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Effect of chitosan-essential oil films on the storage-keeping quality of pork meat products

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Abstract

Edible films based on chitosan were prepared, with and without basil or thyme essential oils, with the aim of assessing their protective ability against lipid oxidation and their antimicrobial activity. Chitosan films had good oxygen barrier properties, which were worsened by essential oil addition, especially when the film equilibrium moisture content increased. Due to the oxygen barrier effect, all the films effectively protected pork fat from oxidation, in comparison to unprotected samples. In spite of the worsening of the oxygen barrier properties, the films with essential oils were more effective than those of pure chitosan, which points to the chemical action of specific antioxidant compounds of the oils. Films were effective to control microbial growth in minced pork meat although the incorporation of essential oils did not improve their antimicrobial activity. Throughout the storage, the films led to colour changes in minced pork meat associated with the conversion of myoglobin into metmyoglobin due to the reduction of the oxygen availability.

Keywords: Chitosan; essential oil; microfluidization; edible coating; pork meat; antioxidant; colour; antimicrobial

1. Introduction

Microbial growth is generally responsible for the spoilage in meats and meat products together with biochemical and enzymatic deteriorations (Devlieghere et al., 2004). In fact, bacterial contamination is one of the main factors determining the loss in fresh-meat quality, since these products are very prone to be contaminated with microorganisms if they are not properly preserved and handled.

Oxygen has a negative effect on the quality of many food products. In the case of the oxidative deterioration of fats and oils, it is responsible for rancid odours and flavours, with a decrease in nutritional quality and safety caused by the formation of potentially toxic compounds (Moure et al., 2001).

The most commonly-used strategy used to extend fresh meat shelf-life is the use of antimicrobial and antioxidant additives of synthetic origin, which are being questioned due to an increasing consumer demand for natural, healthy and safe preservatives. It is reported that essential oils (EO) exhibit their

antimicrobial activity against food borne pathogens by interfering and destabilizing the phospholipid bilayer of the cell membrane, the enzyme systems, and the genetic material of bacteria (Kim et al., 1995; Burt, 2004). Thyme essential oil contains more than 60 ingredients, most of which have important antioxidant and antimicrobial properties (Baranauskiene et al., 2003). Thymol, rosmarinic acid and carvacrol are the most important active compounds of thyme EO (Di Pasqua et al., 2006; Shan et al., 2005). Several *in vitro* studies demonstrated that these compounds exhibit antimicrobial activity against a broad spectrum of both gram negative and gram positive bacteria (Burt & Reinders, 2003; Gaysinsky et al., 2005; Singh et al., 2001). Moreover, studies carried out on minced pork meat (Aureli et al., 1992) and feta cheese (Govaris et al., 2011) showed the strong antibacterial activity of thyme EO against *Listeria monocytogenes*. Basil EO has revealed an antibacterial effect against different bacteria, including common meat spoilage microorganisms such as *Escherichia coli, Enterobacter aerogenes, E. aglomerans* and *L. innocua* (Wan et al., 1998).

In order to achieve effective antimicrobial activity, high concentrations of EOs are generally needed, which might confer inappropriate flavours and odours on the food product (Seydim & Sarikus, 2006). The incorporation of EOs in edible films is an interesting alternative to improve coating functionality, minimizing the problems related with EOs intense aroma (Sánchez-González et al., 2011a). Apart from the possibilities of encapsulating EOs, the use of edible films and coatings has proven to be a useful technique to extend the shelf-life of food products (Vargas et al., 2006; Vargas et al., 2009; Atarés et al., 2011). When the added ingredients exhibit antioxidant properties, the action of such films involves two different mechanisms: the oxygen barrier effect – reducing the oxygen availability in the product- and the specific activity of the incorporated antioxidant agents (Bonilla et al., 2012a).

