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Activity of chitosan-montmorillonite bionanocomposites incorporated with rosemary essential oil: From *in vitro* assays to application in fresh poultry meat

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ABSTRACT

The aim of this work was to develop bionanocomposites based on chitosan/montmorillonite (MMT) incorporated with rosemary essential oil (REO) and to evaluate its activity as primary packaging of fresh poultry meat. Chitosan film form dispersions were incorporated with MMT and different levels of REO (0.5%, 1% and 2% v/v). Good interaction between the phenolic compounds present in the REO, the MMT and the chitosan was observed from FTIR spectra. The incorporation of REO increases film permeability to both water vapour and oxygen, whereas MMT incorporation reduced both permeabilities. However, all the developed films showed oxygen permeability values comparable with EVOH (a gas barrier used in packaging). In the *in vitro* essays, chitosan films with REO demonstrated good antimicrobial activity against *Bacillus cereus* (reduction of 7.2 log) and *Salmonella enterica* (reduction of 5.3 log) and in the migration assay, the total phenolic compounds increased with the level of REO incorporated. In the *in situ* essays, fresh poultry meat was wrapped with the films developed and stored under refrigeration (5 °C ± 2 °C) for 15 days. Compared to control (meat without film), meat wrapped with bioplastics showed reduction of 1.2–2.1 log CFU/g on the total microorganisms count, and active films also succeeded on retarding poultry lipid peroxidation and discoloration. Yet, the incorporation of MMT increased the interaction between the polymer/phenolic compounds/MMT, diminishing the release of the active compounds. In conclusion, the films showed potential to be used by the food industry, once they can substitute the commercial plastic films and extend products (poultry meat) shelf life. When improvement of the mechanical properties is not an issue, chitosan incorporated with REO and without MMT are the most promising materials.

1. Introduction

According to FAO report (2011), roughly one-third of food produced for human consumption is lost or wasted globally, which amounts to about 1.3 billion tons per year. This food waste also represents a huge use of resources and greenhouse gas emission production in vain (FAO, 2011). In low-income countries the causes of food losses and waste are a combination of problems from harvesting techniques, storage and cooling facilities in difficult climatic conditions, to infrastructure, packaging and marketing systems (FAO, 2011).

Food packaging can play an important role in the reduction of food losses and waste by educating the consumers (bring the consumer's attention to the life of a product); increasing the product's shelf life (e.g. through active packaging); and with pack technologies that enable consumers to reseal and re-use the product after opening the packaging (Scott & Butler, 2006).

To meet both the consumers' desire to eat healthily and the longer shelf life required from the food industries the latest research have been focused on developing natural preserving systems rather than those based on artificial additives and chemical preservatives (Scott & Butler, 2006). Therefore, the incorporation of natural preservatives into polymeric materials is emerging as a promising technology, to control the high microbial growth and lipid oxidation occurring at the surface of the majority of solid or semi-solid foods (Kaewprachu, Osako, Benjakul,

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& Rawdkuen, 2015). Furthermore, the growing demand and use of non-biodegradable plastic materials that has resulted in a global waste disposal problem is a world concern (Sadeghi & Mahsa, 2015; Shah, Hasan, Hameed, & Ahmed, 2008; Souza & Fernando, 2016). Biodegradable polymers are, therefore, being highlighted as the future generation of plastic materials capable to minimize the environmental impact of the traditional petroleum based ones (Souza & Fernando, 2016). Consequently, there is an increasing interest in the development of active biopolymer based packaging films to control microbiological safety and quality loss of food packaged through the enhancement of their antimicrobial and antioxidant abilities (Realini & Marcos, 2014; Souza, Rodrigues, Duarte, & Fernando, 2018a).

Rosmarinus officinalis L. (rosemary), an aromatic plant belonging to *Lamiaceae* family, has been used in culinary and medicinal purposes for thousands of years (Ribeiro-Santos et al., 2015). Extracts and essential oil (EO) of this plant are well known for their rich content in phenolic compounds (Brewer, 2011), mainly responsible for the antibacterial and antioxidant properties (Burt, 2004). Together with oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) EO, rosemary essential oil (REO) are reportedly among the most active in preventing food spoilage and pathogens growth (Regnier, Combrinck, & Du Plooy, 2012). Food and Drug Administration and the European Union approved REO as Generally Recognized as Safe (GRAS) and as a food additive (has received recognition as safe and effective antioxidant for food preservation), respectively (Ribeiro-Santos et al., 2015).

Spoilage of meat products occur rapidly and are mainly caused by chemical (protein degradation and lipid oxidation reactions) and microbiological (mostly due to improper handling before and after it is slaughtered) deterioration (Kaewprachu et al., 2015). The modern food industry requires novel and effective approaches to preserve these short shelf life products, and active packaging films, prepared by blending or mixing antimicrobial and antioxidant agents with biopolymeric materials (e.g. starch, proteins, alginates, chitosan and lipids) can play this important role (Khezrian & Shahbazi, 2017; Souza & Fernando, 2016). Food packaging incorporated with REO can, therefore, act retarding the four main mechanisms involved in the deterioration of foods by: (I) inhibiting microbial growth and (II) oxidative deterioration, rancidity; (III) retarding chemical and enzymatic activity; and moisture and/or vapour migration (i.e. through protection from external environment) (Khezrian & Shahbazi, 2017; Ribeiro-Santos, de Melo, Andrade, & Sanches-Silva, 2017). Rosemary essential oil has been studied as an active agent incorporated into different biopolymeric matrices. Examples are its inclusion in cellulose acetate film (Melo et al., 2012) or chitosan coating (Xiao, Zhu, Luo, Song, & Deng, 2010), to preserve foodstuff (refrigerated chicken meat and fresh-cut pears, respectively). Yet, biopolymers, when compared to traditional plastic material, present weaker mechanical properties (Azeredo et al., 2010). To enhance such characteristics, nanofillers, e.g. montmorillonite (MMT), a layered silicate mineral clay, can be added (Souza & Fernando, 2016). Nonetheless, little is known on the effect of incorporating a nanofiller in the antimicrobial and antioxidant activity of REO combined with biopolymers, once very few studies in literature have addressed this issue. Alboofetileh, Rezaei, Hosseini, and Abdollahi (2014) studied the antimicrobial activity of alginate/clay nanocomposite films enriched with different essential oils (but not REO) against three common foodborne pathogens. In the study they found that the antibacterial activity of the essential oils was maintained when incorporated into the nanocomposite film. Abdollahi, Rezaei, and Farzi (2014) studied the influence of chitosan/clay functional bionanocomposite activated with rosemary essential oil on the shelf life of fresh silver carp. The fish fillets were coated with the bionanocomposites enriched with REO (in only one concentration tested) and results confirmed that the functional bionanocomposite coating retarded lipid oxidation and inhibited bacterial deterioration, improving the acceptability of the fillets by about 8 days in comparison with the uncoated samples. Yet, scarce information is known on the functionality of REO, when incorporated at different concentrations, in a chitosan/clay nanocomposite film, applied to fresh poultry meat. Therefore, this study intends to add knowledge on the applicability of this type of novel biodegradable active packaging to fresh poultry meat. Developed films were characterized by Fourier transform infrared spectroscopy (FTIR) and through oxygen and water permeability assays. The active behaviour was also evaluated via *in vitro* characterization (migration of phenolic compounds and antimicrobial tests). Ultimately, this work provides useful information for poultry meat preservation and these films may substitute the non-biodegradable commercial packaging films used.

2. Materials and methods

2.1. Materials and reagents

All chemicals used were of analytical reagent grade and the water purified using Milli-Q system (Millipore, USA). Chitosan (Poly(D-glucosamine)) with 75% of deacetylation and high molecular weight (31–37 kDa), ethanol absolute, 1,1,3,3-tetraethoxypropane (TEP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich (Germany). Rosemary (*Rosmarinus officinalis* L. ct. camphor) essential oil (EO), with food grade classification, was acquired from Biover (Belgium). Glacial acetic acid, glycerol, sodium bromide (NaBr), potassium acetate (CH_3COOK - 99% purity), sodium hydroxide (NaOH), and tween 80 (polyethylene glycol sorbitan monolaurate) were purchased from Alfa Aesar (Germany), while sodium chloride (NaCl), Folin–Ciocalteu Reagent, trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA) and sodium carbonate anhydrous were obtained from PanReac (Spain). Dimethyl sulfoxide (DMSO) was obtained by Fisher Scientific (USA). Tripto-casein soy broth (TSB), tripto-casein agar (TSA), plate count agar (PCA), brilliant green lactose bile broth and mueller hinton agar (MHA) were purchased from Biokar (France). Sodium montmorillonite (Cloisite®Na^+), a natural unmodified nanoclay, was kindly supplied by BYK Additives & Instruments (USA), in this paper it is referred as MMT.

2.2. Experimental design

All experiments were conducted using a completely randomized design with three replications. Films with MMT at 2.5 wt.% and with 4 levels of essential oil (EO) incorporated (0%; 0.5%; 1% or 2% v/v) were tested. This percentage of MMT in the chitosan was chosen based on the work of Wang et al. (2005), which showed that with higher MMT content (5 wt. % and 10 wt.%), the interaction and dispersion within the polymer chain will diminish. Parallel, films without incorporation of MMT with the same levels of REO were tested.

2.3. Film production

Chitosan films were prepared according to Souza et al. (2017) and Dias et al. (2014) with some modification. To produce the film-forming dispersion (FFD), chitosan (1.5% w/v) was dissolved in 1% (v/v) of glacial acetic acid solution in ultrapure water with constant agitation during 24 h at room temperature. Glycerol at the level of 30% (w/w of chitosan) was added as plasticizer in all samples. To produce the films without nanoreinforcement (0% MMT), REO at the levels tested (0%; 0.5%; 1% and 2% v/v in FFD) and tween 80 (0.2% w/v in essential oil), as emulsifier (Abdollahi, Rezaei, & Farzi, 2012a), were incorporated at this stage. The system was homogenized for 5 min with ultraturrax (15000 rpm) (IKA® T18, Germany) followed by degasification using an ultrasound bath (Selecta, Spain) for 5 min (360 W). For the treatments with incorporation of MMT, at the level of 2.5% w/w in chitosan, extra steps of mechanical agitation were performed to supply energy to exfoliate the clay into the chitosan chains. Briefly, the exfoliation process consisted of 3 cycles of 5 min agitation with the ultraturrax (15000 rpm) followed by 15 min (360 W) in an ultrasound bath.

Rosemary essential oil and tween 80 were only incorporated before the last cycle, in the same percentages of the treatments without MMT. The resulting dispersion was then casted in glass molds (18 cm × 25 cm) or in glass Petri Dish (10 cm diameter) and dried for 72 h at room temperature. Dried films were peeled and stored protected from light at 25 °C until evaluation.

2.4. Film characterization

2.4.1. Scanning electron microscopy (SEM)

The scanning electron microscopy micrographs from the surface and cross-section of the films were obtained from Zeiss instrument (Model DSM 962, Germany) under vacuum, accelerated at 3 kV. The morphology of the chitosan film, chitosan + MMT and the films with the incorporation of 2% REO with or without MMT were studied.

2.4.2. Attenuated total reflectance fourier transform infrared (ATR-FTIR) spectroscopy

Attenuated total reflectance Fourier transform infrared spectroscopy spectra of the bionanocomposites was performed using FTIR spectrometer (model PerkinElmer spectrum Two, Perkin Elmer, USA) from 4000 to 650 cm^{-1} with 1 cm^{-1} resolution (Souza et al., 2018a).

