

## Active properties of edible marine polysaccharide-based coatings containing *Larrea nitida* polyphenols enriched extract

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### ABSTRACT

The aim of this work was to develop active edible coatings based on marine polysaccharide matrices and polyphenols-enriched native plant extracts from arid and semiarid regions of Argentina. Initially, five plant extracts were characterized in terms of antioxidant, antibacterial and antiviral activity and the one with better biological properties and no toxicity or genotoxicity, *Larrea nitida* (Ln) extract, was incorporated into agar, alginate or agar/alginate matrices. The Ln extract-containing films were characterized in terms of physico-chemical and polyphenols release performance in food simulants. The incorporation of Ln extract provided darker films, with a more saturated orange-brownish color and with negligible effects on mechanical and barrier properties. Additionally, all the coatings showed antiviral activity when applied to blueberries against murine norovirus (MNV), a cultivable norovirus surrogate. The coatings of agar and Ln extract was able to reduce the infectivity of MNV below the limit of detection after over-night (ON) incubation at 25 °C and after 4 days at 10 °C storage.

These edible polysaccharides coatings containing Ln extract could be an alternative to reduce or eliminate foodborne viruses and protect the food against oxidative process.

### 1. Introduction

Fresh and minimally processed vegetables and fruits market has grown up exponentially because of the changes in consumer's lifestyle. Consumer's preferences towards fresher products with natural additives have made the food industry to focus its efforts on the development of innovative preservation technologies to reduce the growth of both spoilage and foodborne pathogens such as bacteria and human enteric viruses (Dilmaçunal & Kuleaşan, 2018; Prakash, Baskaran, Paramasivam, & Vadivel, 2018). In this regard, edible coatings have recently emerged as one of the most promising technologies for controlling the quality and safety of fresh products while extending their shelf life (Fabra, Falcó, Randazzo, Sánchez, & López-Rubio, 2018; Falcó, Randazzo, Sánchez, López-Rubio, & Fabra, 2019; Guo, Yadav, & Jin, 2017; Majid, Nayik, Dar, & Nanda, 2018).

Edible coatings are based on biodegradable, biocompatible and food-

grade polymers from natural sources, which include polysaccharides, proteins, and lipids. Among these materials, polysaccharides have been widely studied and used for the development of edible coatings due to good film forming availability as well as suitable mechanical and gas barrier properties (Hou et al., 2019). More concretely, research interest in the use of marine polysaccharides (Hou et al., 2019; Kanmani & Whan Rhim, 2014; Shankar & Rhim, 2017; Tavassoli-Kafrani, Shekarchizadeh, & Masoudpour-Behabadi, 2015) such as carrageenan, agar and alginate as biopolymer matrices has increased as they are highly abundant, inexpensive, absorbent, non-toxic and non-immunogenic (Fabra et al., 2018; Oliveira Filho et al., 2019; Shankar & Rhim, 2017). Marine polysaccharides are also attracting great attention because, some of them, have unique antiviral properties to assure the food safety (Falcó et al., 2019). Furthermore, polysaccharide-based coatings present a high potential to serve as vehicles to incorporate active compounds, which greatly contribute to extend product shelf life and to reduce the risk of

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pathogen growth on food surface (Bhardwaj, Alam, & Talwar, 2019). In fact, several works have been carried out in the last decades dealing with active biopolymer matrices containing essential oils and/or natural extracts (Ganiari, Choulitoudi, & Oreopoulou, 2017; Moghimi, Aliahmadi, & Rafati, 2017). Polyphenolic-enriched plant extracts represent an interesting ingredient for the development of edible coatings, mainly due to their natural origin and phytochemical properties, which allows obtaining active materials with the aim of extending the shelf life and the value to the products (Luchese, Brum, Piovesana, Caetano, & Flores, 2017; Mir, Dar, Wani, & Shah, 2018).

*Zuccagnia punctata* Cav., *Larrea divaricata* Cav., *Larrea cuneifolia* Cav., *Larrea nitida* Cav., and *Tetraglochin andina* Ciald. are shrubs that grow in arid and semiarid regions of Argentina and have demonstrated biological activities such as antifungal (Butassi et al., 2015, 2018; Moreno et al., 2018a, b), antibacterial (Zampini, Cudmani, & Isla, 2012), antigenotoxic (Zampini et al., 2008), antioxidant (Álvarez Echazú et al., 2018; Carabajal, Zampini & Isla, 2017; Moreno et al., 2018) and anti-inflammatory (Torres Carro et al., 2017), which have been mainly attributed to their high content in chalcones, flavonoids, phenolic acids, and lignans (Isla et al., 2016; Moreno et al., 2018). However, although several biological properties have been studied, the activity of these plant species against foodborne bacteria and viruses of interest in the food industry has not been previously described and there is not existing literature on their application as active edible coatings.

Concretely, this work compares the potential of alginate and agar on the antiviral activity and their synergetic effect when mixed with natural extracts with potential virucide activity. Although both polysaccharides have excellent film-forming properties, agar forms a slightly viscous fluid that can form thermoreversible gels when the temperature is decreased below a certain temperature and thus, temperatures around 40 °C are needed when applied as an edible coating, fact that could partially limit their application on fresh and perishable fruits like berries. To improve handling properties of the agar-based matrices, alginate was used in the mixture and compared to the counterparts prepared with pure polysaccharide matrices.

Therefore, the main aim of this work was: (i) to characterize and evaluate the most relevant active properties, and the toxicity of the extracts obtained from Argentinian medicinal plants; (ii) to investigate the physicochemical properties of edible polysaccharide-based films containing the most bioactive extract; and (iii) to assess the antiviral efficacy when applied onto the surfaces of blueberries at room and refrigeration temperatures.

## 2. Materials and methods

### 2.1. Reagents

Alginate sodium salt from brown algae (medium viscosity), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich. Agar was obtained from Hispanagar S.A. (Burgos, Spain) and glycerol was purchased from Panreac- Apllichem. Mueller Hinton Broth (MHB), Mueller Hinton Agar (MHA), peptone water and Palcam agar were purchased from Scharlab.

### 2.2. Plant material

The used plant parts were leaves and stems (aerial parts), according to their traditional use. *Zuccagnia punctata* Cav. (Zp), *Larrea divaricata* Cav. (Ld), and *Larrea cuneifolia* Cav. (Lc) were collected in April 2017 at Amaicha del Valle, Tucumán, Argentina at 2000 m above sea level (m.a.s.l.). *Larrea nitida* Cav. (Ln) was collected in April 2017 at Vinchina, La Rioja, Argentina at 3485 m.a.s.l. *Tetraglochin andina* Ciald. (Ta) was collected in February 2017 in Huaca Huasi, Tucumán, Argentina (4300 m.a.s.l.). The plants were identified by Dra. Soledad Cuello and voucher specimens (Zp: LIL 605935; Ld: LIL 614299; Lc: LIL 614829; Ln: LIL

615845; Ta: LIL 610669) were deposited at the Herbarium of Fundación Miguel Lillo (Tucumán, Argentina). The samples were dried in a forced air oven at 40 °C and then they were ground.