Chitosan (CH) is an antimicrobial biopolymer that can be used as a matrix to obtain edible films containing essential oils (Sánchez-González et al., 2010; Bonilla et al., 2012 b). The antibacterial effect of CH-oregano EO films was proved in bologna slices inoculated with *L. monocytogenes* or *E. coli* O157:H7 (Zivanovic et al., 2005). Nevertheless, to the best of our knowledge, neither pure chitosan-based films nor those prepared in combination with thyme or basil essential oils have yet been applied to fresh minced pork meat. The aim of this work was to study the antibacterial and antioxidant properties of edible chitosan films containing basil or thyme essential oils. The films were prepared following two different homogenization treatments and with two essential oil concentrations, and were assessed as to their oxygen permeability under different conditions. Their protective ability against oxidation and bacterial

growth in meat products was evaluated in two cases which can be considered representative of these kinds of products, i.e. pork fat and minced pork meat.

2. Materials and methods

2.1. Reagents

High molecular weight chitosan (Batch 12913CJ/ Batch MKBB0595, Sigma-Aldrich Quimica, Madrid, Spain) was used to prepare the film-forming dispersions (FFD) (0.8 Pa s viscosity, at 1% w/w in 1% w/w glacial acetic acid). Basil and thyme essential oils were provided by Herbes del Molí (Alicante, Spain). Acetic acid, starch, $Mg(NO_3)_2$, KCl, KI, and K_2CO_3 were purchased from Panreac Química, S.A. (Castellar del Vallés, Barcelona, Spain).

2.2. Preparation and characterization of chitosan-based films

High molecular weight CH was dispersed at 1 %wt in an acetic acid solution at 1 % (v/w). To prepare the composite films, basil (B) or thyme (T) essential oils were added to the chitosan solution at 0.5 or 1 wt%. The resulting formulations were referred to as CH, CH:$B_{0.5,}$ CH:$B_{1.0}$ CH:$T_{0.5}$ and CH:$T_{1.0}$. The mixtures were homogenized by means of a rotor-stator (DI25, Yellow Line, IKA®, Germany) at 21,500 rpm for 4 minutes (H1 treatment). Homogenization treatment H2 was performed by using H1 and afterwards high-pressure homogenization at 165 MPa in a single pass by means of a Microfludizer® M110-P processor (Microfludics, Newton, USA). Films were obtained by casting in PTFE plates (15 cm in diameter) and drying at 25 ± 2ºC and 60 ± 2% RH. The surface density of solids in the dry films was 56 mg/cm^2. Films were peeled off from the casting plates and conditioned at 58 ± 1% RH and 10 ± 1ºC for one week. A Palmer digital micrometer (Comecta, Barcelona, Spain) was used to measure film thickness to the nearest 0.001mm at six random positions.

The oxygen permeability tests were conducted following the ASTM D3985 Standard Method (1995), by measuring the oxygen transference rate with an Ox-Tran 1/50 system (Mocon, Minneapolis, USA). The films were exposed to pure nitrogen and oxygen flows on either side. The tests were performed in continuous mode at 10ºC-58% RH, 40ºC-43% RH and 40ºC-83% RH. Oxygen permeability (OP) was calculated by dividing the oxygen transmission rate (OTR) by the difference in oxygen partial pressure between the two sides of the film, and multiplying by the film thickness. Two replicates per formulation were made.

2.3. Protective ability of the films against oxidation of pork fat

Pure fresh pork fat (lard) was purchased in a local market and kept in refrigeration until the experiment was started (less than 24h). Inert cups (60 mm diameter, 48.5 mm depth) were totally filled with fat, which was compressed to ensure the absence of air bubbles. CH, CH:$B_{1.0}$ and CH:$T_{1.0}$ were tested, all of them prepared using the H1 homogenization procedure. Film disks were cut and secured to the cups as a lid. The side of the film in contact with the plate during drying was in direct contact with the fat. The cups were stored at 40ºC-43%RH and 40ºC-83%RH. After 15, 40 and 60 days of storage, the extent of oxidation was evaluated by determining the peroxide value (PV). Pork fat (1 g) was dissolved in a mixture of isooctane and acetic acid (2:3 v/v). 0.5 mL of an oversaturated KI solution were added. The mixture was stirred for 1 minute and 15 mL of distilled water were added. Titration with sodium thiosulfate solution (0.002 N) followed, using starch as indicator. PV was expressed as mEq of oxygen per kilogram of pork fat. All analyses were performed in triplicate.

