Development of flexible bactericidal films based on poly(lactic acid) and essential oil and its effectiveness to reduce microbial growth of refrigerated rainbow trout

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Abstract

The main shortcoming of poly(lactic acid) (PLA) for food packaging applications is its brittleness. The aim of this work was to evaluate the potential use of *Origanum vulgare* L. essential oil (OEO) in PLA-based matrices to obtain developed bio-based films with enhanced mechanical properties as well as antimicrobial performance. The PLA-essential oil composite films were more flexible than neat PLA films; so that up to 6-fold increase in the elongation-at-break values (2.82-16.78%) were acquired when 0.5% (w/w) oil was added to the polymer matrix. The addition of essential oil decreased the PLA glass transition temperature (T_g), as the result of the polymer plasticization, and led to modification of the tensile behavior. AFM results confirmed that the PLA surface microstructure was drastically influenced in terms of roughness parameters (R_a and R_q) when OEO was added. The films' antimicrobial activities were induced by incorporating essential oil, which were more effective against the bacteria in direct contact method than a vapor phase. However, in both of test methods, *Staphylococcus aureus* was the most sensitive bacterium to OEO-containing films, while *Escherichia coli* was the most resistant. Then, efficacy of bioactive film included with 1.5% (w/w) essential oil to reduce microbial growth of rainbow trout during chilled storage was evaluated; the results indicated that antimicrobial PLA film was effective against all the microorganisms tested in this study. These results suggest that the developed PLA films with active substance could be used in designing antimicrobial packaging materials.

Keywords: Antimicrobial film, Flexibility, Poly(lactic acid) PLA, *Origanum vulgare* L., Essential oil, rainbow trout

1. Introduction

Petroleum-based plastics used for food packaging had some disadvantages such as declining oil and gas resources, rising price of crude oil, environmental concerns for their degradation or incineration and global warming (Siracusa, Rocculi, Romani, & Rosa, 2008). In addition, consumer toxicity risks about monomers or oligomers migrating to edible materials present safety problems (Jamshidian, Tehrani, Imran, Jacquot, & Desobry, 2010). These ecological, economic, health and safety challenges have motivated researchers and producers to replace petroleum-based polymers with biodegradable polymers.

One of the most widely used biopolymers is polylactic acid (PLA) which is miscible, biodegradable and thermoplastic, made from renewable sources (Armentano et al., 2015). PLA is the fastest growing market in the plastic industry, credited with a 10 to 30% increase depending on the sources (<http://www.carbios.fr/en/press-releases/show/52>). The increasing interest in developing PLA-based films for food packaging purposes is not only because of the need for substitution of many petroleum-based polymers (Erdohan, Çam, & Turhan, 2013; Mascheroni, Guillard, Nalin, Mora, & Piergiovanni, 2010), but also because of its beneficial physical and mechanical properties (Mascheroni et al., 2010). Nevertheless, practical applications of PLA are often limited by its drawbacks such as low plasticity, low heat resistance, high modulus and fragile behavior (Armentano et al., 2015). Therefore, expansion of PLA functions in the packaging industry requires overcoming these problems (Mascheroni et al., 2010).

Various lipid materials such as oils, fats, fatty acids, and waxes have been mixed with other biopolymer-based film-forming solutions to obtain the desirable properties of component materials to improve gloss, permeability characteristics, strength, flexibility, and general performance of bio-based films (Zhang, Rempel, & McLaren, 2014). In this regard, the inclusion

of plant essential oils in biodegradable film-forming solutions offers an interesting alternative. Essential oils (EOs) similar to other fats, may improve the tensile properties such as flexibility of biopolymer films due to its plasticizing effect. Meanwhile, active compounds like EOs can be added to the films in order to improve their functional properties, such as water vapour permeability, as well as to introduce antimicrobial properties (Teixeira et al., 2014).

The essential oil of *Origanum vulgare* L. (OEO) compared with other essential oils, is considered for its antioxidant and antimicrobial activity (Burt, 2004). These actions are primarily related to two phenols: carvacrol and thymol (main components of this essential oil) and the monoterpene hydrocarbons *p*-cymene and γ -terpinene which exist at low concentrations (Burt, 2004; Hosseini et al., 2016).

Due to the presence of many antimicrobial compounds in EOs, biodegradable films activated with these bactericidal agents are highly useful to minimize the growth of post-processing contaminant microorganisms, extending shelf life of food and improving food safety (Sánchez Aldana, Andrade-Ochoa, Aguilar, Contreras-Esquivel, & Nevárez-Moorillón, 2015).

Fresh fish are highly perishable products and their deterioration is mainly from the biological reactions such as oxidation of lipids, protein degradation or decomposition mediated by endogenous enzymes, and the metabolic activities of microorganisms (Rezaei & Hosseini, 2008).

These activities lead to a short shelf life in fish and other seafood products. Rainbow trout (*Oncorhynchus mykiss*) are highly susceptible to deterioration and microbial spoilage during storage. Given that the shelf life of refrigerated rainbow trout fillet is relatively short and that there is a growing tendency of consumers for fresh fish, the development of new preservation methods, which permit shelf-life extension of fresh fish, is required.

In previous studies, PLA were used as polymer matrices to produce active food packaging films with the combination of natural extracts or essential oils which could come from plants (Erdoğan et al., 2013; Llana-Ruiz-Cabello et al., 2015; Mascheroni et al., 2010; Tawakkal, Cran, & Bigger, 2016). The present study makes a contribution to develop active bio-packaging films using hydrophobic compounds such as essential oil, which could improve the mechanical and physical properties as well as induced antimicrobial function to the PLA films. Moreover, the efficacy of PLA-essential oil film on the microbiological characteristics of rainbow trout during refrigerated storage was evaluated.

2. Materials and methods

2.1. Materials

Polylactic acid (PLA), 2002 D, was purchased in pellet from Natureworks® Co., (USA). Oregano essential oil (100% pure) and chloroform were obtained from NewDirections Aromatics Inc. (Hampshire, UK) and Merck Chemicals Co. (Darmstadt, Germany), respectively. For antimicrobial tests, Tryptic Soy Agar (TSA) and Brain Heart Infusion (BHI) Broth, Violet Red Bile Dextrose (VRBD) Agar and de Man Rogosa Sharpe (MRS) Agar were purchased from Quelab Laboratories Inc. (Montreal, Quebec, Canada). Bacterial stock such as *Staphylococcus aureus* (PTCC 25923), *Listeria monocytogenes* (PTCC 19118), *Escherichia coli* (PTCC 1330) and *Salmonella enteritidis* (PTCC 138) were provided from Persian Type Culture Collection (Tehran, Iran). Sub culturing was carried out on monthly basis to maintain bacterial livability. All strains were preserved in BHI added with 30% glycerol at -20°C when used.