The profile of phenolic compounds of *Larrea* dry extract was previously determined by HPLC-ESI-MS/MS analysis, being identified 2 flavonoids and 10 lignans. Trihydroxy- 6,7'-cyclo lignan was found only in *L. divaricata* and dihydroxy-methoxy-epoxy lignan in *L. cuneifolia* and *L. nitida*, nordihydroguaiaretic acid (NDGA) was found in the three species (Moreno et al., 2018b). In contrast, fifty polyphenolic compounds were previously identified in *Tetraglochin andina* Ciald. extracts which was mainly hydrolysable and condensed tannins (Moreno et al., 2018a) and 23 compounds were identified in *Zuccagnia punctata* Cav. extract, including seven condensed tannins, seven flavonoid glycosides, one flavonoid, five phenolic acid derivatives and three chalcones (Moreno et al., 2018c).

### 2.3. Dry extract preparation

The powdered dried plant material (10 g) was macerated in 200 mL of 60% (v/v) ethanol-water for 1 h with ultrasonic application five times for 10 min. Extracts were filtered, taken to dryness under reduced pressure and then freeze-dried to obtain the plant dry extracts. The extraction yield was determined. The dry extracts were stored at -20 °C until their use.

### 2.4. Antimicrobial activity of extracts

The antimicrobial activity of plant extracts was tested against *Listeria innocua* (Spanish Type Culture Collection CECT 910, ATCC 33090), as surrogated strain of the pathogen *Listeria monocytogenes* and against *Escherichia coli* (CECT 434; ATCC 25922), Gram-positive and Gram-negative bacterial models, respectively. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the plant extracts were determined in MHB. The antibacterial activity of the plant extracts was determined using sterile 96-well plates (Wiegand, Hilpert, & Hancock, 2008). 100 mg of each plant dry extract were diluted in 10 mL of DMSO, and then serial dilutions were made up in 290 µL of sterile MHB (100–500 µg/mL). Control samples with DMSO were prepared to check the non-toxicity of DMSO. Overnight (ON) cultures of the microorganisms in stationary phase measured at 600 nm were diluted in Tryptone Soy Broth and incubated at 37 °C until reaching the exponential phase corresponding with an optical density of 0.2 and a bacterial concentration of 10<sup>6</sup> CFU/mL. 10 µL of the inoculum were added in each well and incubated at 37 °C for 24 h. Depending of the turbidity of the wells, serial dilutions with peptone water were made and plated in petri dishes of MHA. Colonies were counted after incubation at 37 °C for 24 h. Results were expressed as log of colony forming units per milliliter (log CFU/mL) and the logarithmic reduction value (LRV) was calculated with respect to the control samples. The experiment was carried out in triplicate.

### 2.5. Antiviral activity of plant extracts

#### 2.5.1. Virus propagation and cell line

MNV-1 (kindly provided by Prof. H. W. Virgin, Washington University School of Medicine, USA) was propagated and assayed in RAW 264.7 (also provided by Prof. H.W. Virgin). Semi-purified stocks were subsequently produced from RAW cells by centrifugation of infected cell lysates at 660×g for 30 min. Infectious MNV were enumerated by determining the 50% tissue culture infectious dose (TCID<sub>50</sub>) with eight wells per dilution using the Spearman-Kärber method (Pinto, Diez, & Bosch, 1994).

#### 2.5.2. Antiviral effect of plant extracts on MNV

Plant dry extracts were dissolved in 60% (v/v) ethanol-water solution to obtain concentrations of 1 and 10 mg/mL. Each solution was

mixed with an equal volume of MNV suspensions (ca. 5 log TCID<sub>50</sub>/mL), followed by incubation at 37 °C in a shaker for 16 h (ON incubation). Thereafter, Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum was added to stop the reactions. Positive controls were virus suspensions mixed with ethanol 60% under same experimental conditions. Ten-fold dilutions of plant extract-treated and untreated virus suspensions were inoculated into confluent RAW monolayers in 96-well plates. Then, infectious viruses were enumerated by cell culture assays as described above. Each treatment was run in triplicate. The decay of MNV titers was calculated as log<sub>10</sub> (N<sub>x</sub>/N<sub>0</sub>), where N<sub>0</sub> is the infectious virus titer for untreated samples and N<sub>x</sub> is the infectious virus titer for plant extract treated samples (Falcó et al., 2018).

## 2.6. Antioxidant activity of plant extracts

The antioxidant capacity of plant extracts was evaluated through the Trolox equivalent antioxidant capacity (TEAC), using a modification of the original TEAC method (Re et al., 1999). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as antioxidant standard. Each extract was analyzed for ABTS radical scavenging activity to indirectly determine the amount of the extract released. To this, 17 µL of each extract were added to 200 µL of the ABTS<sup>+</sup> solution, and absorbance at 734 nm was registered every minute for 6 min. For calibration, Trolox standards of different concentrations were prepared, and the same procedure was followed. The TEAC of each extract was determined by comparing the corresponding percentage of absorbance reduction at 6 min with the Trolox concentration–response curve. All the determinations were carried out in triplicate using a CLARIO star (BMG LABTECH) spectrophotometer, using 60% ethanol solutions as blanks.

## 2.7. Toxicity assays

### 2.7.1. Mutagenic activity. The ames test

The mutagenic effects of plant dry extracts were evaluated on two *Salmonella Typhimurium* strains (TA98 and TA100). The plate incorporation assay was performed according to Maron and Ames (1983), by adding 0.1 mL of the ON bacterial culture, 0.1 mL of extract at different concentrations (350–1400 µg/plate) and 2 mL of top agar on minimal agar. Negative and positive controls were used simultaneously in each experiment. The positive control was 4-nitro-o-fenilendiamine (10 µL/plate, 1 mg/mL solution) for TA98 and TA100, and the negative control was DMSO (100 µL/plate). The revertant colonies of each plate were counted manually after 48 h of incubation at 37 °C and the mutagenicity relation (His<sup>+</sup> revertant per plate/His<sup>+</sup> spontaneous revertant) was calculated. The extract was considered mutagenic if the number of revertants per plate was more than twice the number of colonies produced on the solvent control plates (spontaneous revertant frequency) or the mutagenicity relation  $\geq 2$ .

### 2.7.2. General toxicity assay. *Artemia salina* test

The acute toxicity levels of plant dry extracts were studied using *Artemia salina* as test organism (Svensson, Mathiasson, Martensson, & Bergström, 2005). To obtain *A. salina* larvae, its cysts were hatched in artificial seawater (NaCl, MgCl<sub>2</sub>·6H<sub>2</sub>O, Na<sub>2</sub>SO<sub>4</sub>, CaCl<sub>2</sub>·2H<sub>2</sub>O, KCl). After 24 h of incubation at 25 °C, the phototropic nauplii have hatched and are in their most sensitive state. Between 10 and 12 nauplii were transferred to microplates containing 100 µL of fresh medium and 3 µL of different concentrations of extract dissolved in DMSO (62.5–1000 µg/mL). Solvent control (DMSO) without extract and a positive control (potassium dichromate) were also assayed. All the plates were incubated for 24 h at 25 °C. Plates were then examined under a magnifying glass and the number of dead (immobile) nauplii in each well was counted. The nauplii were considered immobile if they did not show any forward movement for 10 s. The LC<sub>50</sub> (concentration that kills 50% of the *A. salina* larvae) was calculated.

