J Food Sci Technol
https://doi.org/10.1007/s13197-020-04668-z

ORIGINAL ARTICLE

Oregano essential oil addition in rice starch films and its effects on the chilled fish storage

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Revised: 8 July 2020 / Accepted: 31 July 2020

Abstract Active packaging produced from biodegradable polymers and essential oil could have a great value to food industries. This study aimed to evaluate the effect of the addition of oregano essential oil (OEO) on rice starch films and its application as packaging for fish fillets. Several concentrations of OEO were added to the films, the film added with 4.5% OEO showed lower permeability to water vapor 3.7 g mm kPa^{-1} m^{-2} day^{-1}, intermediate solubility of 24% and high tensile strength (4.4 MPa) while the standard film (without the addition of OEO) presented 8.8 g mm kPa^{-1} m^{-2} day^{-1}, 25.8% and 2.2 MPa, respectively. Packed fish fillets with the active film showed an increase in its shelf life when compared to the standard film. Packaged fish fillets in OEO films showed greater resistance (13.4 N), less oxidation (1.65 mg malonaldehyde/kg of sample) and less microbiological growth 10^7 CFU/g in 6 days of storage, while packaged fish fillets in standard film showed lower resistance (10.4 N), higher oxidation (1.88 mg malonaldehyde/kg of sample) and higher microbiological growth 10^8 CFU/g. Thus, the active packaging developed had the capacity to increase the shelf life of a perishable product that has great interest of food industries.

Keywords Active packaging · Antimicrobial · Antioxidant · Mechanical properties · Shelf life

Introduction

Plastic films show wide application in the food industry because of theirs chemical properties and mechanical resistance keeping the safety of packaged foods. In general, the most commonly used polymers in films are synthetic based but they are associated with serious environmental problems (Uranga et al. 2019). The development of packaging formulated from renewable and biodegradable polymers becomes a way to reduce environmental aggressions (Lee et al. 2015a). Polymers such as proteins, carbohydrates and lipids can be used to prepare films. The characteristics of the films produced are mainly related to the type of polymer used (Lee et al. 2015b).

Starch is a polysaccharide composed mainly of amylose and amylopectin. Although, both are formed by bonds of glucose monomers, what make them different are the linear and branched bonds. Amylose is responsible for the formation of stronger gels and more easily form hydrogen bonds capable of producing films with higher mechanical properties. However, the films properties remain lower when compared to synthetic, being necessary the use of other compounds with higher capacity to supply the deficiencies of these polymers (Wang et al. 2017). The addition of lipids as essential oils increases the quality of the formed film reducing permeability and also the solubility of the material (Pires et al. 2011). Besides modifying the physical properties, the essential oil confers chemical properties to the materials, being responsible for making the packagings active due to their chemical compositions (Burt 2004; Lee et al. 2016).

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13197-020-04668-z) contains supplementary material, which is available to authorized users.

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Published online: 07 August 2020

Oregano essential oil is obtained from *Origanum vulgaris* and it is considered a natural antimicrobial and antioxidant with potential use in conservation processes (Olmedo et al. 2014). Oregano essential oil is considered GRAS and can be used in food products (FDA 2015). The oregano essential oil (OEO) presents in its composition constituents such as tymol and carvacrol. It is believed that such compounds are responsible for providing these extracts bioactivities with antimicrobial and antioxidant action. Its use may be limited due to the intense aroma that may decrease the acceptance if added in several products. A way to use OEO would be as an additive in food packaging, thus contributing to bioactivities during food preservation and minimizing undesirable aroma (Lee et al. 2016; Muriel-Galet et al. 2015).

Catfish (*Genidens genidens*) is a fish that occurs in marine, estuarine and freshwater environments, generally is abundant in coastal waters with muddy and shallow bottom. It is native to South America and in general its capture can represent up to 28,781 t (Maciel et al. 2020). Popularly catfish can be found in Lagoa dos Patos, Brazil and its capture represents economic importance, mainly for artisanal fishing, reaching 29% of the captures in the South region of Brazil. (Walter et al. 2018). Fresh and processed fish are highly perishable because of the microbiological flora inherent in these products, as well as the contamination acquired during handling. The high perishability of these products is an important risk for the health of the consumers besides causing economic losses. The use of active packaging that can increase the shelf life of this product and minimize contamination is of great interest to the food industry (Wu et al. 2014).

In this context, the literature presents several studies on the elaboration of films containing bioactive compounds such as Akhter et al. (2019) that produced starch and pectin films with rosemary and mint essential oils, Gómez-Estaca et al. (2010) developed gelatin and gelatin/chitosa films containing clove, fennel, cypress, thyme, herb-of-the-cross, pine and rosemary, Lee et al. (2015a) elaborated chicken feet protein films added with marjoram, coriander and clove bud oils, Lee et al. (2016) make red pepper seed meal protein films containing OEO, Muriel-Galet et al. (2015) prepared ethylene vinyl alcohol copolymer films added with OEO and green tea extract, Shen and Kamdem (2015) incorporated citronella and cedarwood essential oils in chitosan films, and Teixeira et al. (2014) applied clove, garlic and origanum in fish films. However, complete studies of elaboration of the film since the extraction of the polymer up to its application in the packaged product are scarce. Therefore, this study aimed to verify the effect of OEO addition on films produced with rice starch and to apply them as active packaging in chilled fish fillets.