2.2. GC-MS analysis of oregano essential oil

Essential oil was analyzed in an Agilent 6890 gas chromatograph interfaced to an Agilent 5973N mass selective detector (Agilent Technologies, Palo Alto, USA). Oregano oil was separated by a capillary column HP-5MS (30 m $\times$ 0.25 mm id, 0.25 mm film thickness; Restek, Bellefonte, PA). Essential oil components were identified based on the comparison of their mass spectra with the spectra from Wiley7n.L libraries. The relative percentage for the main components was calculated from the peak areas.

2.3. Preparation of antimicrobial films

PLA-based films were provided by solvent casting method as reported by Jamshidian et al. (2012) with some modifications. Since PLA is very hygroscopic, PLA pellets were previously dried in oven at 90 °C for 3 h. Then, a solution of 3.5% (w/w) PLA was prepared in chloroform and then 0.5, 1 and 1.5% (w/w) of OEO based on PLA dry matter were added to solution and homogenized at 22000 rpm for 1 min using a homogenizer (UltraTurrax IKA, T25, Germany). Afterward, the polymer solution was cast into a glass petri dishes and the solvent was allowed to evaporate in a hood in a dark place over 3 days at ambient temperature. The final thickness of $40 \pm 2 \mu\text{m}$ was determined in all samples except the 1.5% (w/w) OEO formulation concentration, which was $50 \pm 1 \mu\text{m}$.

2.4. Characterization of the films

2.4.1. Mechanical properties

An universal testing machine (TVT-300Xp, Perten, Sweden) was used to determine the tensile strength (TS), elongation-at-break (EAB) and elastic modulus (EM) of the bioactive films according to the ASTM D882-02 standard procedure (ASTM, 2002). Film samples (rectangular

strips of 100 × 20 mm) were conditioned in an environmental chamber for 48 h at 23 ± 2 °C and 53 ± 2% RH before testing. Tests were performed on at least five samples of each material with an initial grip separation and mechanical crosshead speed was set at 50 mm and 5 mm/min, respectively.

2.4.2. *Water vapor permeability (WVP)*

WVP values of films were calculated gravimetrically via ASTM E96-05 method (2005). Circular glass cups with a diameter of 50 and a depth of 10 mm were applied. The samples were cut into circles and sealed onto the cup mouth containing 6 ml distilled water (100% RH, 2.337×10³ Pa vapor pressure at 20 °C), stored in a desiccator at 20 °C and 0% RH (0 Pa water vapor pressure) containing silica gel. The water transported through the film was measured every 2 h during 10 h by the weight loss of the glass permeation cell. The measured WVP of the films was calculated using the following equation:

$$\text{WVP} = (\text{WVTR} \times L) / \Delta p$$

Where WVTR is the water vapor transmission rate (g mm/kPa h m²) calculated from the slope of the straight line divided by the exposed film area (m²), L is average film thickness (mm), and ΔP is the partial water vapor pressure variance (kPa) through two sides of the film. Three replicates of each film were tested.

2.4.3. *Measurement of contact angle (CA)*

CA measurements of water on the surface of samples were carried out by using a sessile drop method with a PG-X goniometer (PG-X, Switzerland). The contact angle was measured by adding a droplet of ultrapure water (~5 μL) onto the film surface with a precision syringe. Five measurements were performed for each film and the average values were reported.

2.4.4. Thermal analysis

Differential scanning calorimetry (DSC) of PLA solvent cast films was performed using a DSC 500-B device (Faith, China). Samples of approximately 10-15 mg (three replicates) were placed into aluminium pans, sealed and scanned over the range of 25 to 200 °C with a heating rate of 10 °C/min. The glass transition temperature (T_g), crystallization temperature (T_c), and melting temperature (T_m) of the PLA films were measured.

2.4.5. Morphological analysis (SEM and AFM)

The morphological examination of active films was performed by scanning electron microscopy (SEM) (VEGA II, TESCAN, Czech Republic). Strips of dry films were immersed in liquid nitrogen and cryo-fractured manually; then the samples were fixed onto aluminum pins by a double-sided tape. The pins with the sample were sputtered with a thin layer of gold in an ion sputter coater (K-450X, EMITECH, England) and placed into a scanning electron microscope for observing the surface and cross-section morphology of the films.

Atomic force microscopy (AFM) (CP-R, Veeco Instruments, USA) was also used to analyze the surface morphology of the films with a $0.2 \times 0.2 \mu\text{m}$ scan size. The images were recorded at ambient atmosphere by triangular cantilever with a spring constant of 50 N/m. Two statistical parameters including average roughness (R_a) and root mean square roughness (R_q) were calculated.

2.5. Fish sample preparation and storage

Fresh water rainbow trout (*Oncorhynchus mykiss*) with an average weight of 250-300 g were purchased from a local aquaculture farm located at Noor, in North of Iran. The fish were killed by asphyxia in air and delivered (packed into an insulated polystyrene box with ice) to the

seafood processing laboratory, within 1 hour of harvesting. Upon arrival, fish were washed with tap water, gutted, headed and then filleted by hand. Two fillets were obtained from each fish after removing the head and bone. To obtain uniform size of samples, skinless fillets were cut into 2×2 cm squares of 1 cm of thickness; and these were wrapped in film incorporated without (PLA) and with OEO (PLA-OEO). Samples that were not covered with any films served as controls (Control). The meat samples were placed into a sterile plastic dish covered with plastic film and stored in refrigerator (4 ± 1 °C).