## 2.8. Development of active films

Six different films were obtained by the solvent casting method: three without the extract and three with Ln extract. Films containing Ln were prepared as follows: 1 g polysaccharide (agar, alginate or agar/alginate mixed in a 1:1 ratio) was dissolved in 100 mL of distilled water using a magnetic stirrer for 30 min at a controlled temperature of 80 °C until they were completely dissolved. Then, solutions were cooled to 40 °C and 50 mg Ln extract dissolved in 0.3 g glycerol was added, mixed with stirring. Ln was previously dissolved in glycerol since it was not soluble in water. The amount of Ln used was selected based on the toxicity results carried out in this work (see section 3.1). The mixture was homogeneously spread over a Teflon plate of 15 cm in diameter and was left to dry in an oven at 37 °C for 48 h. These conditions were established after previous experiments to ensure that homogeneous and continuous films without cracks and/or pinholes were obtained. Control films without Ln extract were also prepared for comparative purposes.

The obtained films were removed from the plates and equilibrated for two days in a desiccator at 20 °C and 53% relative humidity (RH), by using oversaturated solutions of magnesium nitrate-6-hydrate (Panreac Química, SA, Castellar del Vallés, Spain). Film thickness was measured in quintuplicate using a hand-held digital micrometer (Palmer-Comecta, Spain, ±0.001 mm) and the average value was used in mechanical, water vapor and oxygen permeability calculations.

Films' nomenclature was 'Ag' for control agar film, 'Alg' control alginate, 'Ag/Alg' control agar and alginate, 'Ag/Ln' agar film with extract, 'Alg/Ln' alginate film with extract, 'Ag/Alg/Ln' agar and alginate film with extract.

### 2.8.1. Morphological characterization

The microstructural analysis of the cross-sections of the dried films was carried out using Scanning Electron Microscopy (SEM) (Hitachi S-4800). The films (three samples per formulation) were frozen in liquid nitrogen and randomly broken to explore the cross-section of the samples. Films were mounted on M4 Aluminium Specimen Mount and fixed on the support using double-side adhesive tape. Samples were gold–palladium coated and observed using an accelerating voltage of 10 kV and a working distance of 10 mm.

### 2.8.2. Mechanical properties

Tensile properties were determined using a Mecmesin MultiTest universal test machine (Landes Poli Ibérica, S.L., Barcelona, Spain) equipped with a 100-N static load cell, according to ASTM standard method D882-09 18 (ASTM, 2010a). Tensile strength (TS), elongation percentage at break (EAB) and elastic modulus (E) were determined from the stress-strain curves. Equilibrated specimens were mounted in the film extension grips and stretched at 50 mm min<sup>-1</sup> until breaking. Prior to the test, the thickness of the samples was randomly measured at five points. Eight replicates of each formulation were tested.

### 2.8.3. Physicochemical characterization of the films

**2.8.3.1. Water vapor permeability (WVP).** WVP was measured, in triplicate, following the ASTM E96/E96M-10 (ASTM 2010b) gravimetric method for hydrophilic films, using Payne permeability cups of 3.5 cm in diameter (Elcometer SPRL, Hermelle/s Argenteau, Belgium). 5 mL of distilled water was used inside the testing cup to achieve 100% RH on one side of the films, while and oversaturated magnesium nitrate solution was used to control de RH on the other side of the film: 53% RH. During WVP testing, the side of the films in contact with the Teflon plate was placed in contact with the part of the test having the highest RH. Cups with aluminium samples were used as control samples to estimate solvent loss through the sealing. The water vapor transmission rate (WVTR) was measured at 20 °C for each type of film. The WVP was determined from WVTR values, as previously described by Fabra,

Talens, Gavara, and Chiralt (2012).

**2.8.3.2. Oxygen permeability.** Permeability to oxygen (OP) was calculated from oxygen transmission rate (OTR) measurements recorded using an Oxygen Permeation Analyzer M8001 (Systech Illinois, UK). Experiments were carried out in triplicate at 23 °C and 80% RH. The samples were previously purged with nitrogen in the humidity equilibrated test cell, before exposure to an oxygen flow of 10 mL min<sup>-1</sup>. The exposure area during the test was 5 cm<sup>2</sup> for each sample. In order to obtain the oxygen permeability, film thickness and gas partial pressure were considered in each case. Four replicates per formulation were made.

#### 2.8.4. Transparency

The transparency of the films was determined by applying the Kubelka-Munk theory of a multiple dispersion of reflection spectrum, given the reflection spectra of both black and white backgrounds. A spectrophotometer CM-3600d (Minolta Co., Tokyo, Japan) with a 10 mm illuminated sample area was used. Internal transmittance (Ti) of the films was quantified as previously reported by Fabra, Talens, and Chiralt (2009). Moreover, CIE-L\* a\* b\* coordinates were obtained from the reflectance of an infinitely thick layer of the material. Measurements were taken in triplicate for each sample.

#### 2.8.5. In vitro release assays

The release of phenolic compounds from the films containing extract (Ag/Ln, Alg/Ln, Ag/Alg/Ln) was assessed in two different food simulants. Concretely, 50% and 10% (v/v) ethanol solutions were selected as food simulants, according to the Commission Regulation 10/2011 EU on plastic materials and articles intended to be exposed to food (10/2011/EC). For this analysis, the films (pieces of 2 × 2 cm) were immersed in 10 mL of the release medium during different times. Aliquots (0.20 mL) of the supernatant were taken for samples analysis and the released total phenolic compounds (TPC) content was determined in each time interval as described by Singleton, Orthofer, & Lamuela-Raventos (1999) using Folin-Ciocalteu reagent. The aliquot volume was immediately replaced by fresh release medium once it was taken for the analysis. The eluted from the control films without extract (Ag, Alg, Ag/Alg) were considered. The relative percentage was calculated considering the phenolic compounds content of Ln extract used for the film preparation as 100%. Experiments were performed at room temperature (20 °C) in independent triplicates.

**2.8.5.1. Antioxidant activity.** The antioxidant capacity of active films was evaluated through the Trolox equivalent antioxidant capacity (TEAC) according to previously described in 2.7 section using 17 µL of eluted extracts from films. The TEAC of the film samples was determined by comparing the corresponding percentage of absorbance reduction at 6 min with the Trolox concentration–response curve. All the determinations were carried out in triplicate using a CLARIO star (BMG LABTECH) spectrophotometer, using 50 or 10% ethanol solutions as blanks.

#### 2.9. Challenge tests

Locally purchased blueberries were exposed to UV for 15 min in a laminar flow hood to reduce the microbial load. MNV suspension (about ca. 5–6 log TCID<sub>50</sub>/mL) were seeded by distributing 50 µL on the surfaces of fresh blueberries. Inoculated samples were air dried in a laminar flow hood for 60 min. Thereafter each blueberry was immersed for 2 min into Ln coatings and let them dry for 20 min. Finally, samples were stored at 10 and 25 °C. On each sampling day, individual blueberry was placed in a tube containing 5 mL of DMEM supplemented with 10% FCS and shaken for 2 min at 180 rpm to release viral particles from the surface. Blueberries were removed from the DMEM suspension and then

**Table 1**

Antioxidant capacity (TEAC: Trolox equivalent antioxidant capacity) of medicinal plant extracts.

Sample	TEAC (µmol Trolox/g extract)
Tc	3.0 (0.1) <sup>a</sup>
Zp	4.9 (0.3) <sup>b</sup>
Ln	4.5 (0.1) <sup>b,c</sup>
Ld	2.7 (0.2) <sup>a</sup>
Lc	3.9 (0.3) <sup>c</sup>

Zp: *Zuccagnia punctata*; Ld: *Larrea divaricata*; Ln: *Larrea nitida*; Lc: *Larrea cuneifolia*; Ta: *Tetraglochin andina*. Different superscripts indicate significant differences among extracts ( $p < 0.05$ ). Mean values (standard deviation).

viruses were recovered and titrated as described above. Each treatment was performed in triplicate. Positive controls were uncoated blueberries and coated berries without Ln extract in its formulation under the same experimental conditions. The decay of MNV titers was calculated as described above.