Materials and methods

Material

The white rice (*Oryza sativa* L.) and the fish (*Genidens genidens*) were obtained on local market of Rio Grande—RS. The OEO used was obtained from PETITE MARIE with refractive index 1.3644 and density 0.9475 g/mL.

Starch extraction

Rice starch was extracted according to the method described by Wang and Wang (2004). The white rice was grounded and soaked in 0.1% NaOH (1:2 m/v) for 18 h. The filtrate was centrifuged (Hitachi CT6EL, Taiwan) at $1400\times g$ for 10 min. The solid fraction was re-slurried in NaOH 0.1% (1:1 m/v) and centrifuged. After, solid fraction was washed twice with distilled water, centrifuged and neutralized to pH 6.5 (HCl 0.1 M). The starch was washed twice with distilled water and centrifuged, again. Thus, the starch was dried in air circulation oven (Fanem 520, Brazil) at 50 °C for 8 h.

Antioxidant activity

The antioxidant activity of OEO was verified by the reduction of the free DPPH radical (2,2-diphenyl-1-picrylhydrazyl) and the ABTS radical (2,2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid)). For the evaluation of the antioxidant activity in the films only DPPH method was used.

The antioxidant activity of OEO determined by DPPH radical-scavenging was evaluated according to a method described by Rufino et al. (2007a). The OEO was diluted in methyl alcohol in a concentration of 2000 µg/mL. The control solution was prepared with methyl alcohol and the DPPH radical. The percentual of inhibition (I) was evaluated according to Eq. (1):

$$\mathrm{I}(\%) = \frac{(\mathrm{Abs\ c} - \mathrm{Abs\ a})}{\mathrm{Abs\ c}} \times 100 \quad (1)$$

where Abs c is the absorbance of the control for a determined time; and Abs a is the absorbance of the sample at the same times.

The antioxidant activity by the ABTS radical (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) was measured according to the method proposed by Rufino et al. (2007b). A dilution of 500 µg/mL OEO in methyl alcohol was prepared and the control solution was prepared using methyl alcohol and ABTS radical. The percentual of inhibition was calculated according to the equation metioned above.

The antioxidant activity of the films was also determined by the DPPH method, using the methodology described by Maniglia et al. (2014). The antioxidant activity of films was determined using Eq. 1 mentioned above.

Antimicrobial activity

The antimicrobial activity of OEO was determined using the agar dilution method according to the official method M7-A6 NCCLS (2003a). The microorganisms evaluated were *Salmonella enteritidis*, *Escherichia coli* and *Staphylococcus coagulase positive*.

The antimicrobial activity of the films was verified through the halo test method, according to the M2-A8 standard method NCCLS (2003b). It was used a disc of 15 mm of the films. The halos formed in this period were measured in mm using a pachymeter.

Films elaboration

The films were formulated with 3% of rice starch (m/v), 30% of glycerol and 0, 4, 4.5, 5, 6 and 7% (related to the solid content) of OEO. Starch was dissolved in distilled water and the solution was heated at 80 °C under mechanical agitation (Fisatom 713D, Brazil). The glycerol was added and the solution was stirred for 20 min. The filmogenic solution was cooled down to 35 °C and OEO was added and stirred for 20 min. The mixture was placed in Petri dishes and dried in air circulation oven (Biopar S150BA, Brazil) at 40 °C for 8 h. After drying, the films were maintained 24 h in desiccators at 25 °C with relative humidity of 52%.

Films characterization

Opacity

The film opacity was determined using a colorimeter (CR-400 Chroma Meter, Japan). The opacity is the relation between measures in different points of the same film over white and black standard.

Mechanical properties

The mechanical properties were determined using a texture analyzer (TA. XT plus, United Kingdom) based in ATSM D-882-02 official method (ASTM 2002). The thickness was measured using a micrometer and standardized in ± 80 μm, using 15 mL of filmogenic solution in each plate. The tensile strength (TS) and Elongation (E) were determined using the equations:

$$\mathrm{TS(MPa)} = \frac{\mathrm{F}}{\mathrm{A}} \tag{2}$$

$$\mathrm{E}\ (\%) = \frac{\mathrm{df}}{\mathrm{dig}} \times 100 \tag{3}$$

Where F is maximum force (N); A area of strips (m^2); df is final elongation distance (mm); and dig is initial distance between grips (mm).

Solubility in water

The solubility was determined according to the method described by Gontard et al. (1994). The films were cut in squares of 4 cm^2 and dried in oven (DeLeo A15E, Brazil) at 105 °C to measure the initial dry weight. After, the films were immersed in 50 mL of distilled water and were shaken at 175 rpm for 24 h. The films were dried in oven at 105 °C to determine the final dry weight. The equation below was used to determine the solubility (S) in water.

$$\mathrm{S}(\%) = \frac{(\mathrm{Wi} - \mathrm{Wf})}{\mathrm{Wi}} \times 100 \tag{4}$$

where Wi is final dry weight (g); and Wf is initial dry weight (g).

Water vapor permeability (WVP)

The water vapor permeability was determined according to the ASTM E96 standard method (ASTM 1995). The cell was added of anhydrous calcium chloride and closed with the film, blocking the air pass. The permeation cells were conditioned in desiccators with a saturated sodium chloride solution (relative humidity of 75%) and weighted each 24 h for 7 days retirar. The WVP (g mm kPa^{-1} m^{-2} - day^{-1}) was calculated through the equation:

$$\mathrm{WVP} = \frac{(\mathrm{W.T})}{(\mathrm{A.t.}\Delta\mathrm{P})} \tag{5}$$

where W is the weight gain of de cell (g); T is the thickness of de film (mm); A is the permeation area (m^2); t is the time of weight gain (d); and ΔP is the vapor pressure difference across the film (kPa).