2.4. *Protective ability of the films in minced pork meat*

Pork meat was obtained from a local supermarket and was processed immediately after arriving at the laboratory. A whole-muscle cut from one pork carcass was ground with a mincer (Severin Elektrogeräte GmbH, Sundern, Germany) and moulded in Petri dishes to obtain the test samples. The surface of both sides of the samples was coated with the films prepared using H1 and H2. Non-coated and coated samples were placed in PET trays (Cubil, Barcelona, Spain) and were stored at 10ºC. Colour measurements of minced pork meat samples were taken at different storage times by using a spectrocolorimeter CM-3600d (Minolta Co, Tokyo, Japan) with a 30 mm diameter window. Immediately before the measurements, the films were peeled off the coated minced pork samples. CIE-L^* a^* b^* coordinates and chromatic parameters, hue (h^*_{ab}) and chrome (C^*_{ab}), were obtained from the reflection spectra of the samples, using D65 illuminant /10º observer (CIE, 1986). The colour of each sample was measured in quintuplicate.

To evaluate the antimicrobial effect of the films, stock cultures of *E. coli* (CECT101) and *L. inoccua* (CECT 910) were supplied by the Spanish Type Culture Collection (CECT, Burjassot, Spain) and kept frozen (−25°C) in Tryptone Soy Broth (TSB, Scharlab, Barcelona, Spain) supplemented with 30% glycerol (Panreac, Barcelona, Spain). The cultures were regenerated by transferring a loopful of each bacterium into 10 mL of TSB or BHI broth and incubated at 37 °C overnight. A 10 µl aliquot from each overnight culture was again transferred into 10 mL of TSB or BHI broth and grown at 37°C up to the end of the exponential phase of growth. The culture was diluted to obtain 10^3 CFU/mL. This inoculum (1 mL) was poured on the surface of pork meat samples.

To perform the microbiological analyses, 10 g of each coated and non-coated minced meat sample were aseptically obtained and homogenized in a Stomacher with 90 mL of sterile buffered peptone water for 2 min. Aliquots were serially diluted in buffered peptone water and plated out. In non-inoculated meat samples, the total viable counts were determined in Plate Count Agar plates incubated at 37ºC for 24h. Coliform counts were determined using Violet Red Bile Agar plates incubated at 37ºC for 48h. In inoculated samples, dilutions and counts of bacteria were performed by using the specific broth and agar for each bacterium. Every microbial analysis was performed in triplicate in at least two samples per formulation. All the culture media were supplied by Scharlau (Scharlab S.L, Barcelona, Spain).

2.5. Statistical analyses

The results were analysed by means of a multifactor analysis of variance with a 95% significance level using Statgraphics®Plus 5.1. (Manugistics Corp., Rockville, Md., USA). Multiple comparisons were performed through 95% Least Significant Difference intervals (LSD).

3. Results and discussion

3.1. Oxygen permeability of the films

Oxygen permeability (OP) of the films under different conditions are shown in Table 1. These conditions were selected with the aim of simulating the behaviour of the films when applied to refrigerated meat products (10ºC-58%RH). All the films had rather low OP values, in the range of those reported by other authors **(**Di Pierro et al., 2011; Caner et al., 1998; Casariego et al., 2009**).** The good oxygen barrier properties of chitosan films have been previously observed and, in fact, their oxygen permeability has been described as being comparable with those of existing commercial synthetic films, such as polyvinylidene chloride or ethylene vinyl alcohol copolymer films (Casariego et al., 2009). In pure chitosan films, the homogenization procedure had a significant impact on OP ($p<0.05$). The films prepared with H2 procedure were significantly less permeable to oxygen than those prepared with simple homogenization (H1). This is probably due to an improvement in the degree of aggregation of the polymer chains in the chitosan matrix. Bonilla et al. (2012b) studied the microstructure of chitosan-essential oil edible films elaborated with different homogenization procedures and found that pure chitosan H2 films had a more homogeneous structure than those obtained with H1 procedure.