#### 2.10. Statistical analysis

Each experimental value is expressed as the mean ± standard deviation (SD). The statistical analysis of experimental data was performed using InfoStat software (Student Version, 2011). The one-way ANOVA with Tukey post-test at a confidence level of 95% was used to evaluate the significance of differences between groups. The criterion of statistical significance was taken as  $p \leq 0.05$ .

### 3. Results and discussion

#### 3.1. Preparation and characterization of phenolic enriched extracts of medicinal plants

Polyphenolic-enriched extracts are of considerable interest and have received increased attention in recent years due to their bioactive properties. In the first part of this work, the antioxidant and antimicrobial (antiviral and antibacterial) activity against foodborne pathogens (*L. innocua*, *E. coli* and MNV) of the five hydroalcoholic extracts, having a content of TPC between 354.7 and 397.9 mg GAE/g dry extract (Moreno et al., 2018) were analyzed.

The antioxidant activity expressed as mmol Trolox/g extract of the different extracts, is gathered in Table 1. As observed, the antioxidant activity ranged between 2.7 and 4.9 mmol Trolox/g extract, being significantly higher ( $p < 0.05$ ) for Zp, Ln and Lc extracts. It should be noted that the antioxidant activity of the five species was higher than that reported for extracts of other *Larrea* plant species (Aguirre-Joya et al., 2018; Varela, Arslan, Reginato, Cenzano, & Luna, 2016). However, higher antioxidant properties were previously reported for other natural extracts such as green tea and grape seed extracts (Magwebeba et al., 2016; Majchrzak, Mitter, & Elmadfa, 2004).

The microbiological risks of food products even today are one of the main sources of foodborne diseases, being human enteric viruses the most common etiologic agents identified in foodborne outbreaks (Bennett et al., 2018). Furthermore, *Listeria* is another important foodborne pathogen since listeriosis can cause serious problems in newborns, pregnant women, the elderly and immunocompromised individuals, with a high mortality rate of 30% that exceeds that of other common diseases transmitted by food (Realini & Marcos, 2014).

In this work, both *Listeria innocua* and *Escherichia coli* were chosen due to their relevance in foodborne illnesses. The evaluation of antibacterial activity of the five plant extracts against both microorganisms was quantitatively assessed by determining MIC and MBC values and the results are shown in Table 2. Results clearly demonstrated that the antibacterial activity was higher for *L. innocua* than *E. coli* which can be attributed to the differences in the cell wall structure. This can be related

**Table 2**  
Antibacterial activity of Argentinian plant extracts against *L. innocua* and *E. coli*.

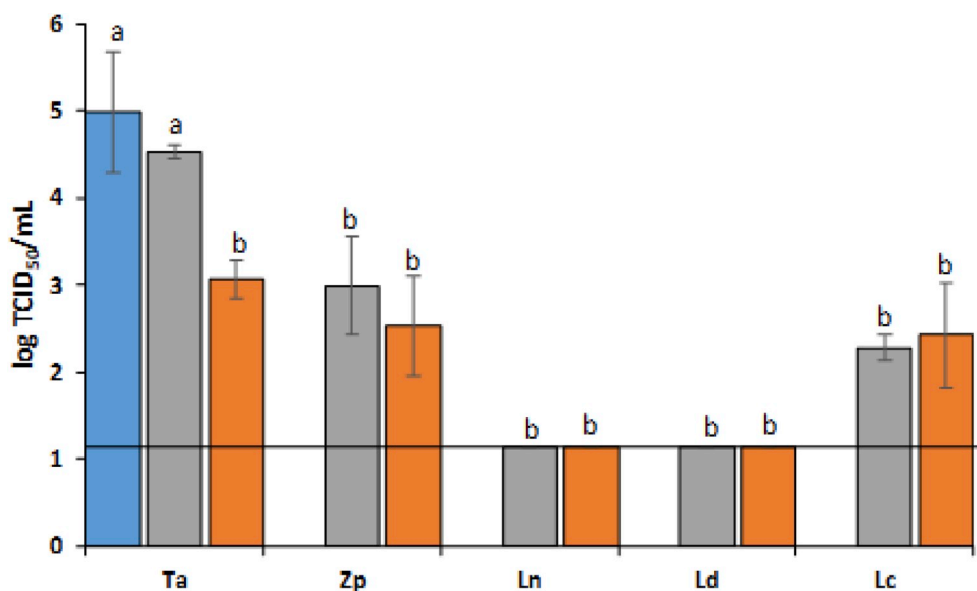
Sample	<i>L. innocua</i>		<i>E. coli</i>	
	MIC ( $\mu\text{g/mL}$ )	MBC ( $\mu\text{g/mL}$ )	MIC ( $\mu\text{g/mL}$ )	MBC ( $\mu\text{g/mL}$ )
Zp	300	>800	>800	>1000
Ld	200	550	800	>1000
Ln	100	350	800	>1000
Lc	300	550	800	>1000
Ta	>500	>700	>1000	>1000

Zp: *Zuccagnia punctata*; Ld: *Larrea divaricata*; Ln: *Larrea nitida*; Lc: *Larrea cuneifolia*; Ta: *Tetraglochin andina*. MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration.

to the fact that Gram-negative microorganisms are less susceptible to the action of the antibacterial compounds since they possess an outer membrane surrounding the cell wall that limit diffusion of hydrophobic compounds through its lipopolysaccharide covering (Harvey, Champe, Fisher, & Strohl, 2007).

As observed, Ln presented stronger antilisterial activity than the other evaluated plant extracts, with MIC and MBC values of 100 and 350  $\mu\text{g/mL}$ , respectively. As compared with literature, Ln extract showed greater or similar antibacterial efficacy than other natural extracts. Cosentino et al. (1999) showed MBC values of commercial thymus oil against *L. monocytogenes* of 0.9 mg/mL, while hydroalcoholic murta extracts presented MBC values between 0.1 and 2.2 mg/mL (López de Dicastillo et al., 2017) and *Cinnamomum javanicum* plant extract had a similar MIC value but higher MBC (0.13 mg/mL and >2 mg/mL, respectively) (Yuan, Wen, & Yuk, 2017).

Moreover, human enteric viruses account for the major causes of foodborne outbreaks in high-income countries and, thus, research interest has recently increased in the identification of natural compounds (i.e. essential oils and polyphenols enriched plant extracts) with antiviral activity (Abdelkebir et al., 2019; Randazzo, Fabra, Falcó, López-Rubio, & Sánchez, 2018). Fig. 1 summarizes the antiviral activity of the evaluated plant extracts against MNV measured at 37 °C where it is clearly evidenced that Ln and Ld were the most effective extracts in reducing the titers of MNV. Incubation of MNV with both plant extracts at concentrations of 0.5 and 5 mg/mL reduced MNV titers to undetectable levels after ON incubation at 37 °C. MNV titers were reduced by 1.9, 2.5, and 2.6 log TCID<sub>50</sub>/mL, after treatment with 5 mg/mL of Ta, Zp, and



**Fig. 1.** Effect of Argentinian plant extracts on MNV infectivity. Blue bar indicates untreated samples, greys bars indicate 0.5 mg/mL plant extract and orange bars 5 mg/mL plant extract. Solid line indicates the detection limit for the TCID<sub>50</sub>/mL assay. Titers are the means  $\pm$  standard deviations of results of three replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Lc extracts, respectively.