Morphology

Films were examined using a scanning electron microscope (JEDL JSM-6610 LV, Japan) (SEM) at 1000 × and electron acceleration of 5 kV. The films were covered with a gold layer (Sputter Coater, SCDO50) to increase the conductivity.

Thermal degradation (TG)

The thermal degradation was determined in thermogravimetric analyzer (Shimadzu DTG 60, Japan) in static atmosphere of air with constant flow rate of 20 mL/min using aluminum pans. The amount of sample used was 3 mg and the temperature range studied was 30–500 °C with a temperature gradient of 10 °C/min.

Fish fillets storage conditions

Two different films were used to pack the fish fillets, the control film (3% starch and 30% glycerol) and the film with 3% starch, 30% glycerol and 4.5% of OEO. The fish selected for the application was the *Genidens genidens*. The fish was filleted, in this stage, the scales, skin, vices, head, tail and spine were removed, only the muscle was kept to use. Amoung of 5 g of fish fillets were packaged using two films of the same treatment, one film at the top and other at the bottom to cover the fish fraction. The packaging containing the fish fraction was sealed using a hot press at 120 °C in order to join the sides of the packaging. For each treatment, 100 g of fish fillets was packaged in total, so it was possible to use the necessary amount of fish fillets for each day of analysis during the 10 days of storage. The fish fillets packaged was kept at 4 °C in a refrigerator. The shelf life study was determined in triplicate at 0, 2, 4 and 6 days of chilled storage (Zinoviadou et al. 2009).

Color

Color parameters of fish fillets were determined using a colorimeter. The parameters measured were luminosity (L), chromaticity a* and b*.

Texture

The texture of fish fillets were evaluated in texture analyzer (TA. XT plus, United Kingdom) using the method described by Jonsson et al. (2001). The fish fillet texture was measured by the shear strength.

Weight loss

The weight loss of packed fish fillets were determined by daily weighing of the same fraction of the packed fish fillets over the storage period.

Determination of total volatile bases nitrogen (TVB-N) and thiobarbituric acid (TBA)

The TVB-N was determined according to described by AOAC (2000). It was used 50 g of fish fillets homogenized with trichloroacetic acid (TCA) 7.5%. The equation below was used to calculate the TVB-N (mg N/100 g).

$$\mathrm{TVB{-}N} = \frac{(\mathrm{VHClu} - \mathrm{VHClb}) \times (\mathrm{N\ HCl} \times 14.01 \times 100)}{5} \quad (6)$$

where V HClu is the volume (mL) of HCl used in the titled sample; V HClb is the volume (mL) used in titled blank; and N HCl is the molarity of HCl.

The TBA was performed according to the method described by AOAC (2000).

Microbiological analyzes

The microorganism determination was examined according to the method described by Zinoviadou et al. (2009). The fish fillets were aseptically homogenized and diluted 10^{-1} to 10^{-6}. Aliquots of 0.1 mL of the dilutions were inoculated separately on the surface of plate count agar (PCA) and incubated at 25 °C for 72 h.

Statistical analysis

All analyses were determined in triplicate with the exception of the mechanical properties that were performed using five replicates. The sample means were compared using ANOVA and Tukey test at 5% significance, except the application film that was compared using T test with 5% significance. The software used was *Statistica* 5.0 (StartSoft Inc., Tulsa, OK, USA), 1999.

Results and discussion

Bioactivity of oregano essential oil

Oregano essential oil was evaluated for its antioxidant capacity through two methods, capturing the radical ABTS and DPPH radical scavenging. The antioxidant action showed an inhibition percentage of 41.7% for the DPPH assay and 15.4% for the ABTS assay. According to Lee et al. (2016) the antioxidant activity of OEO is attributed to the presence of phenolic compounds present in OEO composition. Based on the data of antioxidant activity observed for OEO, this oil has a superior ability to act as an inhibitory agent of propagation stage at the oxidation process.

The OEO was able to inhibit 100% of the microbial growth of the microorganisms *Escherichia coli*, *Staphylococcus coagulase positva* and *Salmonella enteritides* at the concentration of 10^8 CFU/mL. Muriel-Galet et al. (2015) attribute the antimicrobial effect of OEO to carvacrol present in its formulation, which has the ability to destabilize the cell membrane of microorganisms.

The antimicrobial activity of OEO is associated with a series of chemical compounds present in the composition. Among all compounds, phenols specifically act causing a cytoplasmic membrane disturbance, electron flow disruption, proton motor force, active transport and coagulation of cell content. Another important factor is that the essential oils are hydrophobic in nature which provide partitioning of the microbial cell membrane causing cell destabilization and making it more permeable (Burt 2004). The antimicrobial activity of essential oils can also be related to other mechanisms of action, such as disruption of enzymatic systems, impairment of genetic material from microorganisms and formation of fatty acid hydroperoxidase (Chiralt and Atar 2016).

Films characterization

The use of OEO as additive for biodegradable packaging can give these packages different bioactivities such as antimicrobial and antioxidant. However, OEO present has lipidic nature and the addition of lipid compounds in polysaccharide films alters the properties of the films by making them less permeable, soluble and more flexible (Lee et al. 2015b).