2.6. Microbiological analysis

2.6.1. Determination of MIC and MBC of OEO

The inhibitory effects of oregano essential oil against the selected *S. aureus*, *L. monocytogenes*, *E. coli* and *S. enteritidis* isolates were determined by the modified microdilution method (Soković, Glamočlija, Marin, Brkić, & van Griensven, 2010). Briefly, a serial dilution of the oregano essential oil was prepared in a 96-well microtiter plate over the range of 0.06-20 µl/ml (at 10 different concentrations) in BHI medium. Subsequently, 70 µl of essential oil were added to microplate. Bacterial suspensions were adjusted to 1×10^6 CFU/mL in BHI, and 70 µl were added per well of 96-well plates. Plates were incubated at 37 °C for 24 h. The MIC was defined as a lowest concentration which resulted in inhibition of visual growth absorbance was read in an ELISA plate reader at 630 nm (Biotek Instrument Inc, USA). After determination of the MIC, a 5 µl aliquot from each tube that was determined to be non-turbid and the last tube in which turbidity was evident was smeared on TSA and incubated at 37 °C for 24 h. The lowest concentration with no visible growth was defined as the MBC.

2.6.2. Evaluation of antimicrobial activity of the active films

The antimicrobial activity of the active PLA films was carried out using the agar diffusion method based on two approach; direct contact and vapor phase (Du et al., 2009). For this purpose, 10 µl of 10⁶ CFU/ml of each bacterial strain was spread on TSA by swab. Bacterial strains were cultured for 24 h in BHI at 37 °C. In both test methods, direct contact and vapor phase, circular parts of each film (10 mm in diameter) were located on the lids and on the surface of TSA plates, respectively. The diameter of the inhibition area (mm²) around the disc was measured after 24 h of incubation at 37 °C. Films without OEO were treated by using the same protocol and were used as control.

2.6.3. Effect of OEO-incorporated PLA film on microbial changes of trout

To determine the antimicrobial effect of the PLA-based film during storage, trout samples (5 g) were aseptically peeled, added to 45 mL of sterile saline solution (0.85% NaCl) and then homogenized in a stomacher (Wiggen Hauser, D-500, Germany) at room temperature. Decimal dilutions in saline solution were prepared and 1 mL samples of appropriate dilutions were poured or spread to the following media: TSA agar for total viable count (TVC) and psychrotrophic count (PTC), incubated at 30 °C for 2 days and 7 °C for 10 days, respectively; double-layered plates of MRS Agar for lactic acid bacteria (LAB) count, incubated at 37 °C for 72 h (); VRBD agar for *Enterobacteriaceae* count, incubated at 30 °C for 48 h (Volpe et al., 2015). All analyses were performed at 0, 4, 8, 12 and 16 days by triplicate. Microbiological counts are expressed as the log of the colony-forming units per gram (log₁₀ cfu/g) of sample.

2.7. Statistical analysis

ANOVA analysis of variance followed by Duncan's multiple variety tests was used to define significant changes between the treatments at 95% assurance level ($p \leq 0.05$). Average value of three replicates for each sample was achieved and data were reported in the form of average $\pm$ SD.

3. Results and discussion

3.1. GC-MS analysis of essential oil

Sixteen constituents in the essential oil of oregano were determined, with the major components as 81.85% carvacrol, 4.07% *p*-cymene, 3.30% thymol, 2.26% γ -terpinene and 1.52% linalool (Table 1). Carvacrol, the most abundant component of this oil, has also been reported by Baydar, Sagdic, Ozkan, and Karadoga (2004). Several compounds found in this essential oil, such as carvacrol, thymol, camphene, caryophyllene, and γ -terpinene, have known antioxidant properties (Teixeira et al., 2013). Additionally, antibacterial properties have also been described for carvacrol, thymol, *p*-cymene, linalool, α -pinene and α -terpineol (Chorianopoulos et al., 2004). Specially, the components with phenolic structures, such as carvacrol and thymol, were very active against microorganisms (Gutierrez, Barry-Ryan, & Bourke, 2008).

3.2. Mechanical properties of the films

The biodegradable films must be resistant to the usual pressure that happens in transport, application and handling to keep the properties and integrity of foods (Pelissari, Grossmann, Yamashita, & Pineda, 2009). The mechanical properties of the PLA films included with different formulation concentrations of oregano oil are represented in Table 2. Overall, the addition of essential oil caused a significant decrease ($p < 0.05$) in the TS and the EM, while remarkable

increase on EAB was observed. As presented in Table 2, an approximately 67% reduction in the TS values (47.18 to 15.73 MPa) and 58% reduction in EM (1.39 to 0.57 GPa) were attained by adding 1.5% (w/w) of OEO to the PLA films. This was most likely due to plasticizing effect of essential oil; the drop in tensile strength may be caused by the formation of plasticizer-plasticizer interaction which dominates at higher oil contents, resulting in a phase separated structure (Chieng, Ibrahim, Then, & Loo, 2014). A similar effect was observed with olive leaf extract on mechanical properties of PLA-based films (Erdohan et al., 2013).

As formerly mentioned, one of the main shortcomings of PLA in the packaging applications, is low flexibility and its fragile behavior. For that reason, developing films with improved flexibility along with good antimicrobial performance was the main objectives of this research. The pristine PLA film displayed a low EAB value of about 2.8%, which is comparable with the values reported for PLA films in the literature (2-6%) (Armentano et al., 2015; Arrieta, López, Hernández, & Rayón, 2014; Erdohan et al., 2013; Li & Huneault, 2007). In the present study, the PLA film's flexibility markedly increased ($p < 0.05$) after incorporation of essential oil.

According to Table 2, up to 6-fold increase in the EAB values (2.82-16.78%) are obtained when 0.5% (w/w) essential oil was added to the PLA matrices. This may be ascribed by plasticizing role of essential oil included to the polymer matrix resulted in the increase in ductile properties (Ramos, Jimenez, Peltzer, & Garrigos, 2014). Similar results have been reported for PLA formulations with active compounds, such as cinnamaldehyde and/or thymol (Qin, Yang, & Xue, 2015; Ramos et al., 2014).