2.4.3. Water vapour permeability (WVP)

The WVP (mol/m·s·Pa) was calculated according to the gravimetrically method described by Ferreira et al. (2016) at 30 °C. Samples specimens of each treatment were sealed on the top of 45 mm diameter glass cells containing 8 mL of saturated NaCl solution (Relative Humidity (RH) = 76.9%) and then placed inside a desiccator with saturated potassium acetate solution (RH = 22.5%) equipped with a fan to promote air circulation and maintain the driving force during the test. Previous to the test the samples were equilibrated in desiccators at a relative humidity of 76.9% (containing saturated sodium chloride solution). Temperature and the relative humidity inside the desiccator was monitored over test time using a thermohygrometer (Vaisala, Finland). Water vapour flux was measured through weighing the cells at regular time intervals during approximately 24 h, and the WVP calculated by following equation (Eq. (1)):

$$WVP = \frac{N_W \times \delta}{\Delta P_{w.eff}} \quad (1)$$

Where N_w (mol/m^2·s) is the water vapour flux, δ (m) is the film thickness and $\Delta P_{w.eff}$ (Pa) is the effective driving force. Results are the average ± standard deviation of the three replicants analyzed.

2.4.4. Oxygen permeability (OP)

Oxygen permeability was determined according to the methodology described by Ferreira et al. (2016) using a stainless steel cell with two identical chambers separated by the films tested. Previously to the test the specimens were equilibrated at 30 °C and relative humidity of 55% ± 5% (desiccator containing saturated sodium bromide solution). The OP was assessed by pressurizing one of the chambers (feed) up to 0.7 bar with pure oxygen (99.999% purity) (Praxair, Spain), followed by the measurement of the pressure change in both chambers over time, using two pressure transducers (Jumo, Model 404327, Germany). The system was kept at 30 °C using a thermostatic bath (Julabo, Model EH, Germany). The permeability was calculated using equation (2):

$$\frac{1}{\beta} ln\left(\frac{\Delta p_0}{\Delta p}\right) = P\frac{t}{\delta} \quad (2)$$

Where Δp (mbar) is the pressure difference between feed and permeate compartment, P (mol·m/m^2·s·Pa) is the gas permeability, t (s) is the time, δ (m) is the film thickness and β is the geometric parameter of cell (Alves, Costa, & Coelhoso, 2010).

2.5. In vitro active characterization

2.5.1. Migration assay

The release of the active compounds from bio-based films was determined by the specific migration into 95% ethanol solution (fatty food simulant) at 37 °C ± 2 °C during 10 days (Souza et al., 2018a). Periodically, total phenol content present in the simulant media was determined by Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1999), briefly: 1 mL of simulant was mixed with 3 mL of Milli-Q water and 0.25 mL of Folin-Ciocalteu reagent. The system was incubated for 5 min in dark before the addition of 0.75 mL of sodium carbonate solution 5% (w/v), followed by another 60 min incubation in dark. The colorimetric result was measured at 760 nm using spectrophotometer UV/VIS (Spekol 1500, Analytikjena, Germany). A calibration curve was constructed using gallic acid solutions from 0 to 120 mg L^{-1}, and TPC expressed in mg gallic acid equivalent (GAE)·mL^{-1} of simulant.

Also, diffusion coefficients (D) were also calculated from the plot of $M_{F,t}/M_{P,0}$ versus $t^{0,5}$ using initial migration data according to the model based on the Fick's second law described in equation (3) (Chungy, Papadakis, & Yamy, 2002):

$$\frac{M_{F,t}}{M_{P,0}} = \frac{2}{\delta}\left(\frac{Dt}{\pi}\right)^{0,5} \quad (3)$$

Where $M_{F,t}$ (mg GAE) is the amount of migrant in the food simulant at time t, $M_{P,0}$ (mg GAE) is the initial amount of migrant in the packaging film, D (m^2·s^{-1}) is the diffusion coefficient of migrant in the packaging film and δ (m) is the thickness of the packaging film.

2.5.2. Antimicrobial study

Antimicrobial activity of pure REO and bionanocomposites produced was studied by agar diffusion method (Nouri, Yaraki, Ghorbanpour, Agarwal, & Gupta, 2017) against seven foodborne pathogens, namely Gram-negative bacteria (*Escherichia coli* (ATCC8739), *Pseudomonas aeruginosa* (ATCC9027), *Salmonella enterica* (ATCC10708)); Gram-positive bacteria (*Bacillus cereus* (ATCC11778), *Enterococcus faecalis* (ATCC29212), *Listeria monocytogenes* (ATCC15313), *Staphylococcus aureus* (ATCC6538)); and one yeast *Candida albicans* (ATCC10231). Frozen microorganisms were inoculated in TSA or MHA and incubated at their optimum growth temperature (37 °C for all microorganisms except for *B. cereus* and *C. albicans* that were kept at 30 °C) for 16–24 h for bacteria and 48 h for yeast. Subsequently isolated colonies were transferred to 0.85% NaCl solution and the turbidity of the suspension was adjusted to match a 0.5 McFarland turbidity standard, corresponding to 1×10^8 CFU/mL and 1×10^6 CFU/mL for the bacteria or yeast respectively, using a McFarland densitometer (Model Den-1B, Grant Instruments, England). Petri dish (8.5 cm of diameter) containing Mueller Hinton Agar were inoculated by dipping a sterile swab in the microbial suspensions and spreading the bacteria/fungi uniformly. For the REO, wells with 6 mm diameter were cut in the MHA and filled with 50 μL of pure essential oil. For *C. albicans* the oil was also tested diluted in DMSO (1:1). Wells filled with DMSO were used as negative control. For the films, disks with the same size of the wells (6 mm diameter) were cut and placed onto inoculated plate surface and pressed gently. In order to allow the migration of the active compounds to the media, plates were incubated during 24 h in the fridge (5 °C ± 2 °C) prior to the incubation at 37 °C or 30 °C (depending on the microorganism) for 20 h. Disks of sterile paper and commercial plastic films were also used as negative controls.

Absence of microbial growth in the area around or below the films disks/wells (inhibition zones) was regarded as positive antimicrobial activity. Complementary, bionanocomposites were also tested by viable cell colony count (CFU) method (ASTM, 2001; Nouri et al., 2017) against Gram-negative (*S. enterica* (ATCC10708)) and Gram-positive (*B. cereus* (ATCC11778)) foodborne bacteria. Firstly, 0.2 g of each film was

immersed in 4 mL of TSB containing ~10^6 CFU/mL of the tested bacteria. Then the system was kept at 37 °C (*S. enterica*) or 30 °C (*B. cereus*) during 24 h with continuous shaking (150 RPM). Tubes without films were used as control. After the 24 h incubation, 100 μL of serial dilutions were spread onto TSA plates and incubated at the corresponding optimal growing temperature for 16–24 h. The number of viable microorganism colonies were then counted manually and multiplied by the dilution factor. Results were expressed as the number of reductions in the log CFU (colony forming units)/mL, and were calculated according to equation (4):

$$Log\ reduction = \log B - \log A \quad (4)$$

Where B and A are the mean number of bacteria (CFU/mL) in the control samples and treated samples after 24 h incubation, respectively.

2.6. In situ study – shelf life assessment of fresh poultry meat

Minced fresh poultry meat purchased at the local market was used as food matrix to evaluate the preservative properties of the bionanocomposites developed. The meat (approximately 30 g) was wrapped on rectangles (5 cm × 18 cm) of each film and then stored into plastic boxes with screw cap, under refrigeration (5 °C ± 2 °C) for 15 days. Meat unwrapped and wrapped in commercial adherent films (polyvinyl chloride – PVC) were used as controls. Each set was randomly collected and characterized in terms of the physico-chemical and microbiological quality at 0, 3, 7, 10 and 15 days of storage.

2.6.1. Physico-chemical characterization

pH, titratable acidity and moisture were measured according to AOAC method (AOAC, 2016). pH was measured in 5 g of sample, previously stirred for 15 min with 50 mL of ultra-pure water (40 °C), using a digital pH-meter (Crison Instruments, Barcelona, Spain). Titratable acidity was determined by titration with 0.1N NaOH solution, and the results expressed as % (w/w) of oleic acid equivalent, while moisture was determined by oven-drying at 105 °C ± 2 °C. Color was instrumentally determined (CIE-L*a*b* coordinates) on the surface of the meat using CR 410 colorimeter (Minolta Co., Tokyo, Japan) with D65 light source, and visual angle of 10°. Color coordinates range from L = 0 (black) to L = 100 (white), −a (greenness) to +a (redness), and −b (blueness) to +b (yellowness). The standardization of the instrument was done using a white calibration plate. Hue angle was calculated according to equation (5) (Souza et al., 2018b).

$$Hue\ angle = \tan\left(\frac{b^*}{a^*}\right)^{-1} \quad (5)$$

Where b* and a* are the coordinates measured from the samples.

2.6.2. Lipid oxidation

Thiobarbituric acid reactive substances (TBARS) were determined to evaluate the oxidation level of the meat packaged (Rosmini et al., 1996). Briefly, 10 g of the meat were mixed with 20 ml of trichloroacetic acid (TCA) 7.5% (w/v) for 1 h with continuous stirring to extract the malonaldehyde (MDA). The supernatants were filtered, and 5 mL of the filtrate was combined with 5 mL of 0.02M 2-thiobarbituric acid (TBA) and heated (95 °C/30 min) in water bath (Memmert, Germany). The system was cooled in water and the absorbance of the samples was measured at 530 nm in UV/VIS spectrophotometer. Calibration curve using known concentrations of MDA was used to quantify the TBARS. Results were expressed as mg of MDA/kg of meat.

2.6.3. Microbiological growth

Total mesophilic aerobic bacteria (TMAB), psychotropic aerobic bacteria and total coliforms were used to evaluate the microbiological quality of the meat packaged. From series dilutions in saline water (0.85% NaCl solution) the TMAB and psychotropic aerobic bacteria were enumerated on a standard plate count agar after incubation at 30 °C for 72 h or 7 °C for 10 days (ISO, 2013; Lorenzo, Sineiro, Amado, & Franco, 2014). Total coliforms counts were performed in brilliant green lactose bile broth after incubation at 30 °C for 48 h to calculate the most probable number (MPN) and the results were expressed as $\log_{10}$ MPN/g of meat (ISO, 2006).

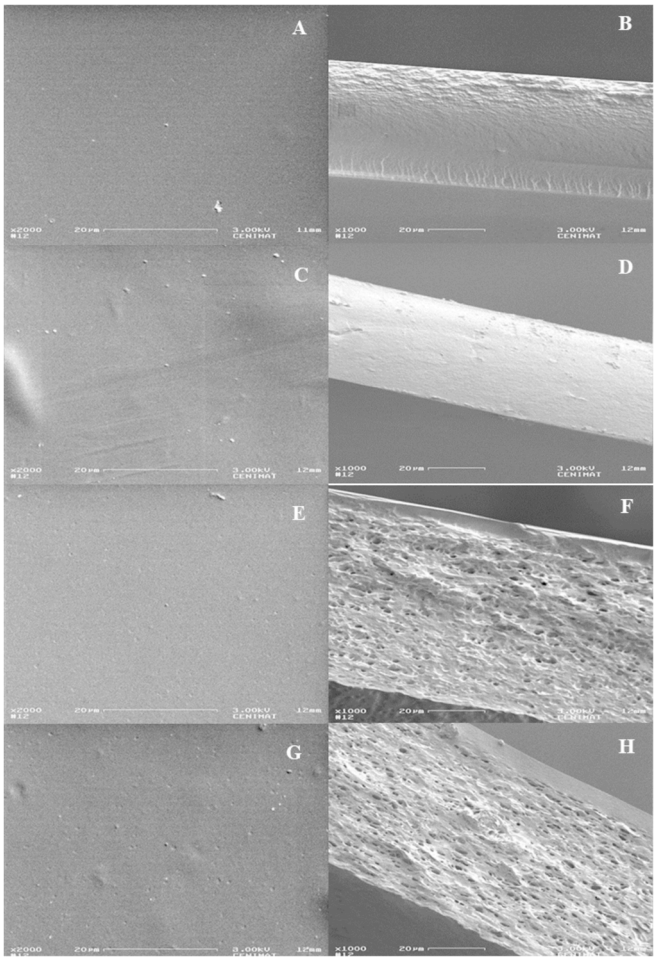

Fig. 1. Scanning electron microscopy micrographs of surface and cross-section, respectively: chitosan film (A and B), chitosan film + MMT (C and D), chitosan film + 2% REO (E and F) and chitosan film + MMT + 2% REO (G and H).