The incorporation of both basil and thyme essential oils slightly affected OP values at 10ºC and 58% RH. When FFD was submitted to H1 treatment, essential oil addition resulted in a small reduction of OP. The addition of thyme EO at the highest ratio led to a significant improvement of the oxygen barrier properties

at both homogenization treatments, as compared to the CH films. A chemical oxygen blocking effect produced by the antioxidant components of the EO could be responsible for this improvement in the oxygen barrier properties. This is in agreement to that observed in previous studies for cornstarch-sodium caseinate films containing α-tocopherol (Jimenez et al., 2003) and chitosan-wheat starch films enriched with thyme or bergamot essential oils (Bonilla et al., 2013).

3.2. Protective ability of the films against oxidation of pork fat

Figure 1a shows the progression of the peroxide values (PV) of pork fat, both uncoated and coated with the three H1 treated films (CH, CH:$B_{1.0}$, CH:$T_{1.0}$**),** throughout storage time at 40ºC-43%RH**.** The results of a parallel experiment, performed at 83% RH, are shown in Figure 1b. As expected, the sample oxidation levels, as well as the oxidation rate, increased in line with storage time. Likewise, the oxidation progressed more intensely in samples stored at 80% relative humidity, especially in uncoated samples. At 60 storage days, statistical analysis revealed that RH have a significant effect on the PV of uncoated samples ($p<0.05$), possibly due the effect of water availability on fat oxidation (Labuza, 1980). On the contrary, in the case of samples coated with chitosan films, RH did not have a significant effect ($p>0.05$) on the PV values, although slightly higher PV values were found at high RH, which may be linked to the increase in the OP of CH films (shown in the legend of figures 1a and 1b). At 40ºC-43%RH all films had low OP, in the range of the values obtained at 10ºC-58% (Table 1). However, at 40ºC-83%RH, OP greatly increased, mainly due to the fact that the film moisture content was higher when equilibrated under much higher RH conditions. The increase in both, temperature and film moisture, implied greater molecular mobility in the system. This favours mass transfer properties, thus affecting the OP of the films, making them more permeable (Han & Gennadios, 2005; Bonilla et al., 2012a). At 83% RH, the incorporation of EOs provoked a significant increase in the OP values, whereas this was observed only as a tendency for films equilibrated at 43% RH. This can be explained by the greater oxygen solubility in the non-polar oil phase, which contributes to increase the transfer rate of the oxygen molecules into the plasticized polymer matrix. This effect of the dispersed liquid oil phases on the OP has been previously observed in different film matrices, such as hydroxy-propyl-methylcellulose (Atarés et al., 2011) and sodium caseinate (Fabra et al., 2012), and it is more appreciable when the continuous matrix favours mass transfer due to its high plasticization level.

In comparison with the uncoated samples, all the edible films had a protective effect against lipid oxidation, at both RH conditions. The latter was more pronounced at longer storage times, when the

process is more advanced. At this storage time, RH had a significant effect on the PV values of unprotected samples and those protected with CH ($p<0.05$). The highest RH promoted the highest PV values, which is related with the highest OP under these conditions, as mentioned above. Generally, PV values were lower in samples coated with films containing essential oils (except at 60 days and 43% RH) as compared to CH films. This reveals the antioxidant chemical effect of the active compounds of the essential oils, since OP values of these films were generally higher. This antioxidant action of the essential oils seemed to disappear at 60 storage days when samples were stored at 43%RH, which could be due to the progressive evaporation of the active compounds in this drier atmosphere or to the progressive oxidation of the antioxidant compounds. No significant differences depending on the oil used were observed.