Opacity

The opacity of the studied films did not present significant difference with the increase of OEO concentration (Table 1). According to the type of application made the addition of oil may influence positively or negatively. Biodegradable film with similar characteristics to the commercial ones requires low levels of opacity, in this way the addition of OEO to the films is not favorable. In the other hand, higher levels of opacity are desirable when the film application is made in a product that demands greater protection against the incidence of light. The lower light transfer to the packaged product decreases the oxidative process. In this research, the high opacity is better, because the fish fillets used for application showed a higher lipid content (9.7% ± 0.58, data not show). According to Fakhouri et al. (2007) factors such as concentration of amylose, glycerol and lipids can alter the opacity levels. In this study only the lipid concentration was varied and the concentrations used were not able to promote changes in the film opacity contents.

Water vapor permability and solubility

The films containing 4.5% of OEO presented the lowest WVP (3.7 g mm kPa^{-1} m^{-2} day^{-1}) (Table 1). According to Zinoviadou et al. (2009) the addition of lipids to the polymer matrices is responsible for several changes such as the reduction of the WVP of the films. In this study, when comparing the standard film with the films containing OEO, it can be seen that the addition of this lipid was responsible for a significant reduction of WVP. However, analyzing only OEO containing formulations the increase in lipid concentrations was not able to consequently decrease WVP. Such behavior can be explained by the difficulty of homogenizing OEO in the film forming solution, so it can be distributed in different ways in the film and modify the characteristics of the polymer chain. Pires et al. (2011) and Wu et al. (2014) mention that OEOs are complex hydrophobic mixtures contributing to increase the hydrophobicity of the films, consequently making homogenization difficult. WVP generally occurs through the hydrophilic portion of the polymer chain and the addition of OEO, even at small concentrations, limits this transference.

Table 1 Characterization of the starch films added with different concentrations of oregano essential oil

Test	OEO (%)	OP (%)	WVP (g mm kPa^{-1} m^{-2} day^{-1})	S (%)	TS (MPa)	E (%)
1	0	$13.9^{a} \pm 0.1$	$8.8^{a} \pm 0.5$	$25.8^{a} \pm 0.6$	$2.2^{c} \pm 0.3$	$138.1^{e} \pm 1.7$
2	4	$14.2^{a} \pm 0.2$	$6.0^{b} \pm 1.4$	$23.8^{b} \pm 1.1$	$3.4^{b} \pm 0.6$	$145.2^{d} \pm 1.7$
3	4.5	$14.1^{a} \pm 0.2$	$3.7^{c} \pm 0.8$	$24.0^{a,b} \pm 1.0$	$4.4^{a} \pm 0.6$	$137.9^{e} \pm 1.3$
4	5	$13.9^{a} \pm 0.2$	$5.9^{b} \pm 1.5$	$19.8^{c} \pm 1.0$	$4.2^{a} \pm 0.3$	$151.1^{c} \pm 1.9$
5	6	$14.1^{a} \pm 0.1$	$5.6^{b} \pm 1.2$	$20.1^{c} \pm 1.1$	$4.7^{a} \pm 0.4$	$178.3^{b} \pm 1.3$
6	7	$14.2^{a} \pm 0.2$	$5.8^{b} \pm 0.8$	$19.0^{c} \pm 1.3$	$4.1^{a} \pm 0.8$	$189.4^{a} \pm 1.5$

Values with different letters in the same column are significantly different ($p < 0.05$)

OEO oregano essential oil, *OP* opacity, *TS* tensile strength, *E* elongation, *WVP* water vapor permeability, *S* solubility

The solubility of the films is also related to WVP. The lipids addition increases the hydrophobic character of the polymer matrix directly. The solubility of the studied films showed a decrease when the OEO concentration was increased. From 5% of OEO, significant changes were observed in relation to solubility. Although the additions of 5, 6 and 7% of OEO increased the hydrophobicity of the film, although part of the OEO added was not effectively incorporated into the films. It is believed that the amount of OEO used was higher than the oil incorporation capacity of film forming solution. Pires et al. (2011) elucidate that the addition of OEO reduces the water absorption by the films, consequently reducing the solubility. According to Fakhouri et al. (2007) solubility is a parameter of great importance, and can indicate the application of the formed films. Packaging with high water solubility may also be desirable in some cases, such as packaging used for filling pre-prepared foods, so that during preparation the packaging may be solubilized together with the food. However, for applications in foods with high moisture content, packaging with low water solubility are indicated, thus the packaging protects the food and does not degrade due to contact with water.

Mechanical properties

The tensile strength is an important parameter in the films production, because they must have resistance to handling. Brittle films are difficult to apply, once they would expose food and wouldn't accomplish with the objective of acting as a protective barrier. The tensile strength indicated that the addition of OEO increased the strength of the film (Table 1). The standard film had the lowest tensile strength value (2.2 MPa), whereas the addition of 4.5% of OEO the tensions showed values higher than 4 MPa.

Pola et al. (2016) and Wang et al. (2011) commented that, in general, the increase of OEO addition to the films promotes reduction in the tensile strength. This can be attributed to the disintegration of the microstructure of the film chain caused by the addition of the lipid. The addition of poorly soluble material in the matrix makes the film discontinuous and uneven, reducing the strength of the material due to lack of cohesion. In general, the oils added in the films are in the liquid phase, so the droplets of the oils that have not been homogenized in the film matrix promote weaker bonds in the polymer creating possible break points. However, such behavior was not verified in this study. The addition of OEO was responsible for almost doubling the resistance values compared to the standard film, indicating that the OEO bound strongly to the polymer matrix in order to interfere in the mechanical properties. According to Akhter et al. (2019) polyphenols tend to make hydrogen bonds with the hydroxyls present in the carbohydrate structure, thus increasing the interfacial adhesion between OEO and starch which makes the material more resistant.