2.7. Statistical analysis

A statistical analysis of data was performed through a one-way analysis of variance using Software OriginLab, version 8.5, and differences among mean values were processed by the Tukey test. Significance was defined at $p < 0.05$.

3. Results and discussion

Bionanocomposites of chitosan/montmorillonite incorporated with different concentrations of rosemary essential oil were successfully casted, resulting in homogeneous yellowish transparent flexible thin films with average thickness of 51 μm.

The surface of the films showed high uniformity as shown in Fig. 1. The incorporation of MMT (Fig. 1C) or REO (with – Fig. 1E – or without MMT – Fig. 1G) did not result in a relevant change in the homogeneity of the film, only some granules and small depressions on its surface were observed.

The cross-sections (obtained from cryogenic fractures) of the control films (Fig. 1B) and Chitosan + MMT (Fig. 1D) show the high homogeneity of the resulting polymeric structure. The incorporation of MMT

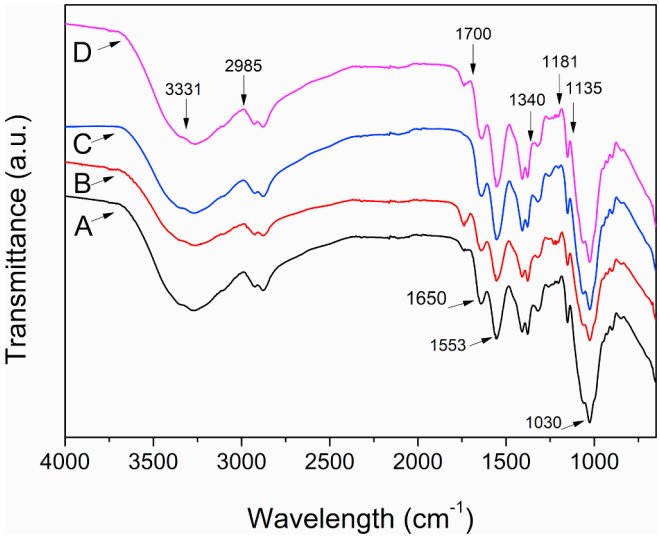

Fig. 2. ATR-FTIR spectra of chitosan (Ch)-based films (A) incorporated with: (B) 2.5% MMT; (C) 2% REO; (D) 2.5% MMT + 2% REO.

seems to have contributed to make the network even more compact, being an indication of good interaction between the polymer and the nanoclay. The incorporation of the REO in the proportion of 2% resulted in an internal spongy structure (Fig. 1F), apparently with some discontinuity. When both REO and MMT were added (Fig. 1H), the spongy structure appears to have slightly decreased, although it is still present. These results corroborate the findings in the permeability studies discussed in section 3.1.

3.1. Film characterization (FTIR, water vapour and oxygen permeability)

Fourier transform infrared spectroscopy is a common technique used to identify chemical composition as well as their inner molecular bonding in materials science (Woranuch & Yoksan, 2013). Beyond the vast applications, it enables the study of the interactions between the different components in composite materials (Paluszkiewicz, Stodolak, Hasik, & Blazewicz, 2011; Pola et al., 2016). Fourier-transform infrared spectroscopy spectra of pristine chitosan film and bio-based films incorporated with 2.5% of MMT and 2% of REO are shown in Fig. 2. All spectra showed major characteristic bands related to chitosan at 3325 cm^{-1} (-OH); 3265 cm^{-1} (-NH asymmetric stretching); 2900 cm^{-1} (C-H bonding of $–NHCOCH_3$ methyl group); 1637 cm^{-1} (amide I); 1550 cm^{-1} (amide II); 1342 cm^{-1} (skeleton vibration involving C-N stretching of amide III); 1375-1408 cm^{-1} ($-CH_2$ bending); 906-1024 cm^{-1} (skeletal vibrations involving the C–O stretching) and 1134 cm^{-1} (asymmetric stretching of the C–O–C bridge) (Silva-Weiss, Bifani, Ihl, Sobral, & Gómez-Guillén, 2013; Souza et al., 2018a; Woranuch & Yoksan, 2013; Ávila, Bierbrauer, Pucci, López-González, & Strumia, 2012). No remarkable differences were observed between the spectra of chitosan film (Fig. 2A) and the other bionanocomposites produced (Fig. 2B, C and 2D), which can be explained by the small amount of nanoclay and/or REO incorporated and by the mode of scanning (ATR mode) used (Souza et al., 2018a).

Multiple functional groups (carboxyl, amide, amine, hydroxyl and carbonyl) present in chitosan create the possibility for new bonding between the polymer and nanofillers or active compounds (Paluszkiewicz et al., 2011). The incorporation of REO (Fig. 2C and D) provoked changes in the intensity of the bands at 1550 cm^{-1} (amide II) and between 3331 and 2985 cm^{-1}, suggesting the formation of the linkage between phenolic compounds present in the essential oil and chitosan, as these bands correspond to stretching of free hydroxyl and to asymmetric and symmetric stretching of the N-H bonds in amino group (Silva-Weiss et al., 2013; Souza et al., 2018a).

In Fig. 2B and D, the subtle changes observed in the intensity of the bands around 1030-1050 cm^{-1} and 1340 cm^{-1} can be attributed to Si-O vibrations bonds from the incorporated MMT (Paluszkiewicz et al., 2011). Changes in peak intensities are related to overlapping of vibrations, in this case of the secondary amide and Si–OR groups, which indicates interactions between the CS matrix and the MMT phase (Paluszkiewicz et al., 2011; Pola et al., 2016).

According to Pola et al. (2016) the incorporation of oregano essential oil in acetate cellulose thin films increased the intensity of absorption spectra as a whole, which correspond to a good interaction between the active compounds and the polymeric matrix that hampered the vibrational movement changing the intensity of the bands. Similar behavior can be observed on the spectra of the bionanocomposites developed with incorporation of REO from 1650 to 1135 cm^{-1} (Fig. 2C and D). Furthermore, the decrease in the absorption intensity of bands at 3450 and 3310 cm^{-1}, corresponding to NH_2 groups, reflects reduction in the amount of amino groups per unit volume capable of forming intermolecular hydrogen bonds with primary OH groups of polysaccharide (Afanas'eva et al., 2013), demonstrating the good entrapment of the phenolic compounds in the chitosan matrix through chemical bonding.

In the development of novel food packaging material, the study of the material's barrier properties is crucial, once it is well known that such characteristics play a major role in determining the shelf life of the product packaged, and reductions in permeability of moisture and gases across the packaging are always targeted (Vilarinho et al., 2018).

Oxygen and water vapour permeability were not statistically affected ($p > 0.05$) with the incorporation of either MMT or REO, except for the samples with or without nanoreinforcement incorporated with 2% of REO that presented increased values on both permeability compared to pristine chitosan (Table 1). Although the differences between treatments did not differ statistically from each other, two trends may be perceived on the material behaviour, namely: (i) decrease in the barrier properties with the incorporation of REO; and (ii) increase with the incorporation of MMT.

Table 1
Water vapour and oxygen permeability in chitosan-based films and diffusion coefficient of phenolic compounds into simulant media.

Film	WVP (10^{-11} mol/m·s·Pa)	O_2 Permeability (10^{-16} mol m/m^2·s·Pa)	Diffusion coefficient (10^{-12} m^2/s)	Maximum total diffused/total incorporated into films
Ch	1.40 ± 0.09^{B*}	0.184 ± 0.074^{B}	No migration	–
Ch + 2.5%MMT	1.75 ± 0.10^{AB}	0.098 ± 0.012^{B}	No migration	–
Ch + 0.5% REO	1.78 ± 0.25^{AB}	0.212 ± 0.091^{B}	1.32 ± 0.49^{B}	0.19
Ch + 2.5%MMT + 0.5% REO	1.90 ± 0.22^{AB}	0.134 ± 0.019^{B}	1.87 ± 0.74^{B}	0.16
Ch + 1% REO	2.10 ± 0.54^{AB}	0.227 ± 0.017^{AB}	4.00 ± 1.18^{B}	0.26
Ch + 2.5%MMT + 1% REO	1.87 ± 0.07^{AB}	0.203 ± 0.024^{B}	0.83 ± 0.16^{B}	0.18
Ch + 2% REO	2.54 ± 0.51^{A}	0.252 ± 0.035^{AB}	20.3 ± 5.1^{A}	0.54
Ch + 2.5%MMT + 2% REO	2.36 ± 0.18^{A}	0.416 ± 0.049^{A}	1.81 ± 1.10^{B}	0.19

**A-B: Different superscripts within the same column indicate significant differences among samples ($p < 0.05$).
Ch: chitosan; MMT: sodium montmorillonite; REO: Rosemary Essential Oil.

Table 2
Antimicrobial study of Rosemary essential oil (REO) and chitosan film incorporated with REO and sodium montmorillonite (MMT).

	Sample code	Gram positive				Gram Negative			Fungi
		B. cereus	*E. faecalis*	*S. aureus*	*L. monocytogenes*	*S. enterica*	*E.coli*	*P.aeruginosa*	*C.albicans*
Inhibition zone (mm) method*	DMSO	0	0	0	0	0	0	0	0
	REO 50%	–	–	–	–	–	–	–	42 ± 0
	REO 100%	21.7 ± 0.6	0	14.0 ± 2.0	22.0 ± 2.0	11.3 ± 1.2	12.7 ± 2.5	0	Total inhibition
	Filter paper	0	0	0	0	0	0	0	0
	Commercial plastic	0	0	0	0	0	0	0	0
	CH	0	0	0	0	0	0	0	0
	CH + MMT	0	0	0	0	0	0	0	0
	CH + 0,5%REO	6	0	6	6	6	6	0	0
	CH + 0,5%REO + MMT	6	0	6	6	6	6	0	0
	CH + 1%REO	6	0	6	6	6	6	0	6
	CH + 1%REO + MMT	6	0	6	6	6	6	0	6
	CH + 2%REO	6	0	6	6	6	6	0	6
	CH + 2%REO + MMT	6	0	6	6	6	6	0	6

	Sample code	Gram positive (*B. cereus*)			Gram Negative (*S. enterica*)		
		CFU/mL	Log (CFU/mL)	Antibacterial activity (Log reduction)	CFU/mL	Log (CFU/mL)	Antibacterial activity (Log reduction)
CFU method	Control	1.7×10^8	8.2	–	6.3×10^8	8.8	–
	CH	< 10	< 1	> 7.2 ± 0.1 ^{A**}	5.3×10^3	3.7	5.3 ± 0.6^{A}
	CH + MMT	1.6×10^2	2.2	6.7 ± 1.0 ^{A}	4.2×10^5	5.6	3.6 ± 0.7^{AB}
	CH + 0,5%REO	< 10	< 1	> 7.2 ± 0.1 ^{A}	9.0×10^3	4.0	5.1 ± 0.9^{A}
	CH + 0,5%REO + MMT	5.3×10^4	4.7	4.0 ± 0.8 ^{AB}	1.1×10^5	5.0	3.9 ± 0.4^{AB}
	CH + 1%REO	1.3×10^3	3.1	6.4 ± 1.6 ^{AB}	3.0×10^3	3.5	5.3 ± 0.1^{A}
	CH + 1%REO + MMT	1.7×10^5	5.2	3.1 ± 0.4 ^{B}	3.3×10^4	4.5	4.3 ± 0.1^{AB}
	CH + 2%REO	1.6×10^5	5.2	4.5 ± 2.4 ^{AB}	1.1×10^7	7.1	3.0 ± 1.4^{B}
	CH + 2%REO + MMT	1.0×10^4	4.0	6.1 ± 2.0 ^{AB}	3.6×10^4	4.6	4.3 ± 0.2^{AB}

*Inhibition zone equal 6 mm means that the microorganism did not grow under the disc film sample.
**A-B: Different superscripts within the same column indicate significant differences among samples ($p < 0.05$).
DMSO: Dimethyl sulfoxide; REO: Rosemary Essential Oil; Ch: chitosan; MMT: sodium montmorillonite.