3.3. Effect of the films on colour development and microbial counts of minced pork meat.

The surface colour of non-coated and coated minced pork meat samples was monitored in terms of CIEL*a*b* colour coordinates obtained from the sample reflection spectra. The samples showed initial values of 49, 65 and 16.3 for luminosity, hue and chrome, respectively. The changes in the colour parameters of non-coated and coated samples during cold storage are reported in Table 2. According to ANOVA, storage time was the factor that had the highest influence on colour changes (higher F-ratio values in the LSD test). Although non-coated samples hardly underwent any colour changes during storage, after 7 storage days they did appear with a more vivid (higher chrome values), red (lower hue values) colour. After 2 storage days, the sample coating scarcely affected the colour but led to a slight decrease in hue (redder samples) and chrome (more vivid colour) values. Nevertheless, after 7 storage days, coated samples suffered a significant increase in luminosity and hue (less red colour) and a significant decrease in chrome values (brownish colour development), which could be a drawback in terms of consumer acceptance. This can be related with the conversion of myoglobin into metmyoglobin due to the low oxygen pressure induced by coating (Mancini and Hunt, 2005). Neither the film composition nor the homogenization conditions of the film-forming solutions were found due to bring about any significant differences in the colour of the coated samples.

The effect of different chitosan-based films on the growth and survival of total aerobic mesophiles and coliform microorganisms at 10ºC is shown in Figure 2. CH films were the most effective at reducing microbial counts and a growth inhibition was observed in both mesophilic and coliform microorganisms through practically the whole storage period. The incorporation of essential oils into the films implied a

reduction in the antimicrobial effectiveness, which suggests that the essential oils used are less effective as antimicrobials against the natural microbial load of minced meat than chitosan. The effective reduction in the chitosan ratio in films containing essential oils can explain the decrease in the antimicrobial effectiveness caused by a dilution effect, as reported by Sánchez-González et al. (2011b).

The effect of chitosan-based films on the growth and survival of *Escherichia coli* and *Listeria inoccua* on inoculated and non-inoculated (control) minced pork meat samples stored at 10° C is shown in Figure 3. In the case of these microorganisms, there were not observed differences between the antimicrobial effect of chitosan films with and without essential oils, which suggests that both polymers and oils exhibited a similar degree of effectiveness. At the end of storage, the films led to a significant reduction in microbial load as compared to non-coated samples (about 2 and 4 log CFU/g, for *L. innocua* and *E. coli*, respectively) which was more marked in samples inoculated with *E. coli.* This coincides with the results found when pure chitosan films were applied to pork meat hamburgers (Vargas et al., 2011) or to intermediate moisture meat products (Rao et al., 2005).

The results obtained reflect the fact that essential oils did not lead to an increase in the antimicrobial effect of the films in minced pork meat, although they can improve the quality preservation through their antioxidant activity. On the other hand, although the total counts of mesophilic and coliform microorganisms are greater when the films contain essential oils, there was not observed a negative effect on the microbial control of *L. inoccua* and *E. coli.*

4. Conclusions

The addition of EOs to CH films did not improve their antibacterial efficiency on meat products. In general, EOs incorporation and homogenization type did not have a clear effect on OP values, all values being very low. However, CH-EOs films effectively protected pork fat against lipid oxidation, which points to the specific chemical action of the oil's antioxidant compounds. The reduction in the oxygen availability in minced meat provoked by sample coating led to the expected changes in colour associated with the conversion of myoglobin into metmyoglobin. However, quality and safety aspects were improved. Therefore, CH- EOs films may be applied to meat products, increasing their shelf-life and safety. Additional works should be carried out to improve the performance of these films.