3.3. Water vapor permeability (WVP) and wettability

Since water is an important factor in reactions leading to food spoilage, so the ratio of WVP are also considered as important aspects in biopolymers-based packaging materials (Jamshidian et

al., 2012). The permeability values of neat PLA films (0.068 g mm/kPa h m²) (Table 2) are comparable to that of solvent cast PLA films (0.054 g mm/kPa h m²) (Erdohan et al., 2013), but much lower than that of another PLA films prepared by the solvent-casting method (0.168 g mm/kPa h m²) (Rhim, Mohanty, Singh, & Ng, 2006). This contrast in WVP can be attributed to the difference in PLA concentration and test conditions (Erdohan et al., 2013). Incorporation of OEO into PLA films decreased the WVP from 0.059 to 0.045 g mm/m² h kPa ($p < 0.05$), but increasing amount of oil did not further influence the permeability ($p > 0.05$). Enhancing effect of the oregano EO on WVP was previously reported for biodegradable films based on starch and chitosan (Pelissari et al., 2009). When compared to the synthetic polymers, the values for active PLA film is 5.5 orders of magnitude lower than that of permeability value noticed in cellophane films (0.248 g mm/kPa h m²), but higher than that observed in LDPE films (0.0072 g mm/kPa h m²) (Péroval et al., 2002).

The contact angle (CA) of water is one of the basic wetting properties of packaging materials, indicating hydrophilic/hydrophobic properties of the material (González & Igarzabal, 2013). Table 2 lists the average CA (°) of water deposited onto PLA films surface. The water contact angle value measured on the pure PLA film (61.2°) is in good agreement with the literature (Arrieta et al., 2014; Rhim et al., 2006). The enhancement of the hydrophobic character of the PLA film surface with the OEO addition was clearly shown by the increase ($p < 0.05$) of water contact angles from 61.2° to the highest value of 94.03° for 1.5% (w/w) oil-incorporated film. This decreased wettability can be attributed to the hydrophobic nature of oregano essential oil.

3.4. Differential scanning calorimetry (DSC)

DSC analysis was accomplished to determine the thermal transitions of the materials developed. DSC thermograms for the glass transition temperature (T_g), as well as crystallization and melting

temperatures (T_c and T_m) are shown in Fig. 1. The incorporation of the antimicrobial agent results in changes in the thermal properties of the films. T_g is defined as a physical change from mode of glass to mode of flexible by heating the amorphous material (Roos & Karel, 1991). While the pristine PLA showed T_g around 44 °C (Llana-Ruiz-Cabello et al., 2015) (Fig. 1a), the values of T_g for the PLA films containing 0.5 and 1.5% (w/w) oregano oil decreased to 38.2 and 36.5 °C, respectively (Fig. 1b and c). Similar behavior was reported in PLA formulations with other antioxidant or antimicrobial agents (Byun et al., 2010; Ramos et al., 2014). According to Ramos et al. (2014) this could be attributed to the plasticizing effect of essential oil and, as a result, an increase in mobility of polymer chains, which improves the flexibility and ductility of PLA films.

The essential oil also facilitates the process of crystallization. As shown in Fig. 1a, neat PLA exhibited crystallization peak at 84 °C. After incorporation of 0.5 and 1.5% (w/w) OEO, the crystallization peak shifted to a lower temperature and a sharp T_c peak appeared at around 73 and 79 °C (Fig. 1b and c). This can be associated to the increased chain mobility at low temperature related with the larger T_g depression (Xiao, Lu, & Yeh, 2009). This T_g reduction enables crystallization to start at an earlier temperature upon heating (Li & Huneault, 2007). Jamshidian et al. (2012) also reported a significant decreased T_c of PLA films with addition of 2% α -tocopherol antioxidant. These authors demonstrated that such effect may be linked to the reduced crystallization induction period due to the presence of oil crystals which acted as crystalline nuclei; that increase the crystallization rate upon heating (Jamshidian et al., 2012). Llana-Ruiz-Cabello et al. (2015) found that the addition of *Allium* spp. extract (Proallium®) to PLA decreases the crystallization temperature of the polymer, and concluded this is because of the plasticizing effect of extract.

The appearance of tertiary peak of PLA-based films, which is assigned to the melting temperature (T_m) of the material, did not show remarkable changes for the active biodegradable films and T_m slightly increased from 166.4 °C (neat PLA) to 168.4 °C with the increase of essential oil amount. According to Dufresne (2008), the melting temperature depends on the crystalline areas, and when this area is extended, the melting temperature will increase.

3.5. Morphological features

Fig. 2. Shows SEM micrographs of the surface and cross-section of the PLA-based films with different OEO contents. The control film (Fig. 2a) had a homogeneous and continuous microstructure with no irregularities such as pores or cracks. As illustrated in Fig. 2b, presence of essential oil in the PLA matrices affected its structure and caused discontinuities in resultant films. This indicates that the interaction between the oregano oil and the PLA is not sufficiently strong to avoid phase separation between the oil and the polymer during the solvent evaporation. In fact, phase separation in the form of PLA microspheres occurs after evaporation of polymer/oil common solvent (i.e. chloroform). As the oil content increased from 0.5 to 1.5% (w/w) (Fig. 2c), lipid droplets were slightly enlarged maybe due to the deformation forces that acted during the polymer chain aggregation by evaporation of solvent (Sánchez-González et al., 2010).

Microscopy image of cross-section structure of the films showed a relatively continuous, compact and homogeneous phase in oil-free films (Fig. 2a). The incorporation of oregano oil reduced the compactness of the film and caused discontinuity within the polymer matrix (Fig. 2b and c). It seems essential oil was evaporated in the time of drying, leading to the formation of free spaces across the films.

AFM imaging is an effective technique used to provide more detailed surface morphological information of the films. The AFM images clearly demonstrate that the surface microstructure of the film is drastically affected by adding OEO. Neat PLA film (Fig. 3a) presents a homogenous smooth morphology with the lowest roughness ($R_a=2.12$ nm and $R_q=3.17$ nm), which is comparable with the results reported by Jamshidian et al. (2012) on PLA thin films. The presence of essential oil led to the significant increases ($p < 0.05$) in the roughness of the films, as indicated by higher R_a and R_q values (Fig. 3b and c). This trend could be associated to the greater development of lipid aggregation and creaming during the drying step, and consequently producing irregularities on the film's surface.

