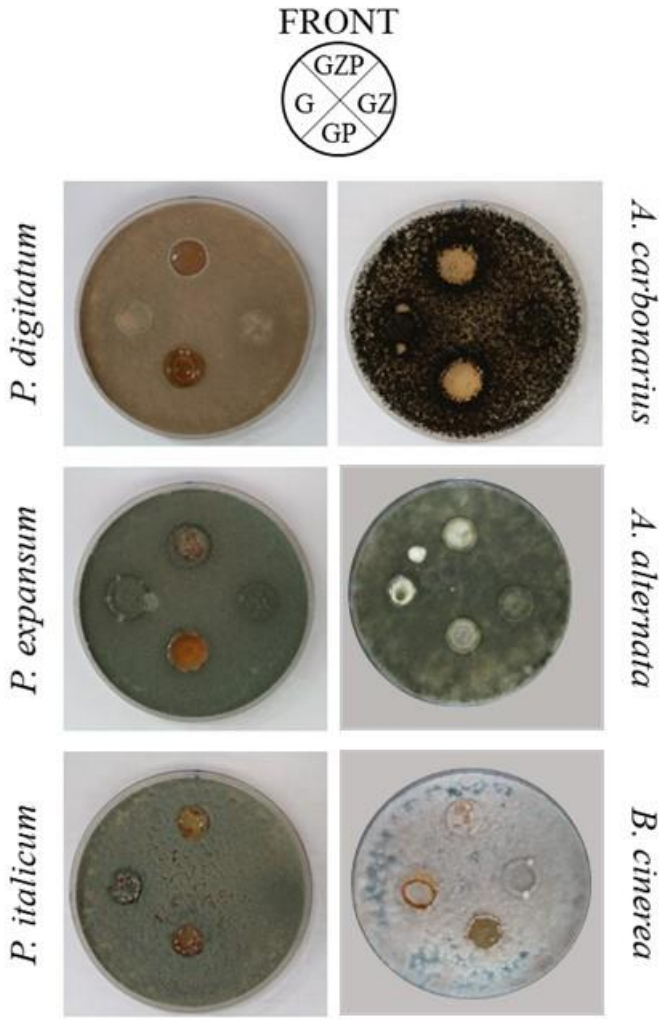


Figure 4. Antifungal activity test of the FFD against different fungi: *Penicillim digitatum* Pd1, *Penicillium expansum*, *Penicillium italicum*, and *Aspergillus carbonarius*, *Alternaria alternata*, and *Botrytis cinerea*. PDA plates were inoculated with 100 µL of a 10^5 conidia/mL for each fungus and 10 -mm discs were place onto the medium. Plates were incubated at 24 ºC for 8 days. Films analyzed: Gel (G), Gel/PEE (GP), Gel/zein (GZ), and Gel/zein/PEE (GZP).

3.3 Fungal decay of uncoated and coated raspberries.

Challenge tests on uncoated and coated raspberries stored at refrigerated temperature were carried out, mimicking the realistic scenario of fresh fruit handing. Figure 5 shows the effect of PEE on the disease incidence in raspberries during 11 days of storage at 5 ºC. After 7 days, all the raspberries fruits from the control group (without the coating) and coated raspberries without PEE showed ~ 90% disease incidence, as it can be observed in Figure 6. In contrast, the decay level of raspberries treated with

PEE-containing coatings were significantly less than that of control sample and those coated with gelatin or gelatin/zein. This can be ascribed to both the higher amount SSD (see Table 4) as compared to their counterparts prepared without PEE and, obviously to the effect of antifungal compound (PEE). After 7 days, the raspberries that were treated with PEE-containing coatings presented a disease incidence of 20 % and 33 % for Gel/PEE and Gel/zein/PEE coatings, respectively. However, for longer storage times, the decay level of raspberries coated with Gel/PEE was higher than that containing Gel/zein/PEE capsules, evidencing the effect of the encapsulation process. In fact, after one-week storage, the lower incidence in non-encapsulated PEE (Gel/PEE) proved the greater availability of the antifungal extract in a short period whereas for longer periods of time, the disease incidence was lower for encapsulated PEE (Gel/zein/PEE). Thus, Gel/zein/PEE can be considered the most efficient antifungal since it had a lower decay level as compared to the control and other treatments.