Previously, the dry extracts used in this study were chemically characterized and two quercetin derivatives flavonoids and 10 major lignans were identified. Trihydroxy-6,7'-cycloignan was found only in Ld extract and dihydroxy-methoxy-epoxylignan in Ln extract, and NDGA was found in extracts obtained from both plant species (Moreno et al., 2018b). Consequently, the high antimicrobial activity of Ln and Ld extracts can be attributed, in part, to the presence of quercetin derivatives and lignans principally NDGA. In fact, the antiviral activity of similar polyphenolic extracts with high concentrations of quercetin has been recently demonstrated (Falcó et al., 2018) and the antiviral activity of NDGA and its derivatives have been previously reported against human immunodeficiency virus, human papilloma virus, herpes simplex virus, influenza virus and Junin virus (Palacio, Cantero, Cusidó, & Goleñowski, 2012).

Therefore, considering that Ln extract showed the highest antimicrobial activity for both *L. innocua* and MNV, and high antioxidant activity, this extract was chosen for the development of active edible coatings. To this end, the toxicity of the Ln extract by means of Ames and

**Table 3**  
Results obtained in the mutagenic activity evaluation assay against *S. Typhimurium* TA98 and TA100 strains.

Sample	$\mu\text{g GAE/plate}$	TA98 (–) S9	T100 (–) S9	MR TA 98	MR TA 100
Ln extract	1400	ND	ND	ND	ND
	700	38 (1)	122 (21)	1.11	1.10
	350	36 (1)	117 (9)	1.05	1.06
Positive control <sup>a</sup>		1222 (109)	723 (171)		
Negative control <sup>b</sup>		34 (2)	110 (16)		

\* MR: mutagenicity relation; ND: not determined. These concentrations affect the viability of the *Salmonella* strains.

<sup>a</sup> Mean number of revertants induced by 4-nitro-o-phenyldiamine (10  $\mu\text{g/plate}$ ).

<sup>b</sup> The number of spontaneous revertant colonies determined without the addition of the samples, only with the vehicle, DMSO. Mean value (standard deviation).

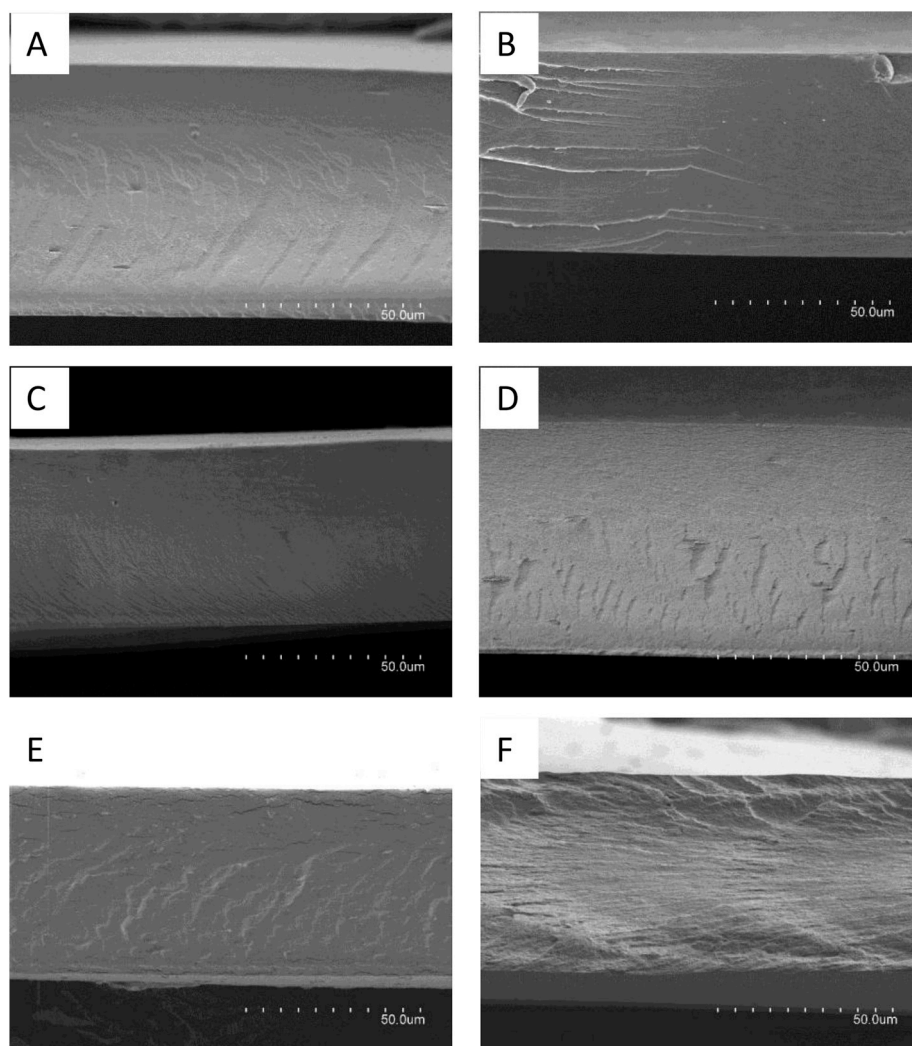


Fig. 2. Cross-section images of the developed films. A: agar control; B: agar/Ln; C: alginate control; D: alginate/Ln; E: agar/alginate control; F: agar/alginate/Ln.

*A. salina* was evaluated in order to evaluate its applicability in food-related products.

Results obtained from the *A. salina* test showed that the Ln extract was not toxic below the concentration of 500 µg/mL. The LC<sub>50</sub> value was of 750 µg/mL, while potassium dichromate used as a positive control exhibited a LC<sub>50</sub> of 30 µg/mL. Table 3 displays the results obtained in the mutagenic activity evaluation assay against *S. typhimurium* TA98 and TA100 strains. As observed, the mutagenicity test using strains of *S. typhimurium* TA98 and TA100 indicated that, up to a concentration of 700 µg/plate, the Ln extract did not induce an increase in the number of spontaneous revertants, showing in all cases MR < 1.5. At the concentration of 1400 µg/plate, the effect against either of both strains could not be determined, given that bacterial viability is affected at that concentration, due to the antimicrobial activity of the extract against *S. Typhimurium*.

Thus, it can be concluded that Ln extract is not toxic or genotoxic at the concentrations tested which would guarantee its safe use and allow its incorporation into active food coatings.

Amongst the active properties tested for the extract, Ln is powerful antiviral against foodborne viruses and thus, it was selected for the development of antiviral edible coatings for berries, which have been identified as important vehicles for the transmission of foodborne viruses, such as human noroviruses. The physicochemical and release properties of the developed active films were characterized, and the antiviral efficiency on blueberries coated with polysaccharide-based

coatings containing Ln was carried out.

### 3.2. Properties of stand-alone polysaccharide-based films

With the aim of developing antiviral edible coatings, three different polysaccharide-based matrices (agar, alginate and agar/alginate mixtures) were used as biopolymer coatings in which the Ln extract was incorporated to confer them active properties. Morphological, physicochemical and functional properties of edible coatings, including mechanical, water vapor/oxygen barrier and optical properties were characterized.