It was expected that with the incorporation of essential oil, an hydrophobic compound, WPV would decrease with the reduction of hydrophilic content of the composite formed (Giannakas, Patsaoura, Barkoula, & Ladavos, 2017), which did not occur. Although the differences were not statistically significant ($p > 0.05$), REO may have acted as a plasticizer (behaviour also reflected in the mechanical properties of the material, evaluated on a previous work (Souza et al., 2018c)), increasing the chitosan polymer chains mobility, thus making the transport of oxygen or water molecules easier through the film (Nouri et al., 2017), which probably explains the results found. Furthermore, from the SEM cross-section images (Fig. 1F and H), it is clear that the incorporation of the REO has changed the structure of the films to a sponge like conformation, even suggesting discontinuity within the internal film structure, which corroborate with the increase of the permeabilities. On the other hand, the presence of dispersed layered silicate into the polymer creates a tortuous path delaying the transport of both water vapour and oxygen through the film (Giannakas et al., 2017; Rhim, Hong, Park, Ng, & Erry, 2006), reducing the permeability, as previously reported in literature (Abdollahi et al., 2012a; Giannakas et al., 2017; Nouri et al., 2017).

The WPV and OP values found are in the same order of magnitude of that referred for chitosan film previously published (WPV = 4.14×10^{-11} mol/m·s·Pa (Ferreira et al., 2016); 1.50×10^{-11} mol/m·s·Pa (Liu, Cai, Jiang, Wu, & Le, 2016); 0.67×10^{-11} mol/m·s·Pa (Pastor, Sánchez-González, Chiralt, Cháfer, & González-Martínez, 2013) and OP = 2.35×10^{-16} mol m/m^2·s·Pa (Ferreira et al., 2016); 0.07×10^{-16} mol m/m^2·s·Pa (Pastor et al., 2013)). Comparing to synthetic films used in the food industry (e.g. 0.01×10^{-11} mol/m·s·Pa for low density poly-ethylene (LDPE) (Ferreira et al., 2014)), the WVP of biopolymers is two or even more orders higher, easily explained by their hydrophilic behaviour. This may limit its use as inner layer sandwiched between hydrophobic materials or as packaging of food with lower water content (Ferreira, Bandarra, Moldão-Martins, Coelhoso, & Alves, 2018). For the oxygen, the developed films showed OP comparable with EVOH (0.24×10^{-16} mol m/m^2·s·Pa (Cerisuelo, Alonso, Aucejo, Gavara, & Hernández-Muñoz, 2012)) one of the best hydrophilic gas barriers used in packaging (Lagaron, Catalá, & Gavara, 2004).

3.2. In vitro active characterization (migration assay and microbiological study)

The migration of TPC followed an "exponential growth to a maximum" type migration profile and reached the equilibrium after the first 3 days of the assay. The higher the amount of REO incorporated, the higher the TPC in the simulant in contact with the films. Furthermore, the incorporation of MMT reduced the diffusion of the phenolic compounds toward the simulant media. Thus the highest diffusion coefficients were reported in the film incorporated with 2% REO (20.3×10^{-12} m^2/s) and 1% REO (4.0×10^{-12} m^2/s) and the lowest in the film with MMT + 0.5% of REO (1.87×10^{-12} m^2/s) and with MMT + 1% REO (0.83×10^{-12} m^2/s) (Table 1). Results are in good agreement with several works in literature (Ramos, Jiménez, Peltzer, & Garrigós, 2014; Souza et al., 2018a). According to Abdollahi et al. (2012a), which also studied chitosan films with MMT and REO, particular arrangement occurs in the film due to the interactions between functional groups of the incorporated compounds with hydroxyl and amino groups in chitosan matrix. Moreover, in the presence of REO, the hydrogen-bonding interaction between chitosan and clay are enhanced. This good interaction can possibly explain the different behaviour with the incorporation of MMT. The net formed between MMT and chitosan entraps the phenolic compounds from REO limiting its diffusion. This is easily observed from the results of maximum total diffused/total incorporated into films, that showed a range of TPC diffusion of only

0.16–0.19, when MMT is present (Table 1).

The antioxidant activity of the simulant media was also studied through radical scavenging method (DPPH) (Brand-Williams, Cuvelier, & Berset, 1995), and after diffusion process the phenolic compounds demonstrated 3–5% of radical scavenging capacity (0.5%–2% REO, independently of MMT incorporation). These small values are related to the small amount of phenolics that migrated to the media, but prove that even after diffusion process the active compounds preserve its bioactivity (Souza et al., 2018a).

In vitro antimicrobial activity of REO and films against foodborne pathogens was also addressed (Table 2). Pure REO presented antimicrobial activity against gram-positive bacteria (*B. cereus, S. aureus* and *L. monocytogenes*), gram-negative (*S. enterica* and *E. coli*) and the fungi *C. albicans*. The greatest inhibitory effect observed was against the yeast, where it was verified a complete inhibition of growth when pure REO was tested, and an inhibition zone with 42 mm of diameter when the assay was performed with diluted (1:1) REO. Concerning the antibacterial activity, results showed that REO was more efficient against gram-positive than against gram-negative bacteria, although inhibition zones of 11.3 and 12.7 mm were recorded for *S. enterica* and *E. coli*, respectively. Good antimicrobial activity of REO is mainly related to its rich content in phenolic compounds (i.e. 1,8-cineole, camphor, eugenol, α-pinene, rosmarinic acid and carnosic acid) (Ribeiro-Santos et al., 2015), and has also been previously reported (Abdollahi, Rezaei, & Farzi, 2012b). The characteristic outer bacteria membrane composition is the main reason for the differences on the resistance of Gram-negative and Gram-positive bacteria. The impermeable lipid membrane which surrounds the Gram-negative cell wall, restricts the diffusion of hydrophobic compounds through the lipopolysaccharide coating, conferring more resistance than the thicker peptidoglycan layer and the diverse negatively functional groups composing the Gram-positive bacteria (Abdollahi et al., 2012b; Nouri et al., 2017).

Films incorporated with REO also presented antimicrobial activity on contact surface underneath the film disks for both Gram-positive and Gram-negative bacteria that was affected by pure REO (Table 2). For the fungi tested, only films incorporated with REO at 1% and 2% presented inhibition zone. Pristine chitosan film did not show activity against the microorganisms evaluated. Sterilized disks of filter paper and commercial plastic were also tested, and neither of them showed any antimicrobial effect, validating that the microorganisms did not grow underneath the film disk due to the antimicrobial activity of the films tested but not to a possible anaerobic condition created when the surface of the agar was covered with the disk films. These results are in good agreement with other published data (Abdollahi et al., 2012b; Siripatrawan & Vitchayakitti, 2016). The decrease activity of REO when incorporated in chitosan films in comparison with the pure REO may be explained either due to the small content present in the specimen, or to the good interaction between phenolic compounds with functional groups of chitosan, which retard the diffusion through the adjacent agar media (Altiok, Altiok, & Tihminlioglu, 2010) and diminish the contact between bacterial cells and polyphenolic molecules (Siripatrawan & Vitchayakitti, 2016).

Excellent antimicrobial activity in growth media against both *B. cereus* and *S. enterica* was also obtained for all films tested, despite the incorporation of REO or MMT (Table 2). Similar with the results of REO in the agar diffusion test, the efficiency of the films was higher against Gram-positive bacteria (*B. cereus*) in the viable cell colony count method. Pristine chitosan film resulted in ~7.2 log and 5.3 log reduction of *B. cereus* and *S. enterica* after 24 h incubation time, respectively. In general, the incorporation of MMT reduced the activity of the films, which corroborates with the lower TPC migration results. The incorporation of REO did not statistically interfere ($p > 0.05$) with the antibacterial activity detected, except in the level of 2% for *S. enterica*.

Chitosan antimicrobial mechanism is related to changes in the cell surface of fungi or bacteria that lead to permeabilization and leakage of intracellular material resulting in cell death, mainly through electrostatic interaction (Verlee, Mincke, & Stevens, 2017). Chitosan binds non-covalently with teichoic acids of peptidoglycan layer of Gram-positive bacteria, while in Gram-negative bacteria it can act as chelator of cation (important nutrients to the cell) disrupting the cell wall integrity or through electrostatic interaction with anionic parts of the lipopolysaccharide at the outer membrane (Nouri et al., 2017; Verlee et al., 2017). The reduction of antimicrobial activity of chitosan with the incorporation of MMT is probably related to the good interaction between the nanoclay and the polymer (as previously discussed in the FTIR results) that reduces the number of functional groups (amino groups) to act in the membrane of microorganisms. Moreover, the structural negative charge of the MMT, counterbalances the positive charges located in the chitosan, leading to a lower electrostatic interaction of the biopolymer with the outer membrane of bacteria, and subsequently to a lower antimicrobial activity. Yet, this has never been reported in literature and therefore needs confirmation with further studies.

Therefore, these results support the potential of such novel material to be used for controlling foodborne pathogens in food preservation.

3.3. *In situ study – shelf life assessment of fresh poultry meat*

3.3.1. *Physico-chemical characterization*

The high content of unsaturated lipids, more prone to oxidation processes, and the development of aerobic spoilage bacteria (i.e. *Pseudomonas spp., Enterobacteriaceae* or lactic acid bacteria, to mention a few) make poultry meat a highly perishable food (Silva, Domingues, & Nerín, 2018). The meat evaluated trended to increase pH values, moisture and hue angle (H*), and decrease the titratable acidity during its shelf life assessment (Table 3). Changes over time were more intense in the meat without packaging protection followed by the meat wrapped in commercial adherent film.

In terms of color, initial values of H* were around 52°, toward reddish tone, and after 15 days refrigerated storage it increased to approximately 70° for meat without wrapping and to 64° for meat wrapped in commercial plastic. Higher angles mean change in the color towards a more yellowish and greenish tone, i.e. a discoloration process. Active films, on the other hand, kept the color (H* between 52° to 57°) through the shelf life period, and did not differ statistically ($p > 0.05$) between treatments through time. Lipid oxidation and the degradation of heme molecules with formation of metmyoglobins are described as the causes of color deterioration during chilled storage of meat products (Ghaderi-Ghahfarokhi, Barzegar, Sahari, Ahmadi Gavlighi, & Gardini, 2017; Melo et al., 2012), therefore, chitosan films have probably avoided this deterioration process. As color is a valuable parameter when accepting or rejecting a product, once consumers will not buy dark or discolored meat (Rojas & Brewer, 2008), these results are promising and demonstrate the potential of the material developed in the preservation of fresh poultry meat.