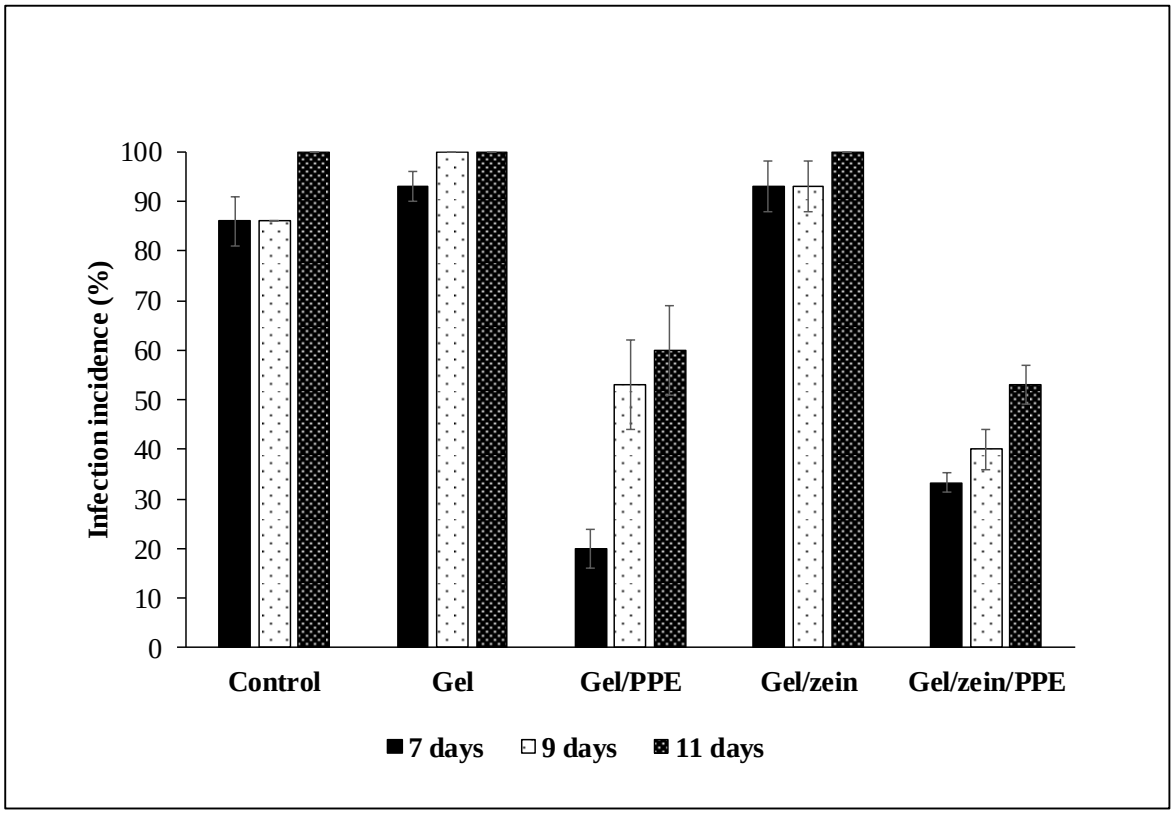


Figure 5. Infection incidence (%) for 11 days storage at refrigerated conditions.