Fig. 2 shows representative SEM micrographs of the cross-section images of the developed polysaccharide-based films after a one-week storage period at 25 °C and 53% RH. Neat alginate or agar films exhibited a quite homogeneous structure with no brittle areas or bubbles, consistent with the formation of a compact arrangement of polymer chains. Both polysaccharides are highly compatible as deduced from the SEM images since no phase separation was observed. Therefore, it can be postulated that interactions between film components (alginate, agar and glycerol) favored the integration of polysaccharides in a homogeneous matrix. In contrast, incorporation of the Ln extract provided a slightly rougher structure, being more accentuated in films prepared with a mixture of both polysaccharides.

Fig. 3 shows typical Ti spectra distribution curves of films, from 400 to 700 nm, as a transparency indicator, which is directly linked with the

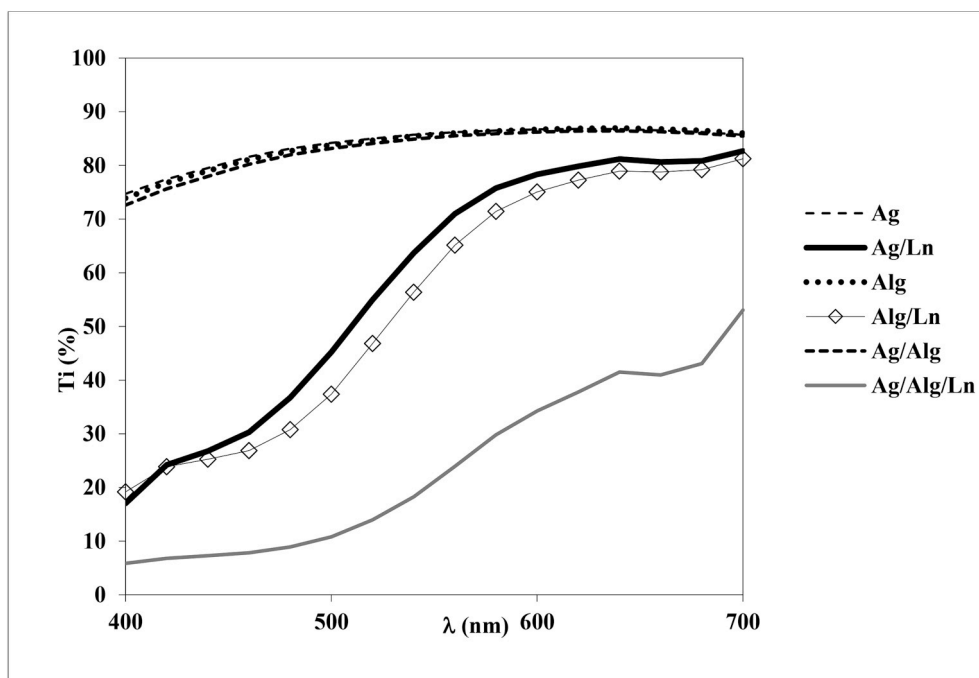


Fig. 3. Spectral distribution of internal transmittance (Ti) of the developed stand-alone films.

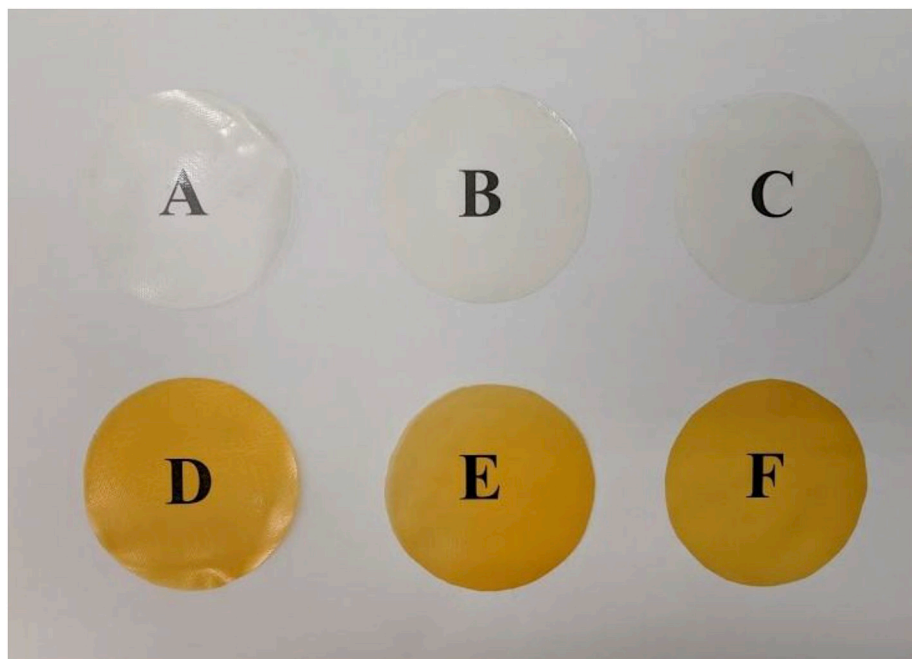


Fig. 4. Contact transparency pictures of the developed stand-alone films. A: agar control; B: alginate control; C: agar/alginate control; D: agar/Ln; E: alginate/Ln; F: agar/alginate/Ln.

internal structure of the developed films (Fabra et al., 2009). An increase in the internal distribution of transmittance is ascribed to an increase in transparency. From the films tested, those prepared with pure polysaccharide were the most transparent, regardless the type of polysaccharide used. In contrast, the presence of the Ln extract significantly reduced the transparency of polysaccharide based films, showing lower Ti values in all the wavelength considered, indicating that these films were more opaque and heterogeneous than their counterparts prepared with pure alginate and/or agar. This can be ascribed not only to the fact that transmittance of the samples is affected by differences in refractive

indices between the neat polysaccharide matrix and the different compounds present in the Ln extract, but also by the light selective absorption of polyphenol compounds of Ln extract at low wavelengths. In fact, although all tested films preserved good contact transparency (see Fig. 4), it was slightly diminished when Ln extract was incorporated in the biopolymer matrices since these polyphenol compounds impart a yellowish color to the films, thus decreasing the hue and Ti values at low wavelengths. Plant extracts commonly provide opacity to polymers and, as a result, films containing extracts are less transparent than films without extracts (Norajit, Kim, & Ryu, 2010). The addition of plant

**Table 4**

Color parameters of the developed stand-alone films.

Films	L*	C* <sub>ab</sub>	h* <sub>ab</sub>
Ag	79.9 (0.3) <sup>a</sup>	20.4 (0.5) <sup>a</sup>	85 (1) <sup>a</sup>
Ag/Ln	52.0 (1.0) <sup>b</sup>	40.2 (0.3) <sup>b</sup>	62 (2) <sup>b</sup>
Alg	78.4 (0.3) <sup>a</sup>	21.7 (0.5) <sup>a</sup>	81 (3) <sup>a</sup>
Alg/Ln	48.1 (0.9) <sup>c</sup>	36.5 (0.5) <sup>c</sup>	57 (1) <sup>c</sup>
Ag/Alg	76.7 (1.5) <sup>a</sup>	20.3 (1.0) <sup>a</sup>	83 (2) <sup>a</sup>
Ag/Alg/Ln	54.4 (1.0) <sup>b</sup>	40.1 (1.5) <sup>b</sup>	63 (2) <sup>b</sup>

L\* lightness, C\*<sub>ab</sub> Chroma, h\*<sub>ab</sub> hue. Different superscripts within a column indicate significant differences among formulations ( $p < 0.05$ ). Mean values (standard deviation).