Regarding the pH, only the samples unwrapped, the samples wrapped in the commercial film and the meat packaged in the pristine chitosan film showed increased pH after 15 days storage time ($p < 0.05$). The pH of all other samples wrapped in the bionanocomposites developed did not differ statistically ($p > 0.05$) between the day zero and the value found on the 15th day of storage, although a slight increase on the value was observed. Between treatments, in the last day assessed, the pH of the meat without wrapping was significantly superior to all other samples, and the pH of pristine chitosan film was equal to chitosan + MMT and commercial film ($p > 0.05$), but also higher than the pH of other samples wrapped with REO ($p < 0.05$). Within the bionanocomposites incorporated with REO no statistically difference was observed (Table 3). Results are in good agreement with previously reported by Melo et al. (2012) and Abdollahi et al. (2014) that assessed the shelf life of chicken meat and fish fillet protected with acetate cellulose film or chitosan solution incorporated with REO, respectively. Complementary, titratable acidity

Table 3
Shelf life study of poultry meat packaged in chitosan-based bionanocomposites – physico-chemical characterization.

Parameters	Days	Unwrapped	Commercial film	Ch	Ch + 0,5%REO	Ch + 1%REO	Ch + 2%REO	Ch + MMT	Ch + MMT + 0,5%REO	Ch + MMT + 1%REO	Ch + MMT + 2%REO
Hue angle (º)	0	55 ± 4^{Ba}	55 ± 1^{Ca}	53 ± 4^{Aa}	52 ± 0^{Ba}	52 ± 0^{Da}	52 ± 0^{Ca}	52 ± 1^{ABa}	52 ± 1^{Ba}	52 ± 1^{Ba}	52 ± 1^{Ba}
	3	58 ± 3^{Bab}	58 ± 1^{Bab}	55 ± 2^{Abc}	57 ± 1^{Aa}	60 ± 1^{Aa}	60 ± 1^{Aa}	52 ± 0^{ABc}	50 ± 0^{Bc}	61 ± 0^{Aa}	52 ± 0^{Bc}
	7	61 ± 4^{ABab}	64 ± 0^{Aa}	53 ± 1^{Acde}	55 ± 1^{Abcde}	58 ± 1^{Bbcd}	59 ± 0^{Aabc}	49 ± 1^{Be}	52 ± 0^{ABde}	57 ± 6^{Abcd}	57 ± 2^{Abcd}
	10	64 ± 5^{ABa}	63 ± 1^{Aab}	49 ± 5^{Ad}	56 ± 1^{Abcd}	56 ± 0^{BCbcd}	57 ± 1^{Bbc}	55 ± 2^{Acd}	54 ± 1^{Acd}	59 ± 2^{Aabc}	59 ± 1^{Aabc}
	15	71 ± 1^{Aa}	64 ± 1^{Ab}	53 ± 2^{Acd}	55 ± 2^{ABcd}	55 ± 1^{Ccd}	55 ± 1^{Bcd}	54 ± 2^{Acd}	53 ± 1^{ABd}	58 ± 2^{Ac}	57 ± 1^{Ac}
pH	0	5.70 ± 0.04^{Ca}	5.96 ± 0.15^{Ca}	5.96 ± 0.3^{Ba}	5.96 ± 0.3^{Aa}	5.96 ± 0.3^{ABa}	5.96 ± 0.3^{Aa}	5.70 ± 0.04^{Aa}	5.70 ± 0.04^{Aa}	5.70 ± 0.0^{Aa}	5.70 ± 0.04^{Ba}
	3	5.72 ± 0.06^{Ccde}	6.19 ± 0.08^{Ca}	6.01 ± 0.2^{Bab}	5.71 ± 0.2^{Acde}	5.54 ± 0.1^{Ce}	5.56 ± 0.1^{Ade}	5.86 ± 0^{Abc}	5.84 ± 0.0^{Abcd}	5.69 ± 0.1^{Acde}	5.80 ± 0^{Bbcde}
	7	6.97 ± 0.2^{Ba}	6.50 ± 0.12^{Bab}	6.33 ± 0^{Bbcd}	6.16 ± 0.2^{Abcd}	6.09 ± 0^{Abcde}	5.80 ± 0.0^{Ade}	6.41 ± 0.6^{Aabc}	5.81 ± 0.1^{Ade}	5.91 ± 0.2^{Acde}	5.58 ± 0.0^{Bde}
	10	7.99 ± 0.1^{Aa}	6.79 ± 0.04^{Bb}	6.51 ± 0.2^{Bbc}	6.08 ± 0.1^{Abc}	5.68 ± 0.1^{BCc}	5.80 ± 0.2^{Ac}	6.35 ± 0.5^{Abc}	6.21 ± 0.4^{Abc}	6.28 ± 0.4^{Abc}	6.37 ± 0.6^{Abc}
	15	8.01 ± 0.39^{Aa}	7.26 ± 0.13^{Ab}	7.14 ± 0.2^{Abc}	6.29 ± 0.2^{Ad}	6.17 ± 0.1^{Ad}	6.00 ± 0.2^{Ad}	6.44 ± 0.3^{Acd}	6.27 ± 0.3^{Ad}	6.06 ± 0.3^{Ad}	6.06 ± 0.3^{Bd}
Titratable Acidity (% oleic acid equivalent)	0	2.19 ± 0.01^{Aa}	1.68 ± 0.22^{Ab}	1.94 ± 0.08^{Aa}	1.94 ± 0.08^{Aa}	1.94 ± 0.08^{Aa}	1.94 ± 0.08^{Aa}	2.19 ± 0.01^{Aa}	2.19 ± 0.01^{Aa}	2.19 ± 0.01^{Aa}	2.19 ± 0.01^{Aa}
	3	2.11 ± 0.03^{Aab}	1.32 ± 0.08^{Bc}	1.13 ± 0.01^{Bc}	1.30 ± 0.24^{ABc}	1.15 ± 0.09^{Cc}	1.25 ± 0.04^{Cc}	2.30 ± 0.58^{Aa}	1.39 ± 0.13^{Cc}	1.64 ± 0.32^{Babc}	1.53 ± 0.09^{Bbc}
	7	0.80 ± 0.16^{Cd}	1.27 ± 0.07^{Bbc}	0.85 ± 0.05^{Bd}	0.87 ± 0.08^{Bd}	1.14 ± 0.08^{Cc}	1.04 ± 0.02^{Dcd}	1.41 ± 0.15^{BCb}	1.80 ± 0.02^{Ba}	1.14 ± 0.06^{Cc}	1.20 ± 0.04^{Cbc}
	10	1.09 ± 0.05^{Bc}	1.15 ± 0.06^{BCc}	1.13 ± 0.18^{Bc}	1.32 ± 0.19^{ABbc}	1.32 ± 0.03^{BCbc}	1.23 ± 0.03^{Cc}	1.74 ± 0.07^{ABa}	1.77 ± 0.12^{Ba}	1.82 ± 0.01^{ABa}	1.57 ± 0.04^{Bab}
	15	0.77 ± 0.04^{Cc}	0.87 ± 0.02^{Cc}	1.22 ± 0.29^{Babc}	1.14 ± 0.43^{Babc}	1.50 ± 0.02^{Bab}	1.59 ± 0.13^{Ba}	0.97 ± 0.03^{Cc}	1.02 ± 0.05^{Dbc}	1.06 ± 0.03^{Cbc}	1.14 ± 0.06^{Cabc}
Moisture (%)	0	75.4 ± 0.4^{Ba}	75.5 ± 0.2^{Ba}	74.9 ± 0.6^{Aa}	74.9 ± 0.6^{Aa}	74.9 ± 0.6^{Aa}	74.9 ± 0.6^{Aa}	75.3 ± 0.5^{Aa}	75.0 ± 0.3^{Aa}	75.0 ± 0.3^{Aa}	75.0 ± 0.3^{Aa}
	3	75.1 ± 0.1^{Bab}	75.8 ± 0.4^{Ba}	73.4 ± 0.1^{BCabcd}	73.1 ± 1.1^{Abcd}	71.8 ± 0.4^{Cd}	72.3 ± 0.6^{Ad}	73.4 ± 0.2^{Aabcd}	74.9 ± 2.3^{Aabc}	71.4 ± 0.5^{Bd}	72.4 ± 0.4^{Bcd}
	7	75.9 ± 0.1^{ABa}	75.1 ± 0.4^{Bab}	72.5 ± 0.1^{Ccd}	73.6 ± 0.5^{Abc}	72.0 ± 0.5^{BCde}	72.0 ± 0.5^{Ade}	72.9 ± 0.8^{Acd}	$72.0 \pm 0.6A^{Bde}$	$70.7 \pm 0.7B^{Ce}$	72.3 ± 0.5^{Bcd}
	10	76.7 ± 0.1^{Aa}	75.6 ± 0.3^{Bab}	74.5 ± 0.3^{ABab}	74.4 ± 1.3^{Aab}	73.5 ± 1.3^{ABCabc}	72.9 ± 2.3^{Abcd}	72.9 ± 2.6^{Abcd}	69.5 ± 0.3^{Bde}	69.1 ± 1.1^{Ce}	70.6 ± 0.1^{Cce}
	15	76.6 ± 0.9^{Aa}	76.7 ± 0.1^{Aa}	73.4 ± 0.8^{Bb}	73.6 ± 0.5^{Ab}	73.7 ± 0.1^{ABb}	73.4 ± 1.0^{Ab}	73.0 ± 1.1^{Ab}	73.7 ± 0.6^{Ab}	72.1 ± 0.2^{Bbc}	70.8 ± 0.5^{Cc}

Superscript letters (A-D): Within each parameter, values in the same column not sharing upper case superscript letters indicate statistically significant differences among days ($p < 0.05$).
Superscript letters (a-g): Within each parameter, values in the same line not sharing lower case superscript letters indicate statistically significant differences among formulations ($p < 0.05$). Thiobarbituric acid reactive substances (TBARS), Total mesophilic aerobic bacteria (TMAB), Colony forming units (CFU), Chitosan (Ch), Sodium Montmorillonite (MMT), Rosemary Essential Oil (REO).

followed the same behavior (i.e. higher pH represents lower acidity) corroborating with the previous reported. For food products rich in proteins and free amino acids that are stored under aerobiosis, the increment on pH may be attributed to the production of volatile basic components due to microbial growth, i.e., their endogenous proteolytic activity that result in the production of basic components (Abdollahi et al., 2014; Melo et al., 2012). Thus, the antimicrobial effect of the coatings might be the explanation for the lower pH found on the samples protected with the active packaging. The antimicrobial effect of the coatings will be discussed in section 3.3.3.

Regarding the moisture content, the active films reduced the moisture of the samples packaged ($p < 0.05$) when compared to the values in the day zero (Table 3). Chitosan is a hydrophilic polysaccharide able to adsorb large amounts of water (Souza et al., 2017), which may explain the reduction in the water content of the meat packaged with the chitosan films. Precooked beef patties stored refrigerated also exhibited higher values of moisture after 3 days storage than precooked beef patties coated in chitosan film (Wu et al., 2000). The authors also attributed the lower values of moisture to the swelling ability of the hydrophilic chitosan film, corroborating with the findings in this work.