3.6. Determination of MIC and MBC of OEO Using micro dilution method

The Minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values of the oregano essential oil against 4 selected microorganisms are depicted in Table 3. It was evident that *S. aureus* was the most sensitive bacterium to OEO, while *E. coli* was the most resistant. On the other hand, lower concentration of essential oil was needed to inhibit the growth of *S.aureus* (MIC 0.125 μ l/ml and MBC 0.3 μ l/ml) and higher concentration was required to inhibit *E.coli* (MIC 0.5 μ l/ml and MBC 1.25 μ l/ml). In general, Gram-negative bacteria are less sensitive to essential oils than Gram-positive bacteria (Burt, 2004), because their cell wall provides a hydrophilic surface (due to the presence of lipopolysaccharide molecules), that limited the distribution of hydrophobic compounds. Soković et al. (2010) was also determined the MIC and MBC of oregano oil against organisms such as *S.aureus*, *L.monocytogenes* and *E.coli* in the range of 0.25-0.5 μ g/mL. The antimicrobial activity of oregano EO is due to the presence of both carvacrol and thymol along with their two precursors monoterpene hydrocarbons, γ -terpinene and *p*-cymene (Nostro et al., 2004).

3.7. Evaluation of antimicrobial activity of active PLA films

Table 4 shows the inhibitory effect of PLA-based antimicrobial films against the four selected bacteria in direct contact and vapor phase methods. The control PLA films (without essential oil) showed no antimicrobial activity against the studied microorganisms. In the case of direct contact test, inclusion of OEO at lowest formulation concentrations (0.5%, w/w) into the PLA matrix showed a weak inhibitory effect against microorganism; as in the case of *E.coli* no inhibitory action was detected (Table 4). However, as the formulation concentrations of essential oil increased, the inhibitory area also significantly increased ($p < 0.05$) and the film containing 1.5% (w/w) oregano oil showed the highest area of inhibition.

As formerly mentioned (see section 3.1.), compositional analysis of oregano essential oil carried out by GC/MS showed a spectrum of 16 compounds with carvacrol (~82%), *p*-cymene (4.07%) and thymol (3.30%) as the major constituents; these terpenoid fractions are responsible for the antimicrobial activity of oregano oil (Corrales et al., 2014). It was demonstrated that carvacrol and thymol are able to disintegrate the outer membrane of Gram-negative bacteria, resulting in leaching out of vital cell components (Burt, 2004). Furthermore, *p*-cymene, the biological precursor of carvacrol, is hydrophobic thus causing the cytoplasmic membrane to expand more than with carvacrol (Ultee, Bennik, & Moezelaar, 2002).

Unlike direct contact method, PLA films containing 0.5% (w/w) OEO were found not to be effective at reducing the microbial growth of the tested bacteria in vapor phase test except for *L. monocytogenes* (Table 4). In the following, although with increasing formulation concentrations of oil (i.e. 1%, w/w) the area of inhibition was observed, but a clear area of inhibition on all bacteria was detected at 1.5% (w/w) essential oil formulation concentrations. As shown in table 4, the inhibitory areas induced by the vapor phase tests were notably smaller for all studied

bacteria (especially in the case of *S. aureus* and *S. enteritidis*) than those observed with direct contact mode. According to Avila-Sosa et al. (2012) antimicrobial activities of the different essential oil vapors could be attained at lesser amounts than when applying the EOs in direct contact with a food surface. Goñi et al. (2009) also demonstrated that when bacteria were subjected to the vapors of essential oils, the inhibitory effects were markedly different from those found by direct contact. However, in both of test methods (i.e. direct contact and vapor phase), *S.aureus* was the most sensitive microorganism, followed by *S. enteritidis* and *L. monocytogenes* whatever the tested formulations. As expected, *E.coli* was the least susceptible strain; it is hypothesized that this result might be due to the presence of outer membrane surrounding the cell wall in gram-negative bacteria, which limits diffusion of hydrophobic substances via its lipopolysaccharide covering (Burt, 2004).

3.8. Microbiological changes of trout

The initial value of total viable counts (TVC) was approximately $2.7 \log_{10}$ cfu/g (Fig. 5a), which is a relatively low bacterial load, in agreement with the results have been reported for fresh trout fillets (Rezaei & Hosseini, 2008; Volpe et al., 2015). In this study, initial bacterial count of $2.7 \log_{10}$ cfu/g indicates good fish quality, considering the microbiological upper limit for fresh fish, proposed by ICMFS (1986). However, such value in the unwrapped fresh trout samples (Control) reached about $11 \log_{10}$ cfu/g at 12 day after refrigerated storage. It is worth nothing that after 12 days of storage, TVC value was about $5.6 \log_{10}$ cfu/g in OEO-PLA wrapped samples, a value below the maximal recommended limit of $7 \log_{10}$ cfu/g for TVC in raw fish. This microbial inhibition of oil-included film has been mainly attributed to oregano's terpenoid fraction comprised of carvacrol, thymol, and *p*-cymene (Hosseini, Rezaei, Zandi, & Farahmandghavi, 2016). The antimicrobial action of phenolic compounds, such as carvacrol and

thymol, are expected to cause structural and functional damages to the cytoplasmic membrane (Sikkema, De Bont, & Poolman, 1995). Similar observations have been reported by Volpe et al. (2015), when coating trout fillets with carrageenan containing essential lemon oil. However, TVC increased up to about $9 \log_{10}$ cfu/g at 16 day after refrigerated storage in oil-PLA wrapped samples (Fig. 5a).

The Gram-negative psychrotrophic bacteria (PTC) are the major group of microorganisms responsible for spoilage of aerobically stored fresh fish at chilled temperatures (Sallam, 2007). The growth pattern of PTC showed a similar trend to TVC in all samples (Fig. 5b). The initial PTC of trout fillets was about $3.3 \log_{10}$ cfu/g at day 0. At day 8, the PTC of control, PLA and OEO-PLA film wrapped samples reached to approximately 7.8, 7.3 and $5.8 \log_{10}$ cfu/g, respectively. Essential oil-film wrapping resulted in 2.1 and $1.5 \log_{10}$ cfu/g reductions in PTC counts of cold stored fish ($p < 0.05$) as compared with control and PLA film wrapped samples, respectively. Carvacrol, as the major component of OEO in the present study (see section 3.1.), disintegrates the outer membrane of the Gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to adenosine triphosphate (Burt, 2004).

Initial lactic acid bacteria (LAB) counts for trout samples were approximately $1.9 \log_{10}$ cfu/g (Fig. 5c). Similar initial results were reported for rainbow trout fillet by Volpe et al. (2015) ($2 \log_{10}$ cfu/g). LAB reached final (day 16) counts of 6.3 and $6.5 \log_{10}$ cfu/g for control and PLA-wrapped samples, respectively, whereas significantly ($P < 0.05$) lower populations of approximately $4.35 \log_{10}$ cfu/g were recorded for samples wrapped with essential oil-incorporated PLA films. The low LAB counts in this study were expected since LAB tends to grow slowly at refrigeration temperatures (Sallam, 2007).