The elongation showed in Table 1 indicated that the increase of OEO in the film formulation increased the elongation capacity of the films, with the exception of test 3 which showed the same elongation as the standard. Comparing the standard film with the film containing 7% of OEO, the elongation increased approximately 50%. The increased flexibility of films due to the addition of essential oils has also been verified by other authors in the literature (Pola et al. 2016 and Wang et al. 2011). The increased extensibility of polysaccharide films through the addition of essential oils is due to the strong intermolecular interactions of the polymer chains with the essential oils fatty acids. The bonds reduce free volume and increase the mobility of polysaccharide chains (Akhter et al. 2019).

The studied films with concentrations of 5, 6 and 7% of OEO, though they have shown to have high elongation at break, were not selected for application because the surface of the film was slightly oily. Although the film has been formed, it is believed that a small part of the added oil has migrated to the surface of the film. Regarding the results of optical, mechanical and microstructural properties, permeability to water vapor and solubitity, the film containing 4.5% OEO was similar to the other treatments in terms of opacity and solubility. However, it presented a lower WVP (3.7 g mm kPa^{-1} m^{-2} day^{-1}) compared to the others and a high tensile strength (4.4 MPa). Films with greater resistance and lower WVP can be applied to products with higher humidity, such as fish fillets, as they are able to maintain the characteristics of the products and preserve their shelf life.

Morphology

The SEM was performed with 1000 × approximation and with an incidence of 5 kV, due to the fragility of OEO added films, which degraded when the incidence of electrons was concentrated in a specific part of the film, changing the real characteristics of the films. Both micrographs of the evaluated films exhibited irregular, roughness and a lack of homogeneity, besides presenting pores in its structure. However, when comparing the figures, Fig. 1a presents a surface with less surface roughness and cracks than Fig. 1b. The incorporation of OEO in the films can increase the homogeneity of the films and promote more interaction among polymer, plasticizer and additive making the films more homogeneous and less porous. According to Medina-Jaramillo et al. (2017) packages that present greater homogeneity and surfaces, such as less pores and cracks, may show more efficient mechanical and barrier properties. According to Romani et al. (2017) this

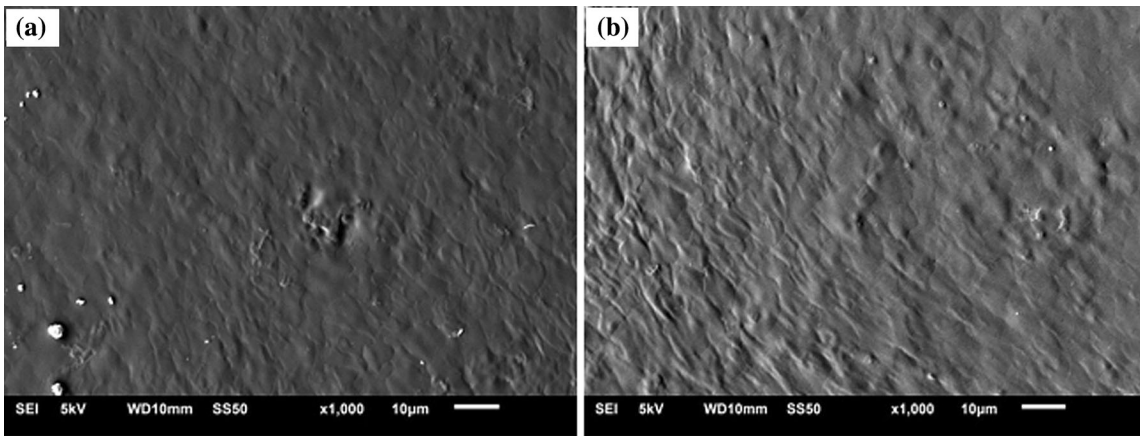

Fig. 1 Micrography of the films **a** added of oregano essential oil and **b** standard film

effect can be attributed to the strong intermolecular interaction, entanglement and continuous phase formation of the polymeric matrix, and also improved interfacial interactions among the components of the film and the essential oil.

In general, it is believed that the starch films obtaining process was not able to fully gelatinize the starch and to promote a complete homogenization with both glycerol and oil, generating the roughness and granules present on the surface. According to Vicentino et al. (2011) the presence of roughness on the surface indicates that there was no good interaction between the phenolic compounds and fatty acid chains presents in OEO, which is not interesting when it is desired to obtain films with good barrier properties.

Thermogravimetry (TG)

The TG curve obtained for OEO showed that the oil was fully degraded before it reaches 150 °C (Fig. 2a). OEO is composed of a several of volatile compounds that begin to degraded at temperatures below 100 °C. The TG of the starch showed small degradation at temperatures around 100 °C, resulting from the liberation of water contained in the matrix. The greatest thermal degradation with a weight loss of 50% was suffered by the starch around 320 °C. When the films elaborated in this study were observed, they showed maximum degradation between 280 and 320 °C. The behavior of the TG curves of the films was similar to the starch, since this is the constituent polymer of the films. However, for both films a decrease of weight loss was observed before the maximum degradation temperature (280 °C) due to the degradation of the constituents of the films (water, glycerol and OEO in the film that contained the additive).