3.3.2. Lipid oxidation

The initial TBARS value was approximately 0.15 mg MDA/kg meat which intensively increased over time (Table 4). Poultry meat without protection reached 2.03 mg MDA/kg meat after 15 days of storage, while samples wrapped on active films kept the levels of MDA much lower, around the initial values. In the active films without nanoreinforcement the final content of MDA did not statistically differ from the initial values of TBARS ($p > 0.05$). Samples packaged in films with MMT and REO presented lower oxidation compared to control samples, although with final values higher than in day zero ($p < 0.05$). Lipid oxidation is one of the most detrimental processes in foodstuffs products, leading to modifications on the original flavour (off-flavour development/rancid odours) and taste that are most associated with consumer depreciation and rejection of food (Kaewprachu et al., 2015; Vilarinho et al., 2018). According to Sheard et al. (2000), the threshold of off-odour perception by consumers corresponds to a TBARS value of 0.5 mg MDA/kg sample for pork patties. Analysing the results, unwrapped samples reached the off-odour threshold at the 7th day of storage, while meat wrapped in commercial plastic, chitosan + MMT and chitosan + MMT + 0.5REO and 2%REO only on day 10. Meat packaged in films with REO but without MMT did not reached the 0.5 mg MDA/kg sample after the 15 days storage. The retarding on the lipid oxidation may be attributed to few factors: (i) the good oxygen barrier properties of chitosan film, as discussed before, which avoid the free radical initiation chain reaction in lipid oxidation; (ii) chitosan ability of cations chelation (Abdollahi et al., 2014); and (iii) the phenolic compounds present in the REO that act as primary antioxidant by donating electrons to stabilize free radicals and interrupt the oxidation chain reaction (Melo et al., 2012). Similar results were previously reported with different biopolymers incorporated with essential oils applied in a meat and fish products, namely: chitosan solution with REO coating fresh silver carp (Abdollahi et al., 2014), cellulose acetate film with REO in chicken breast (Melo et al., 2012), chitosan film with Thyme EO in fish fillets (Yang et al., 2015). However, in the work of Abdollahi et al., 2014, the fish coated with chitosan + MMT + REO (1.5%) did not differ in the level of MDA from the samples protected with the pristine chitosan coating or incorporated with MMT, which demonstrate that the bionanocomposites are more efficient in the film form, moreover, even with lower amount of essential oils added. The lower activity of films reinforced with MMT may be attributed to the good interaction between the components of the bionanocomposites that avoided the migration of the active compounds, thus reducing its preservative ability.

Table 4
Shelf life study of poultry meat packaged in chitosan-based bionanocomposites – microbiological and thiobarbituric acid reactive substances characterization.

Parameters	Days	Unwrapped	Commercial film	Ch	Ch + 0,5%REO	Ch + 1%REO	Ch + 2%REO	Ch + MMT	Ch + MMT + 0,5%REO	Ch + MMT + 1%REO	Ch + MMT + 2%REO
TBARS (mg MDA/kg meat)	0	0.15 ± 0.01^{Ca}	0.15 ± 0.01^{Ba}	0.14 ± 0.01^{Ba}	0.14 ± 0.0^{BCa}	0.14 ± 0.01^{Ba}	0.16 ± 0.03^{ABa}	0.15 ± 0.02^{Ba}	0.15 ± 0.01^{Ca}	0.15 ± 0.01^{Ca}	0.15 ± 0.01^{Ba}
	3	0.16 ± 0.01^{Cb}	0.14 ± 0.02^{Bb}	0.18 ± 0.02^{Bb}	0.34 ± 0.01^{Aa}	0.22 ± 0.03^{Aa}	0.21 ± 0.02^{Aab}	0.23 ± 0.14^{BCab}	0.16 ± 0.0^{Cb}	0.16 ± 0.0^{Cb}	0.13 ± 0.01^{Bb}
	7	0.94 ± 0.20^{Ba}	0.10 ± 0.01^{Bc}	0.26 ± 0.02^{Abc}	0.34 ± 0.02^{Abc}	0.15 ± 0.00^{Bc}	0.28 ± 0.01^{Abc}	0.43 ± 0.2^{ABCb}	0.14 ± 0.02^{Cc}	0.30 ± 0.07^{Bbc}	0.15 ± 0.01^{Bc}
	10	1.54 ± 0.33^{Aa}	0.64 ± 0.24^{Abc}	0.16 ± 0.01^{Bcd}	0.22 ± 0.01^{Bcd}	0.14 ± 0.03^{Bcd}	0.20 ± 0.05^{Bcd}	0.96 ± 0.28^{ABb}	0.60 ± 0.02^{Abcd}	0.21 ± 0.03^{BCcd}	0.53 ± 0.1^{Abcd}
	15	2.03 ± 0.28^{Aa}	0.55 ± 0.10^{Ac}	0.26 ± 0.01^{Ac}	0.24 ± 0.0^{Bc}	0.21 ± 0.01^{Ac}	0.28 ± 0.11^{Ac}	1.15 ± 0.54^{Ab}	0.43 ± 0.02^{Bc}	0.70 ± 0.03^{Abc}	0.62 ± 0.03^{Abc}
TMAB (Log_{10} CFU/g meat)	0	5.2 ± 0.3^{Da}	5.2 ± 0.3^{Ca}	5.2 ± 0.3^{Ca}	5.2 ± 0.3^{Ca}	5.2 ± 0.3^{Ca}	5.2 ± 0.3^{Ca}	5.2 ± 0.3^{Ca}	5.2 ± 0.3^{Ba}	5.2 ± 0.3^{Ca}	5.2 ± 0.3^{Ca}
	3	8.5 ± 0.5^{Cab}	8.7 ± 0.0^{Ba}	6.4 ± 0.3^{Bef}	6.3 ± 0.0^{Bf}	5.7 ± 0.1^{Bfg}	6.0 ± 0.0^{Bf}	$7.0 \pm 0.5B^{de}$	5.1 ± 0.1^{Bg}	7.7 ± 0.0^{Bcd}	7.8 ± 0.1^{Bbc}
	7	9.9 ± 0.4^{Ba}	9.5 ± 0.3^{Aa}	8.2 ± 0.4^{Ab}	7.7 ± 0.4^{Ab}	7.5 ± 0.2^{Ab}	7.4 ± 0.4^{Ab}	8.4 ± 0.5^{Ab}	8.4 ± 0.2^{Ab}	8.1 ± 0.5^{ABb}	7.8 ± 0.1^{Bb}
	10	10.1 ± 0.1^{Aa}	9.6 ± 0.1^{Aa}	8.9 ± 0.4^{Ab}	8.1 ± 0.4^{Abc}	8.0 ± 0.3^{Ac}	8.0 ± 0.5^{Ac}	8.4 ± 0.2^{Abc}	8.2 ± 0.0^{Abc}	8.5 ± 0.1^{Abc}	8.5 ± 0.1^{Abc}
Total coliforms (Log_{10} MPN/g meat)	0	1.9 ± 0.5^{Ca}	1.9 ± 0.5^{Ca}	1.9 ± 0.5^{Ba}	1.9 ± 0.5^{Ca}	1.9 ± 0.5^{Ca}	1.9 ± 0.5^{Ba}	1.9 ± 0.5^{Ca}	1.9 ± 0.5^{Ba}	1.9 ± 0.5^{Ba}	1.9 ± 0.5^{Aa}
	3	3.9 ± 0.5^{Bab}	3.4 ± 0.0^{Babcd}	2.4 ± 0.1^{Bd}	3.3 ± 0.1^{Babcd}	3.2 ± 0.2^{Ba}	3.4 ± 0.0^{Aabcd}	3.6 ± 0.4^{Aabc}	4.0 ± 0.3^{Aa}	2.6 ± 0.4^{ABcd}	2.9 ± 0.4^{Abcd}
	7	5.2 ± 0.2^{Aa}	3.9 ± 0.5^{Bb}	4.0 ± 0.2^{Ab}	4.2 ± 0.2^{Ab}	3.7 ± 0.4^{ABb}	3.7 ± 0.4^{Ab}	2.6 ± 0.0^{Bc}	3.6 ± 0.1^{Ab}	2.6 ± 0.1^{ABc}	2.9 ± 0.3^{Ac}
	10	5.6 ± 0.4^{Ab}	6.4 ± 0.1^{Aa}	3.4 ± 0.1^{Acd}	3.6 ± 0.4^{ABcd}	4.1 ± 0.2^{Ac}	3.0 ± 0.4^{Ade}	3.1 ± 0.2^{ABde}	2.4 ± 0.1^{Be}	3.2 ± 0.1^{Ad}	3.0 ± 0.3^{Ade}

Superscript letters (A-D): Within each parameter, values in the same column not sharing upper case superscript letters indicate statistically significant differences among days ($p < 0.05$).
Superscript letters (a-g): Within each parameter, values in the same line not sharing lower case superscript letters indicate statistically significant differences among formulations ($p < 0.05$). Thiobarbituric acid reactive substances (TBARS), Total mesophilic aerobic bacteria (TMAB), Colony forming units (CFU), Most probable number (MPN), Chitosan (Ch), Sodium Montmorillonite (MMT), Rosemary Essential Oil (REO).

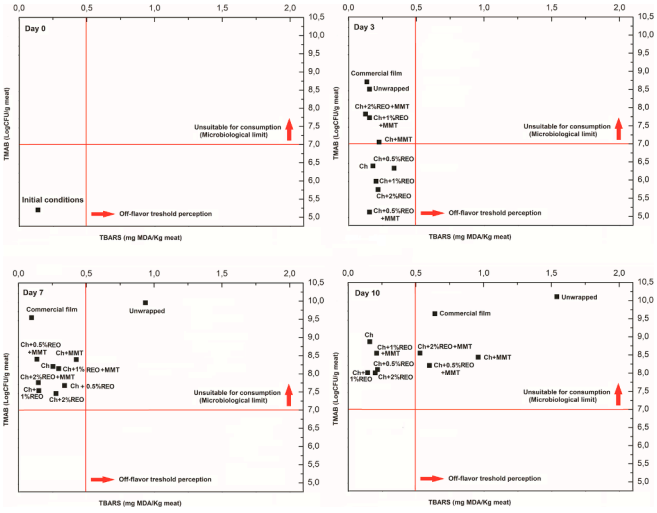

Fig. 3. Comparative analysis of lipid oxidation and microbiology study of poultry meat packaged in chitosan (Ch)-based film incorporated with montmorillonite (MMT) and different levels of rosemary essential oil (REO) over the shelf life time.

3.3.3. Microbiological quality

As expected, total mesophilic aerobic bacteria increased over storage time for all treatments tested (Table 4). Comparing to unwrapped meat, active films reduced around 2 $\log_{10}$ CFU/g in the counting of aerobic bacteria, representing a gain on the quality and safety of the product packaged. Although the differences between the samples packaged with the active films were not statistically significant ($p > 0.05$), in nominal value, chitosan film without REO showed higher values than the others added with the natural extracts. One more time, the incorporation of MMT diminished the preservative effect of the bionanocomposites by resulting on higher TMAB. Abdollahi et al. (2014) also evaluated bionanocomposites of chitosan/MMT/REO but as edible coating in the shelf life extension of fish fillets. The authors reported a lower TMAB in the food protected with the active coating, and a further protection in the samples where REO was also incorporated, which coincide with the results of this work. Although these authors have not tested the bionanocomposites in the film form, and neither with only the incorporation of REO, as it was tested in this work. In terms of the psychotropic aerobic bacteria (results not shown), the behaviour was similar to the one observed for TMAB. Regarding growth, at days 0 and 3, psychotropic aerobic bacteria showed lower counts than TMAB (approximately 0.95 of the total aerobic bacteria incubated at 30 °C), but at 7 and 10 days of shelf life the opposite was observed (1.15 of the total aerobic bacteria incubated at 30 °C). As most of psychrophilic strains are spoilage microorganisms and some are pathogenic (e.g. *L. monocytogenes*, *Yersinia enterolitica,* to mention a few), these results are promising once the active films enhance the security of the product in the same time that retard its deterioration (Coma, 2008).