Enterobacteriaceae, a hygiene indicator, were also a part of the microflora of fresh rainbow trout (Mexis et al., 2009). Initial *Enterobacteriaceae* counts were about $2.4 \log_{10}$ cfu/g, and reached a value of about 8.5, 8.4 and $6.9 \log_{10}$ cfu/g after 16 days of storage in control, PLA and OEO-PLA wrapped samples, respectively (Fig. 5d). Such a slow growth of *Enterobacteriaceae* is also reported by Volpe et al. (2015) for filleted trout stored in a refrigerator (0-4 °C), likely a consequence of a slow growth rate presented by these bacteria with respect to other Gram-negative psychrotrophic spoilers. Conclusively, the lower bacteria counts were observed in essential oil treated samples compared to the control during refrigerated storage, indicating the remarkable effect of essential oils on the preservation of trout.

4. Conclusion

In this research, pliable antimicrobial PLA film was developed and characterized. Several analytical techniques were used to evaluate the effects of incorporating oregano essential oil on the PLA film properties. Tensile tests emphasized that the inclusion of essential oil produced plasticization because of oil-incorporated PLA films presented enhanced flexibility. The contact angle decreased with essential oil addition, therefore, PLA-essential oil composite films have a more hydrophobic surface than pure PLA ones. DSC results revealed that the addition of OEO into the PLA matrix decreases the glass transition temperature of the films. Microstructural observations (SEM and AFM) clearly demonstrated a re-organization of the surface of the composite films due to the presence of essential oil. Either by direct contact or by vapor phase method, the developed films displayed a notable bactericidal effect against four bacteria studied. Moreover, the application of PLA-based films with oregano EO on rainbow trout delayed the growth of microorganisms; this antimicrobial activity remained throughout the studied period and contributed to extend the shelf-life of refrigerated trout. According to all these results, it can

be said that the addition of oregano oil may offer good perspective for antimicrobial flexible films, and therefore suitable for biodegradable food packaging applications.

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References

- Armentano, I., Fortunati, E., Burgos, N., Dominici, F., Luzi, F., Fiori, S., et al. (2015). Bio-based PLA-PHB plasticized blend films: Processing and structural characterization. *LWT - Food Science and Technology*, 64, 980-988.
- Arrieta, M. P., López, J., Hernández, A., & Rayón, E. (2014). Ternary PLA-PHB Limonene blends intended for biodegradable food packaging applications. *European Polymer Journal*, 50, 255-270.
- ASTM (2002). Standard test method for tensile properties of thin plastic sheeting (D882-02). In Annual Book of ASTM Standards. American Society for Testing Materials, Philadelphia, PA.
- ASTM (2005). Standard test method for water vapor transmission of materials (E96-05). In Annual Book of ASTM Standards. American Society for Testing Materials, Philadelphia, PA.
- Avila-Sosa, R., Palou, E., Jiménez Munguía, M. T., Nevárez-Moorillón, G. V., Navarro Cruz, A. R., López-Malo, A. (2012). Antifungal activity by vapor contact of essential oils added to amaranth, chitosan, or starch edible films. *International Journal of Food Microbiology*, 153, 66-72.

- Baydar, H., Sagdic, O., Ozkan, G., & Karadogan, T. (2004). Antibacterial activity and composition of essential oils from *Oreganum*, *Thymbra* and *Satureja* species with commercial importance in Turkey. *Food Control*, 15, 169-172.
- Burt, S. (2004). Essential oils: Their antibacterial properties and potential application in foods A review. *International Journal of Food Microbiology*, 94, 223-253.
- Byun, Y., Kim, Y. T., & Whiteside, S. (2010). Characterization of an antioxidant polylactic acid (PLA) film prepared with α -tocopherol, BHT and polyethylene glycol using film cast extruder. *Journal of Food Engineering*, 100, 239-244.
- Chiang, B. W., Ibrahim, N. A. Then, Y. Y., & Loo, Y. Y. (2014). Epoxidized vegetable oils plasticized poly(lactic acid) biocomposites: mechanical, thermal and morphology properties. *Molecules*, 19, 16024-16038.
- Chorianopoulos, N., Kalpoutzakis, E., Aliogiannis, N., Mitaku, S., Nychas, G. J., & Haroutounian, S. A. (2004). Essential oils of *Satureja*, *Origanum*, and *Thymus* species: Chemical composition and antibacterial activities against foodborne pathogens. *Journal of Agricultural and Food Chemistry*, 52, 8261-8267.
- Corrales, M., Fernández, A., & Han, J. H. (2014). Antimicrobial Packaging Systems. In J. H. Han (Eds.), *Innovations in Food Packaging* (pp.133-170). San Diego: Elsevier Academic Press.
- Du, W. X., Olsen, C. W., Avena-Bustillos, R. J., McHugh, T. H., Levin, C. E., & Friedman, M. (2009). Effects of allspice, cinnamon, and clove bud essential oils in edible apple films on physical properties and antimicrobial activities. *Journal of Food Science*, 74, 372-378.
- Dufresne, A. (2008). Polysaccharide nanocrystal reinforced nanocomposites. *Canadian Journal of chemistry*, 86, 484-494.