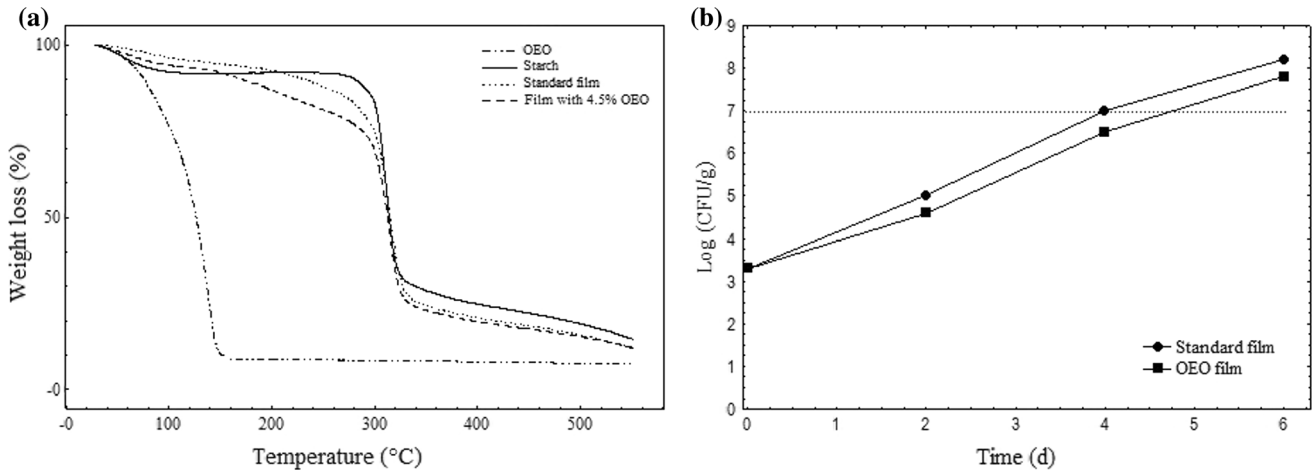

Fig. 2 **a** Thermogravimetry of oregano essential oil, rice starch, oregano essential oil film and standard film and **b** microorganism growth curve obtained for packed fish fillets with oregano essential oil films and standard films

According to Lee et al. (2015a) the thermal degradation amplitude of films containing essential oil is lower than the films without these components. The interaction of the polymer with the active compound promotes weaker inter and intramolecular bonds capable to originate a more fragile film and less stable thermally.

Antimicrobial and antioxidant activity of films

The film added with 4.5% of OEO showed 8.5 mm total inhibition halo, when evaluated against a concentration of 1 to 2 $\times$ 10^8 CFU/mL for all tested microorganisms. The antimicrobial and antioxidant activities of the OEOs are attributed to the presence of phenolic compounds in the composition of the oils, such as carvacrol and thymol. These compounds may attack the phospholipid cell membrane of the microorganisms causing increased permeability and cytoplasm flow, or may react with enzymes present on the cell wall of microorganisms (Burt 2004; Wu et al. 2014). In general, gram-positive microorganisms are more susceptible to antimicrobial action than gram-negative microorganisms. This is due to the fact that gram-negatives have an outer membrane that protects the cell wall and makes it difficult for the diffusion of compounds (Burt 2004).

DPPH analysis of OEO films (4.5%) presented a percentage of antioxidant activity of 18.9 $\pm$ 1.3%. The evaluation of the behavior of OEO films in relation to the DPPH radical stability was performed measuring the OEO ability present in the film to donate hydrogen to stabilize the DPPH radical, consequently reducing the oxidative process. OEO is composed by compounds such as rosmarinic acid, caffeic acid, coumaric acid, kerceitna, thymol and carvacrol which are responsible for the antioxidant activity of oregano, then these compounds stabilize the DPPH radical due to hydrogen donation (Boroski et al. 2012). However, when added in polymeric matrices several factors can influence the antioxidant activity of OEO, such as, OEO compatibility with the polymer, chemical composition of OEO, active compounds, release of active components, interactions between components present in the films, among others. In polymeric starch and glycerol bases, the ease of water penetration into the polymer structure facilitates the release of OEO active compounds activating the antioxidant action (Piñeros-Hernandez et al. 2017).

The percentages of inhibition of the DPPH radical obtained when evaluating only the OEO and the OEO film were different. The inhibition generated by the OEO was higher than that obtained with the film containing OEO, although they were evaluated at similar concentrations. OEO in its liquid format become more available to promote oxidative and microbial inhibitions, whereas OEO bound to the polymer matrix may have reduced performance and present change during the film obtaining process.

Teixeira et al. (2014) while studying the preparation of fish protein films added of different essential oils, verified a reduction of the antioxidant activity when this oils were added to the polymer matrix. This behavior can be justified by the partial loss of volatile compounds in the production process of the films and the probable interaction of the oil with the polymers.

Shelf life of chilled fish fillets

The film selected (3% of starch, 30% of glycerol and 4.5% (m/v) of OEO) was applied as an active packaging to extend the shelf life of chilled fish fillets. Figure 3a shows the films selected for application, Fig. 3b the fish used to measure the shelf life and Fig. 3c shows how the films were packaged for shelf life evaluation. During storage, weight loss, color (Table 2), muscle shear force (Table 3), lipid oxidation (TBA) (Table 3) and fish freshness (TBV-N) (Table 3) were evaluated.