Regarding the total coliforms, these microorganisms grown up throughout the storage time ($p < 0.05$) for all samples, protected or not by the films, except for the meat packed in the films chitosan + MMT + 0.5% or 2% REO ($p > 0.05$) (Table 4). Comparing the unprotected meat and those packed in commercial film with the other samples wrapped in the active chitosan films, it was concluded that the chitosan films were responsible for a decrease of the total coliform growth between 2.3 and 4.0 log cycles. The lowest contaminations were recorded for the samples protected by the chitosan films incorporated with REO and MMT, although the differences were not significant among the different active films ($p > 0.05$). These results are consistent with those presented for TMAB and reinforce the explanation that the REO enhances the antimicrobial activity of chitosan films. Furthermore, these findings corroborate with previously reported by Souza et al. (2018b) for chicken breast patties protected in chitosan film incorporated with ginger essential oil (GEO). According to these authors, the presence of the active compounds from the natural extract potentialized the protection effect of the chitosan film, resulting in an extension of the product shelf life time. However, comparing the microbial growth of the meat protected with the films incorporated with REO (this work) and with GEO (Souza et al., 2018b), the samples protected in the former showed lower contaminations than the poultry meat wrapped in the latter, demonstrating that this novel film has more potential than the material incorporated with ginger essential oil. This behaviour is also in line with the *in vitro* antimicrobial study, once the rosemary essential oil here studied demonstrated superior activity (inhibited both gram-positive and -negative bacteria) than the ginger essential oil (only inhibited gram-positive bacteria) (Souza et al., 2018b).

Antimicrobial activity of films produced is therefore related to both chitosan and the presence of phenolic compounds derived from REO, as discussed before in the *in vitro* antimicrobial activity. For bovine meat, a counting of 10^7 CFU/g meat was set as the maximum TMAB accepted before the product get unsuitable for consumption (Cannarsi, Baiano, Marino, Sinigaglia, & Del Nobile, 2005). This maximum contamination is also considered the limit for raw fish (Abdollahi et al., 2014). Thus, considering this also the limit for the poultry meat, the samples packaged in chitosan, chitosan + 0.5%REO, chitosan + 1%REO, chitosan + 2%REO and chitosan + 0.5%REO + MMT extended the shelf life of the food analysed once only exceeded the limit on the 7th day of storage, while the other samples reached that on the 3rd day (Table 4). Analysing together the TBARS and TMAB results over time (Fig. 3), it is easy to visualize the effect of the films on the extension of the product shelf life. Unwrapped samples are since the 3rd day of analyse on the rejection zone while meat protected with active films are on acceptable ones.

Finally, this novel technology can complement the necessary intake of phenolic compounds (add nutritional value to the diet) once it enables not only the release of such active compounds to the food, but also due to the edible character of the films (Medina-Jaramillo, Ochoa-Yepes, Bernal, & Famá, 2017), which is an extra advantage to the bionanocomposites developed.

4. Conclusions

Production of the films were successfully achieved, resulting in homogeneous thin flexible films. Good interaction between the incorporated compounds was observed in FTIR spectra, which helped to understand the activity behavior either *in vitro* or *in situ*, of the active packaging developed. Overall, sodium montmorillonite diminished the bioactivity ability of the films, either antimicrobial or antioxidant, which was reflected in the inhibition of the liquid media growth of both Gram-positive and Gram-negative bacteria assessed, and also in the physico-chemical values of the meat packaged in these films. Apparently, MMT trapped the phenolic compounds avoiding its release towards the food or the media in contact with the active films. On the other hand, the nanoreinforcement improved the barrier properties (both WPV or OP) of the material developed, helping to diminish the negative effect of the incorporation of essential oil on this property. Together with the good oxygen barrier properties and the presence of phenolic compounds from REO, the bionanocomposites developed acted as a good vehicle for extending food self-life and food quality improvement of meat products. They retarded the lipid oxidation, the microbiological growth of spoilage bacteria, the meat discoloration and the increase of the pH values. Yet, future works can improve the functionality of this novel bionanocomposite by testing and evaluating the right proportion of MMT with the rate of REO added to chitosan, e.g. by lowering the amount of MMT added in order to increase the amount of REO released to the foodstuff.

A further advantage of this novel technology lays on the extra nutritional value added to the product packaged once powerful bioactive

compounds migrate towards the food and may complement the consumers diet, therefore have potential to be implemented by the food industry, helping to reduce the gap between research and industrial exploitation.

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References

Abdollahi, M., Rezaei, M., & Farzi, G. (2012a). A novel active bionanocomposite film incorporating rosemary essential oil and nanoclay into chitosan. *Journal of Food Engineering, 111*(2), 343–350. https://doi.org/10.1016/j.jfoodeng.2012.02.012.

Abdollahi, M., Rezaei, M., & Farzi, G. (2012b). *Improvement of active chitosan film properties with rosemary essential oil for food packaging.* 847–853. https://doi.org/10.1111/j.1365-2621.2011.02917.x.

Abdollahi, M., Rezaei, M., & Farzi, G. (2014). Influence of chitosan/clay functional bionanocomposite activated with rosemary essential oil on the shelf life of fresh silver carp. *International Journal of Food Science and Technology, 49*(3), 811–818. https://doi.org/10.1111/ijfs.12369.

Afanas'eva, N. V., Petrova, V. a., Vlasova, E. N., Gladchenko, S. V., Khayrullin, a. R., Volchek, B. Z., et al. (2013). Molecular mobility of chitosan and its interaction with montmorillonite in composite films: Dielectric spectroscopy and FTIR studies. *Polymer Science - Series A, 55*(12), 738–748. https://doi.org/10.1134/S0965545X13120018.

Alboofetileh, M., Rezaei, M., Hosseini, H., & Abdollahi, M. (2014). Antimicrobial activity of alginate/clay nanocomposite films enriched with essential oils against three common foodborne pathogens. *Food Control, 36*(1), 1–7. https://doi.org/10.1016/j.foodcont.2013.07.037.

Altiok, D., Altiok, E., & Tihminlioglu, F. (2010). Physical, antibacterial and antioxidant properties of chitosan films incorporated with thyme oil for potential wound healing applications. *Journal of Materials Science: Materials in Medicine, 21*(7), 2227–2236. https://doi.org/10.1007/s10856-010-4065-x.

Alves, V. D., Costa, N., & Coelhoso, I. M. (2010). Barrier properties of biodegradable composite films based on kappa-carrageenan/pectin blends and mica flakes. *Carbohydrate Polymers, 79*(2), 269–276. https://doi.org/10.1016/j.carbpol.2009.08.002.

AOAC (2016). *Official methods of analysis of the association of official analytical chemists* (20th ed.). .

ASTM (2001). *Standard methods for determining the antimicrobial activity of immobilized antimicrobial agents under dynamic contact conditions.* ASTM - America Society Standard Testing and Materials (E2149-1).

Ávila, A., Bierbrauer, K., Pucci, G., López-González, M., & Strumia, M. (2012). Study of optimization of the synthesis and properties of biocomposite films based on grafted chitosan. *Journal of Food Engineering, 109*(4), 752–761. https://doi.org/10.1016/j.jfoodeng.2011.11.009.

Azeredo, H. M. C., Mattoso, L. H. C., Avena-Bustillos, R. J., Filho, G. C., Munford, M. L., Wood, D., et al. (2010). Nanocellulose reinforced chitosan composite films as affected by nanofiller loading and plasticizer content. *Journal of Food Science, 75*, N1–N7. https://doi.org/10.1111/j.1750-3841.2009.01386.x.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food Science and Technology, 28*, 25–30.

Brewer, M. S. (2011). Natural antioxidants: Sources, compounds, mechanisms of action, and potential applications. *Comprehensive Reviews in Food Science and Food Safety, 10*(4), 221–247. https://doi.org/10.1111/j.1541-4337.2011.00156.x.

Burt, S. (2004). Essential oils: Their antibacterial properties and potential applications in foods - a review. *International Journal of Food Microbiology, 94*(3), 223–253. https://doi.org/10.1016/j.ijfoodmicro.2004.03.022.

Cannarsi, M., Baiano, A., Marino, R., Sinigaglia, M., & Del Nobile, M. A. (2005). Use of biodegradable films for fresh cut beef steaks packaging. *Meat Science, 70*(2), 259–265. https://doi.org/10.1016/j.meatsci.2005.01.006.

Cerisuelo, J. P., Alonso, J., Aucejo, S., Gavara, R., & Hernández-Muñoz, P. (2012). Modifications induced by the addition of a nanoclay in the functional and active properties of an EVOH film containing carvacrol for food packaging. *Journal of Membrane Science, 423*(424), 247–256. https://doi.org/10.1016/j.memsci.2012.08.021.

Chungy, D., Papadakis, S. E., & Yamy, K. L. (2002). Simple models for assessing migration from food-packaging ® lms. *Food Additives & Contaminants, 19*(6), 611–617. https://doi.org/10.1080/02652030210126638.

Coma, V. (2008). Bioactive packaging technologies for extended shelf life of meat-based products. *Meat Science, 78*(1–2), 90–103. https://doi.org/10.1016/j.meatsci.2007.07.035.

Dias, M. V., Machado Azevedo, V., Borges, S. V., Soares, N. D. F. F., Barros Fernandes, R. V., Marques, J. J., et al. (2014). Development of chitosan/montmorillonite nanocomposites with encapsulated α-tocopherol. *Food Chemistry, 165*, 323–329. https://doi.org/10.1016/j.foodchem.2014.05.120.

FAO (2011). *Global food losses and food waste - extent, causes and prevention. Rome.* Retrieved from http://www.fao.org/docrep/014/mb060e/mb060e.pdf.

Ferreira, A. R. V., Bandarra, N. M., Moldão-Martins, M., Coelhoso, I. M., & Alves, V. D. (2018). FucoPol and chitosan bilayer films for walnut kernels and oil preservation. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology, 91*(December 2017), 34–39. https://doi.org/10.1016/j.lwt.2018.01.020.

Ferreira, A. R. V., Torres, C. A. V., Freitas, F., Reis, M. A. M., Alves, V. D., & Coelhoso, I. M. (2014). Biodegradable films produced from the bacterial polysaccharide FucoPol. *International Journal of Biological Macromolecules, 71*, 111–116. https://doi.org/10.1016/j.ijbiomac.2014.04.022.

Ferreira, A. R. V., Torres, C. A. V., Freitas, F., Sevrin, C., Grandfils, C., Reis, M. A. M., et al. (2016). Development and characterization of bilayer films of FucoPol and chitosan. *Carbohydrate Polymers, 147*, 8–15. https://doi.org/10.1016/j.carbpol.2016.03.089.

Ghaderi-Ghahfarokhi, M., Barzegar, M., Sahari, M. A., Ahmadi Gavlighi, H., & Gardini, F. (2017). Chitosan-cinnamon essential oil nano-formulation: Application as a novel additive for controlled release and shelf life extension of beef patties. *International Journal of Biological Macromolecules, 102*, 19–28. https://doi.org/10.1016/j.ijbiomac.2017.04.002.

Giannakas, A., Patsaoura, A., Barkoula, N. M., & Ladavos, A. (2017). A novel solution blending method for using olive oil and corn oil as plasticizers in chitosan based organoclay nanocomposites. *Carbohydrate Polymers, 157*, 550–557. https://doi.org/10.1016/j.carbpol.2016.10.020.

ISO (2006). *ISO 4831:2006(en) Microbiology of food and animal feeding stuffs — horizontal method for the detection and enumeration of coliforms — most probable number technique*.

ISO (2013). *ISO 4833-1:2013 Microbiology of the food chain – Horizontal method for the enumeration of microorganisms – Part 1: Colony count at 30 degrees C by the pour plate technique*.