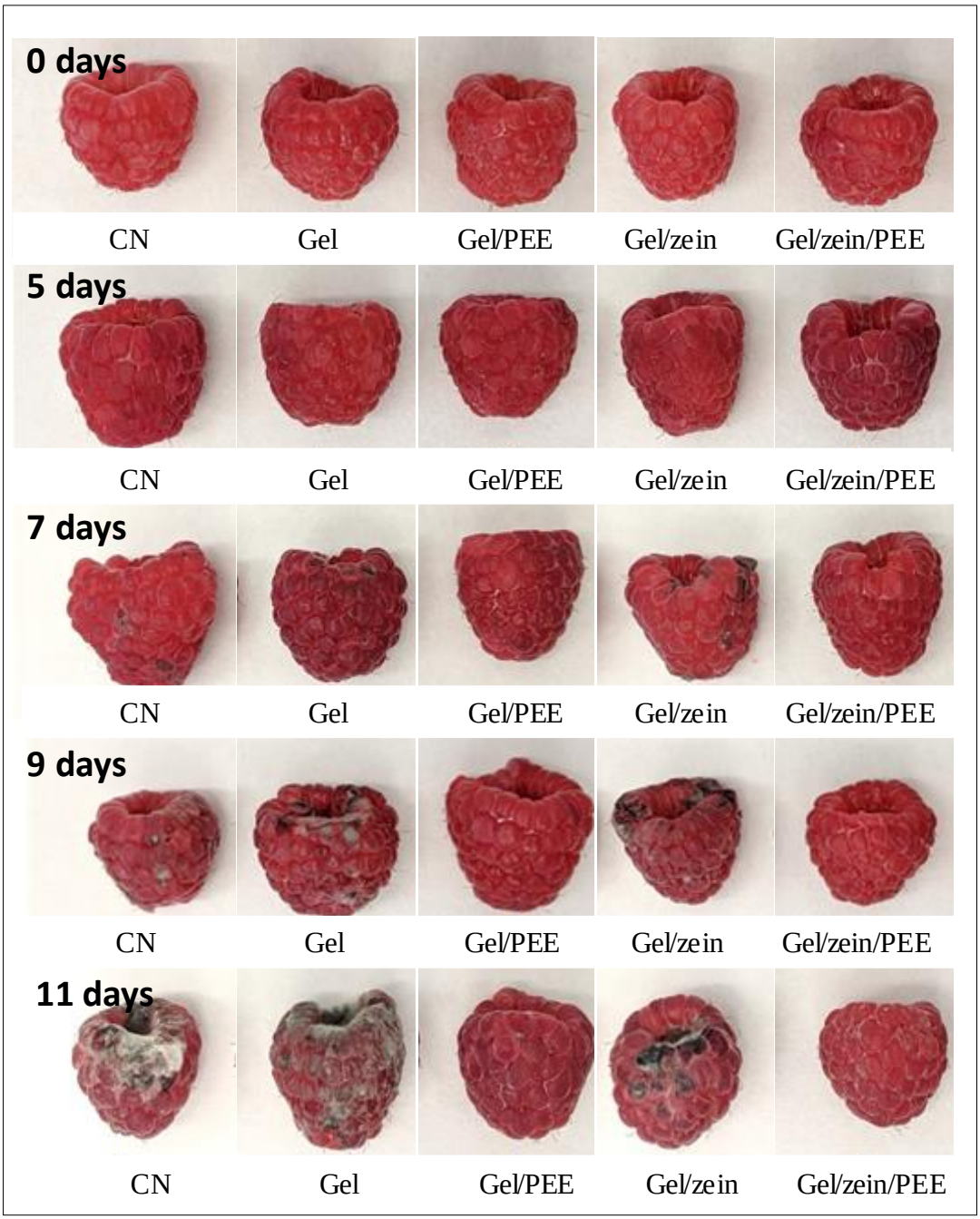


Figure 6. Detailed images of uncoated (CN) and coated raspberries for 11 days storage at refrigerated conditions.

Table 4. Surface solid density (SSD) and infection incidence of non-coated and coated raspberries.

	SSD (g/m^2)
Control	-
Gel	6.4 (0.4)[a]
Gel/PEE	8.7 (0.5)[b]
Gel/zein	6.8 (0.3)[a]
Gel/zein/PEE	8.2 (0.4)[b]

Mean value (standard deviation). Different superscripts within a column indicate significant differences among coating ($p < 0.05$).

4. CONCLUSIONS

In this work, an Argentine propolis ethanolic extract (PEE) has been successfully used to develop antifungal edible coatings. First, the antifungal activity of PEE was evaluated against different fungal pathogens such as *P. digitatum, P. expansum, P. italicum, A. alternata, A. carbonarius*, and *B. cinerea*, being all of them sensitive towards the PEE. Among them, the strongest effect was observed for *P. digitatum* and *B. cinerea*, with MIC values of 0.14 and 0.17 mg/mL, respectively.

The addition of PEE to gelatin films did not notably affect the microstructural and water barrier properties but significantly affected the mechanical behavior, turning the films more elastic and stretchable than control Gel and Gel/zein stand-alone films. In addition, the films incorporating PEE showed remarkable antifungal properties against the major fungal pathogens of fruit and vegetables, as it has been clearly demonstrated either in *in-vitro* and *in-vivo* assays carried out with the films. Nevertheless, the encapsulation of PEE into zein capsules seemed to induce a better preservation of the raspberries in terms of fungal decay as compared to their counterparts prepared by direct incorporation of PEE into the gelatin coating. According to these results, gelatin films containing encapsulated PEE into zein nanocapsules may have great potential to be applied as novel and GRAS edible coatings to achieve the goal of extending the shelf life of raspberries.

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