The fish used in the application was characterized in terms of its proximal composition (supplementary material, Table S1) and was chosen for the application due to its high lipid content of 9.7%. One of the objectives of the study was to verify a possible action of the OEO added to the starch films in extend the shelf life, then the food product for the application needed to present relevant fat content.

The weight loss was measured during 7 days, thus for packed fish fillets with OEO added film a value of 28.5% $\pm$ 0.5 was obtained and for packed fish fillet with standard film the observed value was 29.4% $\pm$ 1.5. Statistically there was no difference, the addition of OEO was not able to retard the exudation of the product.

The parameter L* (Table 2) refers to the lightness when 0 is black and 100 is white, so after the days of storage both fish fillets decreased their luminosities indicating the browning of the product. However, for fish fillet packed with OEO film, browning was less pronounced than for fish fillet packed with the standard film. Parameter a* (Table 2) represents the color spectrum from green to red. Both treatments had reduced color intensity from red to green, which indicates deterioration of the packaged material. The same was observed for parameter b* (Table 2), which varies from blue to yellow and showed a reduction in the color intensity from yellow to blue (dark), demonstrating the deterioration of the packed material. Cortez-Vega et al. (2012) mention that color parameters are indicative of fish deterioration, since appearance is the main sensory factor observed by consumers at the time of purchase. Lorenzo et al. (2014) further mention that during the storage period the coloring of the meat is altered due to the oxidative degradation suffered by some nitrosopigments, the authors

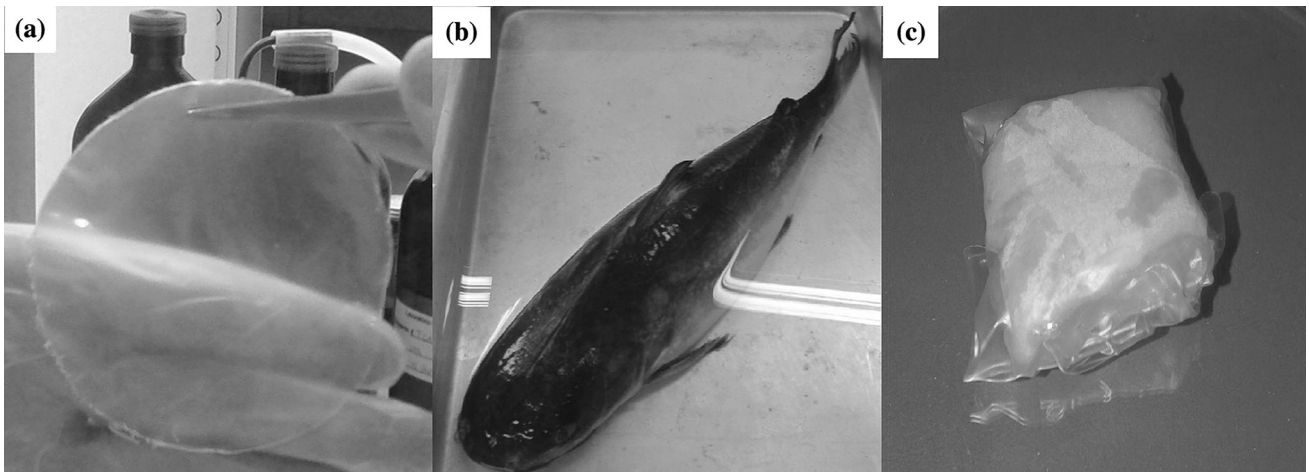

Fig. 3 **a** Film selected for application (3% starch, 30% glycerol and 4.5% OEO), **b** fish used in the application test and **c** application of the film to fish fillets

Table 2 Color evaluation of the packed fish fillets with OEO film and standard film

Days	OEO films			Standard film		
	L*	a*	b*	L*	a*	b*
0	$61.8^{a,A} \pm 3.3$	$14.1^{a,B} \pm 0.7$	$9.7^{a,,C} \pm 1.3$	$61.8^{a,A} \pm 3.3$	$14.1^{a,B} \pm 0.7$	$9.7^{a,C} \pm 1.3$
2	$58.1^{a,A} \pm 4.0$	$9.0^{b,A} \pm 1.3$	$9.7^{a,A} \pm 0.8$	$51.5^{b,B} \pm 1.6$	$8.1^{b,A} \pm 1.3$	$9.9^{a,A} \pm 0.6$
4	$51.8^{b,A} \pm 1.8$	$9.2^{b,A} \pm 0.9$	$7.6^{b,A} \pm 1.4$	$52.8^{b,A} \pm 2.4$	$5.1^{c,B} \pm 0.9$	$7.1^{b,A} \pm 0.7$
6	$48.5^{b,A} \pm 2.5$	$3.5^{c,A} \pm 0.5$	$6.8^{b,A} \pm 0.2$	$42.7^{c,B} \pm 8.6$	$3.3^{d,A} \pm 0.4$	$7.5^{b,A} \pm 0.7$

Values with different lowercase letters in the same column are significantly different ($p < 0.05$) and values for the same analysis with different capital letters in the same line are significantly different ($p < 0.05$)

OEO oregano essential oil

Table 3 Shear force (N), volatile bases nitrogen (TVB-N) values and thiobarbituric acid (TBA) data obtained for the packed fish fillets with OEO film and standard film