**Table 5**Mechanical properties<sup>a</sup> of the stand-alone coatings.

Sample	TS (MPa)	E (MPa)	EAB (%)
Ag	27.5 (6.8) <sup>a</sup>	992 (52) <sup>a</sup>	19.7 (7.1) <sup>a</sup>
Ag/Ln	19.5 (2.6) <sup>a,b</sup>	970 (30) <sup>a</sup>	14.5 (3.5) <sup>a</sup>
Alg	22.4 (9.1) <sup>a,b</sup>	793 (94) <sup>b</sup>	17.8 (10.9) <sup>a</sup>
Alg/Ln	10.3 (2.1) <sup>b</sup>	784 (24) <sup>b</sup>	10.6 (3.2) <sup>a</sup>
Ag/Alg	24.0 (8.3) <sup>a,b</sup>	615 (41) <sup>c</sup>	23.7 (4.3) <sup>a</sup>
Ag/Alg/Ln	12.9 (3.9) <sup>a,b</sup>	477 (42) <sup>c</sup>	21.4 (5.1) <sup>a</sup>

<sup>a</sup> Elastic modulus (E), tensile strength (TS), and elongation at break (EAB) of films equilibrated at 53% relative humidity for one week. Different superscripts within a column indicate significant differences among formulations ( $p < 0.05$ ). Mean values (standard deviation).

extracts in films provides an adequate barrier to light, which is important to prevent the degradation of light sensitive components (Mir et al., 2018). In fact, previous works have reported a greater protection against UV light in biopolymer matrices containing plant extracts as compared to their counterparts prepared without extracts (Abdollahi, Damirchi, Shafafi, Rezaei, & Ariaii, 2019; Fabra et al., 2018; Mir et al., 2018).

From the reflectance spectra of an infinite thickness film, Lightness (L\*), hue (h\*<sub>ab</sub>) and Chroma (C\*<sub>ab</sub>) were obtained as well as the total color differences ( $\Delta E$ ) with respect to the neat polysaccharide films (Table 4). Incorporation of Ln extract provided darker (lower L\*) films, with a more saturated (higher C\*<sub>ab</sub>) orange-brownish color (lower h\*<sub>ab</sub>).

Tensile properties are useful parameters to describe the mechanical behavior of films and are closely related to their internal structure. Table 5 displays the tensile parameters (elastic modulus (E), tensile strength (TS), and elongation at break (EAB)) of the tested films prepared with and without Ln extract. Neat polysaccharide films were highly resistant and stiff with values in the range of those previously reported in the literature (Barbut & Harper, 2019; Kanmani & Rhim, 2014; Shankar & Rhim, 2017), being agar-based films stiffer (higher E values) than alginate or agar/alginate films. It is observed that the incorporation of Ln extract did not significantly modify the stretchability and stiffness of the films, although a tendency to decrease the mean TS values was observed for Ln-containing edible coatings. This suggests that the Ln extract had a weakening effect on the films, hence showing the interruption effect of the extract in the cohesion of the polysaccharide network. Similar effects have been reported in protein and polysaccharide-based films incorporating hydrophobic compounds (Fabra, Talens, & Chiralt, 2008; Khwaldia, Banon, Desobry, & Hardy, 2004). The small differences observed in the present work could be explained by the low amount of Ln added into the polysaccharide matrices.

Barrier properties, usually described by their permeability values, are important because they will affect oxidation and respiration rates in the enclosed foods and thus, they are the most important factors to evaluate the effectiveness of edible coatings. Table 6 gathers water vapor and oxygen barrier properties data of developed edible films. WVP values ranged between 7.8 and 8.5 g Pa<sup>-1</sup> s<sup>-1</sup>m<sup>-2</sup> for neat polysaccharide matrices, in agreement with those found in literature for

**Table 6**

Water vapor and oxygen permeability of the developed stand-alone films.

Sample	WVP 10 <sup>-10</sup> (g Pa <sup>-1</sup> s <sup>-1</sup> m <sup>-2</sup> )	OP 10 <sup>-20</sup> (m <sup>3</sup> m m <sup>-2</sup> s <sup>-1</sup> Pa <sup>-1</sup> )
Ag	8.47 (0.27) <sup>a</sup>	13.95 (3.69) <sup>a</sup>
Ag/Ln	6.10 (1.77) <sup>b</sup>	7.47 (1.48) <sup>b</sup>
Alg	8.30 (0.52) <sup>a</sup>	1.77 (0.79) <sup>c</sup>
Alg/Ln	6.04 (0.10) <sup>b</sup>	4.16 (0.03) <sup>b,c</sup>
Ag/Alg	7.83 (0.19) <sup>a,b</sup>	1.89 (0.27) <sup>c</sup>
Ag/Alg/Ln	6.06 (0.17) <sup>b</sup>	3.76 (1.45) <sup>b,c</sup>

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

agar-based films (Roy, Rhim, & Jaiswal, 2019) although they were slightly lower than those previously reported for alginate-based films (Rhim, 2004; Wang, Shankar, & Rhim, 2017). Interestingly, the incorporation of Ln extract significantly reduced the WVP probably due to the higher hydrophobic nature of the extract (which was not soluble in water).

Oxygen permeability (OP) was measured at 53% RH and the results are also summarized in Table 6. The OP values for the neat alginate and agar-based films agree with that reported in the literature (Rojas-Gräu et al., 2007; Zhang et al., 2019). The high permeability values obtained can be related to the fact that, at high RH, hydrophilic films tend to lose their gas barrier properties due to the increase of polymer chain mobility (Forsell, Lahtinen, Lahelin, & Myllärinen, 2002). It is noticeable that neat agar-based films were much more permeable to oxygen than their counterparts prepared with alginate (either Alg or Ag/Alg). Curiously, the incorporation of Ln extract lead to an increase in the OP values, except in the case of agar matrices where it was significantly decreased by approximately 46.45%, showing no significant differences ( $p < 0.05$ ) with their counterparts prepared with alginate and Ln. Thus, similar OP values were observed for Ln-containing edible films.