Kaewprachu, P., Osako, K., Benjakul, S., & Rawdkuen, S. (2015). Quality attributes of minced pork wrapped with catechin-lysozyme incorporated gelatin film. *Food Packaging and Shelf Life, 3*, 88–96. https://doi.org/10.1016/j.fpsl.2014.11.002.

Khezrian, A., & Shahbazi, Y. (2017). Application of nanocompostie chitosan and carboxymethyl cellulose films containing natural preservative compounds in minced camel's meat. *International Journal of Biological Macromolecules,* 1–13. https://doi.org/10.1016/j.ijbiomac.2017.08.117.

Lagaron, J. M., Catalá, R., & Gavara, R. (2004). Structural characteristics defining high barrier properties in polymeric materials. *Materials Science and Technology, 20*(1), 1–7. https://doi.org/10.1179/026708304225010442.

Liu, Y., Cai, Y., Jiang, X., Wu, J., & Le, X. (2016). Molecular interactions, characterization and antimicrobial activity of curcumin–chitosan blend films. *Food Hydrocolloids, 52*, 564–572. https://doi.org/10.1016/j.foodhyd.2015.08.005.

Lorenzo, J. M., Sineiro, J., Amado, I. R., & Franco, D. (2014). Influence of natural extracts on the shelf life of modified atmosphere-packaged pork patties. *Meat Science, 96*(1), 526–534. https://doi.org/10.1016/j.meatsci.2013.08.007.

Medina-Jaramillo, C., Ochoa-Yepes, O., Bernal, C., & Famá, L. (2017). Active and smart biodegradable packaging based on starch and natural extracts. *Carbohydrate Polymers, 176*(May), 187–194. https://doi.org/10.1016/j.carbpol.2017.08.079.

Melo, A. A. M. De, Geraldine, R. M., Silveira, M. F. A., Lopes, M. C., Silva, C., Fernandes, T. H., et al. (2012). Microbiological quality and other characteristics of refrigerated chicken meat in contact with cellulose acetate-based film incorporated with rosemary essential oil. *Brazilian Journal of Microbiology,* 1419–1427.

Nouri, A., Yaraki, M. T., Ghorbanpour, M., Agarwal, S., & Gupta, V. K. (2017). Enhanced antibacterial effect of chitosan film using Montmorillonite/CuO nanocomposite. *International Journal of Biological Macromolecules, 109*, 1219–1231. https://doi.org/10.1016/j.ijbiomac.2017.11.119.

Paluszkiewicz, C., Stodolak, E., Hasik, M., & Blazewicz, M. (2011). FT-IR study of montmorillonite–chitosan nanocomposite materials. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 79*(4), 784–788. https://doi.org/10.1016/j.saa.2010.08.053.

Pastor, C., Sánchez-González, L., Chiralt, A., Cháfer, M., & González-Martínez, C. (2013). Physical and antioxidant properties of chitosan and methylcellulose based films containing resveratrol. *Food Hydrocolloids, 30*(1), 272–280. https://doi.org/10.1016/j.foodhyd.2012.05.026.

Pola, C. C., Medeiros, E. A. A., Pereira, O. L., Souza, V. G. L., Otoni, C. G., Camilloto, G. P., et al. (2016). Cellulose acetate active films incorporated with oregano (*Origanum vulgare*) essential oil and organophilic montmorillonite clay control the growth of phytopathogenic fungi. *Food Packaging and Shelf Life, 9*, 69–78. https://doi.org/10.1016/j.fpsl.2016.07.001.

Ramos, M., Jiménez, A., Peltzer, M., & Garrigós, 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. https://doi.org/10.1016/j.foodchem.2014.04.026.

Realini, C. E., & Marcos, B. (2014). Active and intelligent packaging systems for a modern society. *Meat Science, 98*(3), 404–419. https://doi.org/10.1016/j.meatsci.2014.06.031.

Regnier, T., Combrinck, S., & Du Plooy, W. (2012). Essential oils and other plant extracts as food preservatives. *Progress in food preservation* (pp. 539–579). Oxford, UK: Wiley-Blackwell. https://doi.org/10.1002/9781119962045.ch26.

Rhim, J.-W., Hong, S.-I., Park, H.-M., Ng, P. K. W., & Erry, P. K. W. N. G. (2006). Preparation and characterization of chitosan-based nanocomposite films with antimicrobial activity. *Journal of Agricultural and Food Chemistry, 54*(16), 5814–5822. https://doi.org/10.1021/jf060658h.

Ribeiro-Santos, R., Carvalho-Costa, D., Cavaleiro, C., Costa, H. S., Albuquerque, T. G., Castilho, M. C., et al. (2015). A novel insight on an ancient aromatic plant: The rosemary (*Rosmarinus officinalis* L.). *Trends in Food Science & Technology, 45*(2), 355–368. https://doi.org/10.1016/j.tifs.2015.07.015.

Ribeiro-Santos, R., de Melo, N. R., Andrade, M., & Sanches-Silva, A. (2017). Potential of migration of active compounds from protein-based films with essential oils to a food and a food simulant. *Packaging Technology and Science, 30*(12), 791–798. https://doi.org/10.1002/pts.2334.

Rojas, M. C., & Brewer, M. S. (2008). Effect of natural antioxidants on oxidative stability of frozen, vacuum-packaged beef and pork. *Journal of Food Quality, 31*(2), 173–188. https://doi.org/10.1111/j.1745-4557.2008.00196.x.

Rosmini, M. R., Perlo, F., Pérez-Alvarez, J. A., Pagán-Moreno, M. J., Gago-Gago, a, López-Santoveña, F., et al. (1996). TBA test by an extractive method applied to "paté". *Meat Science, 42*(1), 103–110. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22060305.

Sadeghi, G. M. M., & Mahsa, S. (2015). Compostable polymers and nanocomposites — a big chance for planet earth. *Recycling materials based on environmentally friendly techniques*InTechhttps://doi.org/10.5772/59398.

Scott, B., & Butler, P. (2006). *Packaging technologies with potential to reduce the amount of food thrown away.* http://www.wrap.org.uk/content/packaging-technologies-potential-reduce-amount-food-thrown-away/, Accessed date: 10 January 2018.

Shah, A. A., Hasan, F., Hameed, A., & Ahmed, S. (2008). Biological degradation of plastics: A comprehensive review. *Biotechnology Advances, 26*(3), 246–265. https://doi.org/10.1016/j.biotechadv.2007.12.005.

Sheard, P. R., Enser, M., Wood, J. D., Nute, G. R., Gill, B. P., & Richardson, R. I. (2000). Shelf life and quality of pork and pork products with raised n-3 PUFA. *Meat Science, 55*(2), 213–221. https://doi.org/10.1016/S0309-1740(99)00145-X.

Silva-Weiss, A., Bifani, V., Ihl, M., Sobral, P. J. A., & Gómez-Guillén, M. C. (2013). Structural properties of films and rheology of film-forming solutions based on chitosan and chitosan-starch blend enriched with murta leaf extract. *Food Hydrocolloids, 31*(2), 458–466. https://doi.org/10.1016/j.foodhyd.2012.11.028.

Silva, F., Domingues, F. C., & Nerín, C. (2018). Control microbial growth on fresh chicken meat using pinosylvin inclusion complexes based packaging absorbent pads. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology, 89*(May 2017), 148–154. https://doi.org/10.1016/j.lwt.2017.10.043.

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-ciocalteu. *Methods in Enzymology, 299*(1999), 152–178.

Siripatrawan, U., & Vitchayakitti, W. (2016). Improving functional properties of chitosan films as active food packaging by incorporating with propolis. *Food Hydrocolloids, 61*, 695–702. https://doi.org/10.1016/j.foodhyd.2016.06.001.

Souza, V. G. L., & Fernando, A. L. (2016). Nanoparticles in food packaging: Biodegradability and potential migration to food—a review. *Food Packaging and Shelf Life, 8*, 63–70. https://doi.org/10.1016/j.fpsl.2016.04.001.

Souza, V. G. L., Fernando, A. L., Pires, J. R. A., Rodrigues, P. F., Lopes, A. A. S. S., & Fernandes, F. M. B. (2017). Physical properties of chitosan films incorporated with natural antioxidants. *Industrial Crops and Products, 107*(February), 565–572. https://doi.org/10.1016/j.indcrop.2017.04.056.

Souza, V. G. L., Pires, J. R. A., Rodrigues, P. F., Lopes, A. A. S., Fernandes, F. M. B., Duarte, M. P., et al. (2018c). Bionanocomposites of chitosan/montmorillonite incorporated with *Rosmarinus officinalis* essential oil: Development and physical characterization. *Food Packaging and Shelf Life, 16*, 148–156. https://doi.org/10.1016/j.fpsl.2018.03.009.

Souza, V. G. L., Pires, J. R. A., Vieira, É., Coelhoso, I., Duarte, M., & Fernando, A. (2018b). Shelf life assessment of fresh poultry meat packaged in novel bionanocomposite of chitosan/montmorillonite incorporated with ginger essential oil. *Coatings, 8*(5), 177. https://doi.org/10.3390/coatings8050177.

Souza, V. G. L., Rodrigues, P. F., Duarte, M. P., & Fernando, A. L. (2018a). Antioxidant migration studies in chitosan films incorporated with plant extracts. *Journal of Renewable Materials, 6*(5), 548–558. https://doi.org/10.7569/JRM.2018.634104.

Verlee, A., Mincke, S., & Stevens, C. V. (2017). Recent developments in antibacterial and antifungal chitosan and its derivatives. *Carbohydrate Polymers, 164*, 268–283. https://doi.org/10.1016/j.carbpol.2017.02.001.

Vilarinho, F., Andrade, M., Buonocore, G. G., Stanzione, M., Vaz, M. F., & Sanches Silva, A. (2018). Monitoring lipid oxidation in a processed meat product packaged with nanocomposite poly(lactic acid) film. *European Polymer Journal, 98*(November), 362–367. https://doi.org/10.1016/j.eurpolymj.2017.11.034.

Wang, S. F., Shen, L., Tong, Y. J., Chen, L., Phang, I. Y., Lim, P. Q., ... Liu, T. X. (2005). Biopolymer chitosan/montmorillonite nanocomposites: Preparation and characterization. *Polymer Degradation and Stability, 90*(1), 123–131. https://doi.org/10.1016/j.polymdegradstab.2005.03.001.

Woranuch, S., & Yoksan, R. (2013). Eugenol-loaded chitosan nanoparticles: I. Thermal stability improvement of eugenol through encapsulation. *Carbohydrate Polymers, 96*(2), 578–585. https://doi.org/10.1016/j.carbpol.2012.08.117.

Wu, Y., Rhim, J. W., Weller, C. L., Hamouz, F., Cuppett, S., & Schnepf, M. (2000). Moisture loss and lipid oxidation for precooked beef patties stored in edible coatings and films. *Journal of Food Science, 65*(2), 300–304. https://doi.org/10.1111/j.1365-2621.2000.tb15997.x.

Xiao, C., Zhu, L., Luo, W., Song, X., & Deng, Y. (2010). Combined action of pure oxygen pretreatment and chitosan coating incorporated with rosemary extracts on the quality of fresh-cut pears. *Food Chemistry, 121*(4), 1003–1009. https://doi.org/10.1016/j.foodchem.2010.01.038.

Yang, F., Hu, S., Lu, Y., Yang, H., Zhao, Y., & Li, L. (2015). Effects of coatings of polyethyleneimine and thyme essential oil combined with chitosan on sliced fresh channa argus during refrigerated storage. *Journal of Food Process Engineering, 38*(3), 225–233. https://doi.org/10.1111/jfpe.12155.