Days	Shear force (N)		TVB-N (mg N/100 g of sample)		TBA (mg MA/kg of sample)	
	OEO film	Standard film	OEO films	Standard film	OEO films	Standard film
0	$61.4^{a,A} \pm 3.1$	$61.4^{a,A} \pm 3.1$	$2.56^{a,A} \pm 0.8$	$2.56^{a,A} \pm 0.8$	$0.14^{a,A} \pm 0.01$	$0.14^{a,A} \pm 0.01$
2	$43.0^{b,A} \pm 2.7$	$47.0^{b,A} \pm 1.7$	$2.54^{a,A} \pm 0.7$	$2.92^{a,A} \pm 0.6$	$0.64^{b,A} \pm 0.01$	$0.57^{b,B} \pm 0.01$
4	$25.2^{c,A} \pm 2.8$	$17.9^{c,B} \pm 2.2$	$1.72^{a,A} \pm 0.6$	$1.90^{a,A} \pm 0.6$	$0.86^{c,A} \pm 0.01$	$0.95^{c,B} \pm 0.01$
6	$13.4^{d,A} \pm 1.2$	$10.4^{d,B} \pm 1.0$	$2.33^{a,A} \pm 1.0$	$2.24^{a,A} \pm 0.6$	$1.65^{d,A} \pm 0.01$	$1.88^{d,B} \pm 0.02$

Values with different lowercase letters in the same column are significantly different ($p < 0.05$) and values for the same analysis with different capital letters in the same line are significantly different ($p < 0.05$)

OEO oregano essential oil, *TVB-N* total volatile bases nitrogen, *N* nitrogen, *TBA* thiobarbituric acid, *MA* malonaldehyde

studied the foal meat storage and verified similar results for color behavior.

The shear force (Table 3) of the analyzed packed fish fillets indicates that the fish fillets packed with the standard film showed a higher strength loss than the fish fillets packed with the OEO film over the storage days. According to Suárez-Mahecha et al. (2007) muscle firmness is also an important freshness indicative, and softening indicates deterioration and diminution of meat quality, which can be attributed to an effect of microbiological, enzymatic and oxidative origin. As mentioned by Dhananjayan et al. (2006) the microbial action can cause the resistance loss of the material, due to the use of nutrients for its growth that reduce the resistance of the fiber.

Regarding the TVB-N no significant difference was verified in the days of analysis for the fish fillets packed in

both films (Table 3). Shen and Kamdem (2015) mentioned that the total nitrogen bases are compounds obtained during the process of microbiological deterioration. Usually is used to measure the quality index of fish fillet conservation during the storage period. According to ICMSF (1986) the maximum value allowed for TVB-N is 30 mg N/100 g of sample. The data obtained are within the limit established by the legislation. Authors such as Gonzaga Junior et al. (2015) and Soccol et al. (2005) studied the shelf life of the fish fillets for long time and also did not verify differences in TVB-N contents.

Values below the acceptable limit of TVB-N (30 mg N/100 g) are related to the freshness of the fish fillets due to the low amount of secondary metabolites formed during microbiological growth. Some studies such as Gonzaga Junior et al. (2015) and Reesha et al. (2015) did not associate the increase of TVB-N with microbiological growth above the established limit. Reesha et al. (2015) verified the microbiological degradation of the fish fillets above the allowed limit 10^6 CFU/g during 15 days of storage, however the TVB-N still maintained below 30 mg N/100 g. Although TVB-N is just an indicative of freshness, the determination of the overall quality of the fish fillets should be determined according to a set of parameters such as those evaluated in this study.

The determination of TBA is performed with the purpose of verifying the behavior of the shelf life of the products through lipid oxidation. The values of TBA are presented when the products are stored in the presence of oxygen (Gonzaga Junior et al. 2015). According to Gonzaga Junior et al. (2015) 1.5 mg of malonaldehyde per kg of fish fillet would be the maximum acceptable oxidation. In this study, on the 4th day of storage (Table 3) the fish fillets remained within the limit established for both tests, and on the 6th day of storage the fish fillets were oxidized. However, the packaged fish fillets with OEO film presented lower levels of malonaldehyde, consequently, indicated less oxidation than the product packed with the film without the antioxidant agent. According to Lee et al. (2016) the packaged product containing low amount of TBA suggests that the packaging was able to maintain the quality of the meat.

During the storage, the packed fish fillets with the standard film showed higher microbial growth than the packed fish fillets with OEO film, evidencing the antimicrobial activity present due to the addition of OEO. The initial microbial count in the packed fish fillets in both treatments was to 10^4 CFU/g (Fig. 2b). The ICMSF (1986) establishes a maximum limit for microorganism growth of 10^7 CFU/g. Thus, the packed fish fillets with the standard film exceeded the maximum counting limit on the 4th day of storage, while packed fish fillets with OEO film remained within the limit by the same storage time (Fig. 2b). Fish fillet is a food highly perishable with short shelf life. The use of antimicrobial and antioxidant agent as OEO in the package composition can improves the shelf life of the product.

Conclusion

The addition of OEO promoted improvements in the characteristics of the films. The films showed an increase in the tensile strength and elasticity and reduced the solubility in water and water vapor permeability. The film with better properties was produced with 3% (w/v) of starch, 30% of glycerol (in relation of the starch weight) and 4.5% of OEO (in relation to the solids weight). The shelf life study of packed fish fillet showed few differences when comparing to the product packed with standard film and with OEO film, but indicated the trend that OEO film can protected for a longer time the packed fish fillets.

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