- Erdohan, Z. Ö., Çam, B., & Turhan, K. N. (2013). Characterization of antimicrobial polylactic acid based films. *Journal of Food Engineering*, 119, 308-315.
- Goñi, P., López, P., Sánchez, C., Gómez-Lus, R., Becerril, R., & Nerín, C. (2009). Antimicrobial activity in the vapor phase of a combination of cinnamon and clove essential oils. *Food Chemistry*, 116, 982-989.
- González, A., & Igarzabal, C. I. A. (2013). Soy protein-Poly (lactic acid) bilayer films as biodegradable material for active food packaging. *Food Hydrocolloids*, 33, 289-296.
- Gutierrez, J., Barry-Ryan, C., & Bourke, P. (2008). The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology*, 124, 91-97.
- Hosseini, S. F., Rezaei, M., Zandi, M., & Farahmandghavi, F. (2016). Development of bioactive fish gelatin/chitosan nanoparticles composite films with antimicrobial properties. *Food Chemistry*, 194, 1266-1274.
- [Http://www.carbios.fr/en/press-releases/show/52](http://www.carbios.fr/en/press-releases/show/52). (November, 2014).
- ICMFS. (1986). International Commission on Microbiological Specifications for Foods, Sampling for Microbiological Analysis: Principles and Scientific Applications, second ed., vol. 2. University of Toronto Press, Toronto.
- Jamshidian, M., Tehrany, E. A., Imran, M., Akhtar, M. J., Cleymand, F., & Desobry, S. (2012). Structural, mechanical and barrier properties of active PLA-antioxidant films. *Journal of Food Engineering*, 110, 380-389.
- Jamshidian, M., Tehrany, E. A., Imran, M., Jacquot, M., & Desobry, S. (2010). Polylactic acid: production, applications, nanocomposites, and release studies. *Comprehensive Reviews in Food Science and Food Safety*, 9, 522-571.

- Li, H., & Huneault, M. A. (2007). Effect of nucleation and plasticization on the crystallization of poly(lactic acid). *Polymer*, 48, 6855-6866.
- Llana-Ruiz-Cabello, M., Pichardo, S., Jiménez-Morillo, N. T., Bermúdez, J. M., Aucejo, S., González-Vila, F. J., et al. (2015). Molecular characterisation of a bio-based active packaging containing *Origanum vulgare* l. Essential oil using pyrolysis gas chromatography-mass spectrometry. *Journal of the Science of Food and Agriculture*. doi: 10.1002/jsfa.7502.
- Llana-Ruiz-Cabello, M., Pichardo, S., Baños, A., Núñez, C., Bermúdez, J. M., Guillamón, E., et al. (2015). Characterisation and evaluation of PLA films containing an extract of *Allium* spp. to be used in the packaging of ready-to-eat salads under controlled atmospheres. *LWT-Food Science and Technology*, 64, 1354-1361.
- Mascheroni, E., Guillard, V., Nalin, F., Mora, L., & Piergiovanni, L. (2010). Diffusivity of propolis compounds in Polylactic acid polymer for the development of anti-microbial packaging films. *Journal of Food Engineering*, 98, 294-301.
- Mexis, S. F., Chouliara, E., and Kontominas, M. G. (2009). Combined effect of an oxygen absorber and oregano essential oil on shelf life extension of rainbow trout fillets stored at 4 °C. *Food Microbiology*, 26, 598-605.
- Nostro, A., Blanco, A.R., Cannatelli, M.A., Enea, V., Flamini, G., Morelli, I., et al. (2004). Susceptibility of methicillin-resistant staphylococci to oregano essential oil, carvacrol and thymol. *FEMS Microbiology Letters*, 230, 191-195.
- Pelissari, F. M., Grossmann, M. V., Yamashita, F., & Pineda, E. A. G. (2009). Antimicrobial, mechanical, and barrier properties of cassava starch-chitosan films incorporated with oregano essential oil. *Journal of Agricultural and Food Chemistry*, 57, 7499-750.

- Péroval, C., Debeaufort, F., Despre, D., Voilley, A., 2002. Edible arabinoxylan-based films. 1. Effects of lipid type on water vapour permeability, film structure, and other physical characteristics. *Journal of Agricultural and Food Chemistry*, 50, 3977-3983.
- Ramos, M., Jimenez, A., Peltzer, M., & Garrigos, M. C. (2014). Development of novel nano-biocomposite antioxidant films based on poly (lactic acid) and thymol for active packaging. *Food Chemistry*, 162, 149-155.
- Rezaei, M., & Hosseini, S. F. (2008). Quality assessment of farmed rainbow trout (*Oncorhynchus mykiss*) during chilled storage. *Journal of Food Science*, 73, 93-96.
- Rhim, J. -W., Mohanty, A. K., Singh, S. P., & Ng, P. K. W. (2006). Effect of the processing methods on the performance of polylactide films: thermocompression versus solvent casting. *Journal of Applied Polymer Science*, 101, 3736-3742.
- Roos, Y., & Karel, M. (1991). Plasticizing effect of water on thermal behavior and crystallization of amorphous food models. *Journal of Food Science*, 56, 38-43.
- Sallam, K. I. (2007). Antimicrobial and antioxidant effects of sodium acetate, sodium lactate, and sodium citrate in refrigerated sliced salmon. *Food Control*, 18, 566-575.
- Sánchez-González, L., González-Martínez, C., Chiralt, A., & Cháfer, M. (2010). Physical and antimicrobial properties of chitosan-tea tree essential oil composite films. *Journal of Food Engineering*, 98, 443-452.
- Sánchez Aldana, D., Andrade-Ochoa, S., Aguilar, C. N., Contreras-Esquivel, J. C., & Nevárez-Moorillón, G. V. (2015). Antibacterial activity of pectic-based edible films incorporated with Mexican lime essential oil. *Food Control*, 50, 907-912.
- Sikkema, J., De Bont, J., & Poolman, B. (1995). Mechanisms of membrane toxicity of hydrocarbons. *Microbiological reviews*, 59, 201-222.

- Siracusa, V., Rocculi, P., Romani, S., & Rosa, M. D. (2008). Biodegradable polymers for food packaging: a review. *Trends in Food Science & Technology*, 19, 634-643.
- Soković, M., Glamočlija, J., Marin, P. D., Brkić, D., & van Griensven, L. J. L. D. (2010). Antibacterial effects of the essential oils of commonly consumed medicinal herbs using an in vitro model. *Molecules*, 15, 7532-7546.
- Tawakkal, I. S. M. A., Cran, M. J., Bigger, S. W. (2016). Release of thymol from poly(lactic acid)-based antimicrobial films containing kenaf fibres as natural Filler. *LWT - Food Science and Technology*, 66, 629-637.
- Teixeira, B., Marques, A., Pires, C., Ramos, C., Batista, I., Saraiva, J. A., et al. (2014). Characterization of fish protein films incorporated with essential oils of clove, garlic and origanum: physical, antioxidant and antibacterial properties. *LWT - Food Science and Technology*, 59, 533-539.
- Teixeira, B., Marques, A., Ramos, R., Neng, R. N., Nogueira, M. F. J., Saraiva, J. A., et al. (2013). Chemical composition and antibacterial and antioxidant properties of commercial essential oils. *Industrial Crops and Products*, 43, 587-595.
- Ultee, A., Bennik, M. H. J., & Moezelaar, R. (2002). The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Applied and Environmental Microbiology*, 68, 1561-1568.
- Qin, Y., Yang, J., & Xue, J. (2015). Characterization of antimicrobial poly(lactic acid)/poly(trimethylene carbonate) films with cinnamaldehyde. *Journal of Materials Science*, 50, 1150-1158.