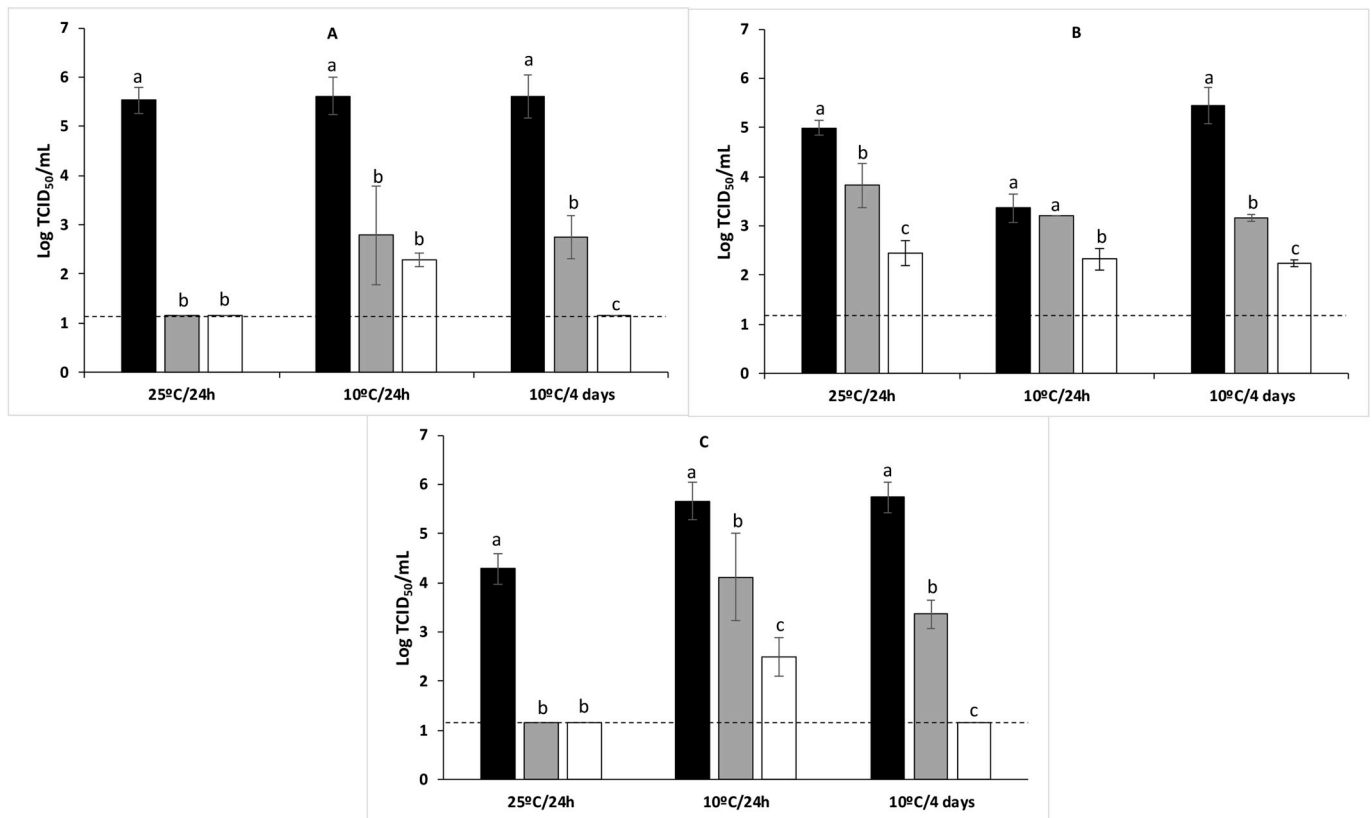
The different behavior of Ln extract when it was incorporated into polysaccharide matrices can be explained by changes in the film microstructure, thickness, void volume in the biopolymer structure and different arrangement of the biopolymer chain. In fact, there is a controversy in the literature concerning the barrier properties behavior, based on the type of hydrocolloid and the nature of plant extract. For instance, Nouri and Mohammadi Nafchi (2014) also reported an increase in OP values due to the incorporation of betel leaves extract in starch-based films. In contrast, Akhtar et al. (2013) reported a decrease in OP values of HPMC films when a commercial plant extract (betacyanins) were added. They attributed this improvement in the OP barrier properties to availability of free hydroxyl groups of natural extract to interact with the HPMC matrix by hydrogen bonds, giving a more compact structure of polymer matrix. On the other hand, Ekrami, Emam-Djomeh, Ghoreishy, Najari, and Shakoury (2019) did not find significant differences of OP values of Salep mucilage films with or without different amounts of pennyroyal extracts.

Although it is outside the scope of this paper, the release of Ln extract from the developed polysaccharide based films was assessed in two different media (Supplementary Material Fig. S1) and the antioxidant activity of the Ln extract released to the simulant media was quantified after 14 days by the ABTS assay for all the polysaccharide matrices, showing a positive correlation between the Ln released and the antioxidant activity.

### 3.3. Challenge tests

Ln extract with demonstrated antiviral activity has been postulated as potential candidate to develop antiviral coatings. In order to broaden the use of natural compounds for the development of antiviral coatings, agar and alginate-based coatings, with and without Ln extract, were used to coat fresh blueberries artificially inoculated with MNV and stored at 10 °C (ON and 4 days) and 25 °C (ON). The appearance of the uncoated and coated samples are given in Fig. S2 of Supplementary





**Fig. 5.** Reduction of MNV titers (log TCID<sub>50</sub>/mL) on blueberries after treatment coatings at different temperatures and storage times. (A: Agar coating; B: Alginate coating; C: Agar/Alginate coating).

\*Black bars: virus control; Grey bars: coating control; White bars: coating Ln.

\*\*Dashed lines depict the detection limit.

\*\*\*Each column represents the average of triplicates. Within each column for each storage condition, different letters denote significant differences between treatments ( $P < 0.05$ ).

material. Neat agar coatings (without Ln extract) reduced the infectivity of MNV below the limit of detection after ON storage at 25 °C while MNV titers were reduced by 2.54 and 2.88 log after ON and 4 days at 10 °C storage (Fig. 5A) since viruses usually persist better at lower temperatures than at higher temperatures. These results are not entirely surprising since other biopolymeric matrices such as carrageenan also exert antiviral activity on coatings application (Falcó et al., 2019). Furthermore, the efficacy of the coatings containing Ln extract was not improved at 25 °C in agar-based films (Fig. 5A), probably due to the intrinsic antiviral activity of the agar. In contrast, the incorporation of Ln in Alg and Alg/Ag edible coatings increased the antiviral activity of the coatings at 10 °C. Alg/Ln edible coatings reduced MNV titers by 1.37, 0.88 and 0.92 logs compared to neat alginate coatings, after ON at 25 °C, ON at 10 °C and 4 days at 10 °C (Fig. 5B).

#### 4. Conclusions

Plant species that grow in arid and semiarid regions of Argentina were proposed as active compounds for the development of active edible coatings for food applications. Amongst them, Ln extract showed the most promising antimicrobial and antioxidant properties, being a potential antiviral compound for foodborne viruses. Furthermore, it was proved not to be toxic or genotoxic at 500 µg/mL, being this concentration selected for the development of antiviral edible coatings based on marine polysaccharides.

The incorporation of Ln extract did not significantly modify the stretchability and stiffness of the films, although a tendency to decrease the mean tensile strength values was observed for Ln-containing edible coatings, suggesting that the Ln extract had a weakening effect on the

films. Water vapor barrier properties were significantly improved in Ln-containing films probably due the higher hydrophobic nature of the extract (which was not soluble in water) as compared to the neat polysaccharide based matrices. However, although the effect that the Ln extract had on the oxygen permeability values depended on the polysaccharide matrices, similar OP values were observed for Ln-containing edible films.

These antiviral edible coatings containing Ln could be an alternative in the strategies followed to reduce or eliminate human enteric viruses, since they were proved to effectively reduce the titers of MNV in artificially contaminated blueberries.

#### Declaration of competing interest

The authors declare that there are no conflicts of interest.

#### CRediT authorship contribution statement

**María Alejandra Moreno:** Formal analysis, Methodology, Writing - original draft. **Hyllenne Bojorges:** Formal analysis, Methodology. **Irene Falcó:** Methodology. **Gloria Sánchez:** Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing - review & editing. **Gracia López-Carballo:** Methodology. **Amparo López-Rubio:** Conceptualization, Data curation, Supervision, Resources, Writing - review & editing. **Iris Catiana Zampini:** Methodology. **María Inés Isla:** Conceptualization, Resources, Writing - review & editing. **María José Fabra:** Conceptualization, Data curation, Supervision, Resources, Project administration, Writing - original draft, writing - review & editing.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodhyd.2019.105595>.

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