- Volpe, M. G., Siano, F., Paolucci, M., Sacco, A., Sorrentino, A., Malinconico, M., et al. (2015). Active edible coating effectiveness in shelf-life enhancement of trout (*Oncorhynchus mykiss*) fillets. *LWT - Food Science and Technology*, 60, 615-622.
- Xiao, H., Lu, W., & Yeh, J. -T. (2009). Crystallization behavior of fully biodegradable poly(lactic acid)/poly(butylene adipate-co-terephthalate) blends. *Journal of Applied Polymer Science*, 112, 3754-3763.
- Zhang, Y., Rempel, C., & McLaren, D. (2014). Edible Coating and Film Materials: Carbohydrates. In J. H. Han (Eds.), *Innovations in Food Packaging* (pp.133-170). San Diego: Elsevier Academic Press.

Fig. 1.

(i)

(ii)

(a)

(b)

(c)

Fig. 2.

Fig. 3.

Fig. 4.

Figure captions

Fig. 1. DSC thermograms of (a) neat PLA and PLA-based films containing (b) 0.5, and (c) 1.5% (w/w) oregano essential oil.

Fig. 2. SEM micrographs of (a) neat PLA film, and PLA-based films containing (b) 0.5, and (c) 1.5% (w/w) oregano essential oil for (i) surface and (ii) cross-section.

Fig. 3. AFM topographic images of (a) neat PLA, and PLA-based films containing (b) 0.5, and (c) 1.5% (w/v) oregano essential oil. Mean values with different superscript in small letters for R_a and in capital letters for R_q are significantly different ($p < 0.05$).

Fig. 4. Total viable counts (TVC) (a), psychrotrophic bacteria count (PTC) (b), lactic acid bacteria (LAB) (c), and *Enterobacteriaceae* (d) of unwrapped trout samples (Control), PLA film wrapped samples (PLA) and samples wrapped with PLA film containing oregano essential oil (PLA-OEO). Points represent mean values of three determinations $\pm$ standard error.

Table 1

Chemical composition of volatiles
in oregano essential oil.

Components	RT/min	(%)
α -Pinene	3.95	0.36
β -Pinene	4.51	0.64
Limonene	4.98	0.32
<i>p</i> -Cymene	5.07	4.07
1,8-Cineole	5.18	1.24
γ -Terpinene	5.49	2.26
Linalool	5.95	1.52
Camphene	6.59	0.69
Endo-borneol	6.82	0.75
4-Terpinene	6.93	0.41
α -Terpineol	7.07	0.36
Thymol	8.05	3.30
Carvacrol	8.16	81.85
Myrcene	9.04	0.42
Caryophyllene	9.43	1.44
Linalyl acetate	9.73	0.36

Table 2

Tensile strength (TS), elongation-at-break (EAB), elastic modulus (EM), water vapor permeability (WVP) and contact angle (CA) of PLA-based films containing different amounts of oregano essential oil.

OEO (% w/w)	TS (Mpa)	EAB (%)	EM (Gpa)	WVP (g mm/kPa h m ²)	CA (°)
0 (neat PLA)	47.18 ± 6.01 ^a	2.82 ± 0.46 ^b	1.39 ± 0.15 ^a	0.068 ± 0.001 ^a	61.20 ± 0.81 ^c
0.5	18.66 ± 1.39 ^b	16.78 ± 2.85 ^a	0.79 ± 0.00 ^b	0.045 ± 0.003 ^b	83.46 ± 1.55 ^b
1	18.36 ± 0.75 ^b	15.45 ± 0.98 ^a	0.77 ± 0.03 ^b	0.051 ± 0.002 ^{bc}	93.10 ± 0.87 ^a
1.5	15.73 ± 1.10 ^b	14.18 ± 0.77 ^a	0.57 ± 0.04 ^b	0.062 ± 0.007 ^a	94.03 ± 0.45 ^a

Values are presented as means ± standard deviation. Different letters in the same column indicate significant differences ($p < 0.05$).

Table 3

MIC and MBC values of oregano essential oil against four tested bacterial strains.

Bacteria	<i>S.aureus</i>	<i>L. monocytogenes</i>	<i>E.coli</i>	<i>S. enteritidis</i>
MIC (µl/ml)	0.125	0.2	0.5	0.2
MBC (µl/ml)	0.3	0.4	1.25	0.4

Table 4

Antibacterial activity of PLA-based films containing different amounts of oregano essential oil in overlay and vapor phase test.

OEO (% w/w)	Inhibition Area (mm ²)			
	<i>S.aureus</i>	<i>L. monocytogenes</i>	<i>E.coli</i>	<i>S. enteritidis</i>
<u>Direct contact</u>				
0 (neat PLA)	ND ^{*c}	ND ^c	ND ^b	ND ^c
0.5	28.8 ± 0.1 ^c	7.6 ± 0.1 ^c	ND ^b	4.4 ± 0.1 ^c
1	428.6 ± 0.2 ^b	43.2 ± 0.3 ^b	42.1 ± 0.3 ^b	278.9 ± 0.3 ^b
1.5	722.5 ± 0.1 ^a	239.2 ± 0.2 ^a	230.5 ± 0.3 ^a	710.2 ± 0.3 ^a
<u>Vapor-phase</u>				
0 (neat PLA)	ND ^b	ND ^b	ND ^b	ND ^b
0.5	ND ^b	3.4 ± 1.01 ^b	ND ^b	ND ^b
1	35.1 ± 0.4 ^b	49.5 ± 0.3 ^b	7.6 ± 0.4 ^b	17.0 ± 0.2 ^b
1.5	248.6 ± 0.8 ^a	148.9 ± 0.20 ^a	147.8 ± 0.1 ^a	241.3 ± 0.8 ^a

Values are presented as means ± standard deviation. Different letters in the same column indicate significant differences ($p < 0.05$).

* ND: Not detected.