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Table 1: Oxygen permeability (cm^3 mm m^{-2} atm^{-1} day^{-1}) under refrigerated conditions and at intermediate relative humidity (10°C-58%RH) of all the films obtained by both homogenization procedures (rotor-stator: H1; or additionally microfluidized: H2). Average values and standard deviations in brackets.

Film	Homogenization	
	H1	H2
CH	0.15 (0.04)^{x,bc}	0.091 (0.009)^{y,bc}
CH:$B_{0.5}$	0.094 (0.009)^{x,ab}	0.115 (0.002)^{x,ab}
CH:$B_{1.0}$	0.142 (0.012)^{x,bc}	0.0938 (0.0008)^{x,bc}
CH:$T_{0.5}$	0.117 (0.010)^{x,c}	0.149 (0.011)^{x,c}
CH:$T_{1.0}$	0.118 (0.006)^{x,a}	0.053 (0.002)^{y,a}

Different letters indicate significant differences ($p<0.05$) due to film formulation (a, b, c, d) or due to homogenization treatment (x, y). Chitosan: CH, Basil: B, Thyme: T. Sub-index in film formulation stands for chitosan:essential oil ratio.

Table 2. Luminosity (L*), hue (h^*_{ab}) and chrome (C^*_{ab}) values of refrigerated minced pork meat samples coated with the different films obtained by both homogenization procedures (rotor-stator: H1; or additionally microfluidized: H2). Average values and standard deviations in brackets.

		L^*		**h^*_{ab}**		**C^*_{ab}**	
	Storage time (days)	**2**	**7**	**2**	**7**	**2**	**7**
	Non-coated	47.9 (0.4)	48.4 (1.3)	62.6 (1.3)	57 (3)	16.3 (0.6)	17.86 (1.00)
H1	CH	44.9 (0.2)	51.0 (0.8)	57.1 (0.3)	66.5 (1.4)	19.9 (0.8)	16.1 (0.4)
	CH:$B_{0.5}$	45.00 (0.3)	49.9 (0.4)	55.9 (0.5)	65.0 (0.7)	19.7 (0.3)	16.5 (0.2)
	CH:$B_{1.0}$	46 (0.5)	50.0 (0.6)	57.0 (0.2)	62.5 (0.6)	20.27 (0.14)	15.67 (0.11)
	CH:$T_{0.5}$	44.8 (0.4)	49.2 (1.1)	55.59 (1.02)	63.98 (1.07)	20.4 (0.3)	17.4 (0.3)
	CH:$T_{1.0}$	44.9 (0.3)	49.7 (0.5)	56.0 (0.2)	64 (2)	20.3 (0.5)	16.74 (0.18)
H2	CH	45 (0.8)	50.2 (1.3)	59.6 (0.8)	66.14 (2.09)	17.6 (0.4)	16.8 (0.5)
	CH:$B_{0.5}$	44.5 (0.7)	49.3 (0.8)	59.2 (1.8)	63.3 (0.9)	18.96 (0.71)	16.8 (0.2)
	CH:$B_{1.0}$	45.9 (0.9)	50.8 (0.8)	57.8 (1.9)	63.6 (0.4)	19.45 (0.80)	15.7 (1.2)
	CH:$T_{0.5}$	46.6 (0.4)	50.8 (0.2)	59.5 (1.8)	66.4 (1.9)	19.62 (0.78)	16.1 (0.6)
	CH:$T_{1.0}$	47.6 (0.5)	51.5 (0.7)	61.3 (3.2)	65.8 (1.9)	17.8 (0.4)	16.1 (0.2)

F-ratio values for L^* (homogenization: 24.30, film: 8.51, time: 1112.46); h^*_{ab} (homogenization: 34.64, film: 4.74, time: 415.23) and C^*_{ab} (homogenization: 47.62, film: 5.61,time: 672.09).
Chitosan: CH, Basil: B, Thyme: T. Sub-index in film formulation stands for chitosan:essential oil ratio.

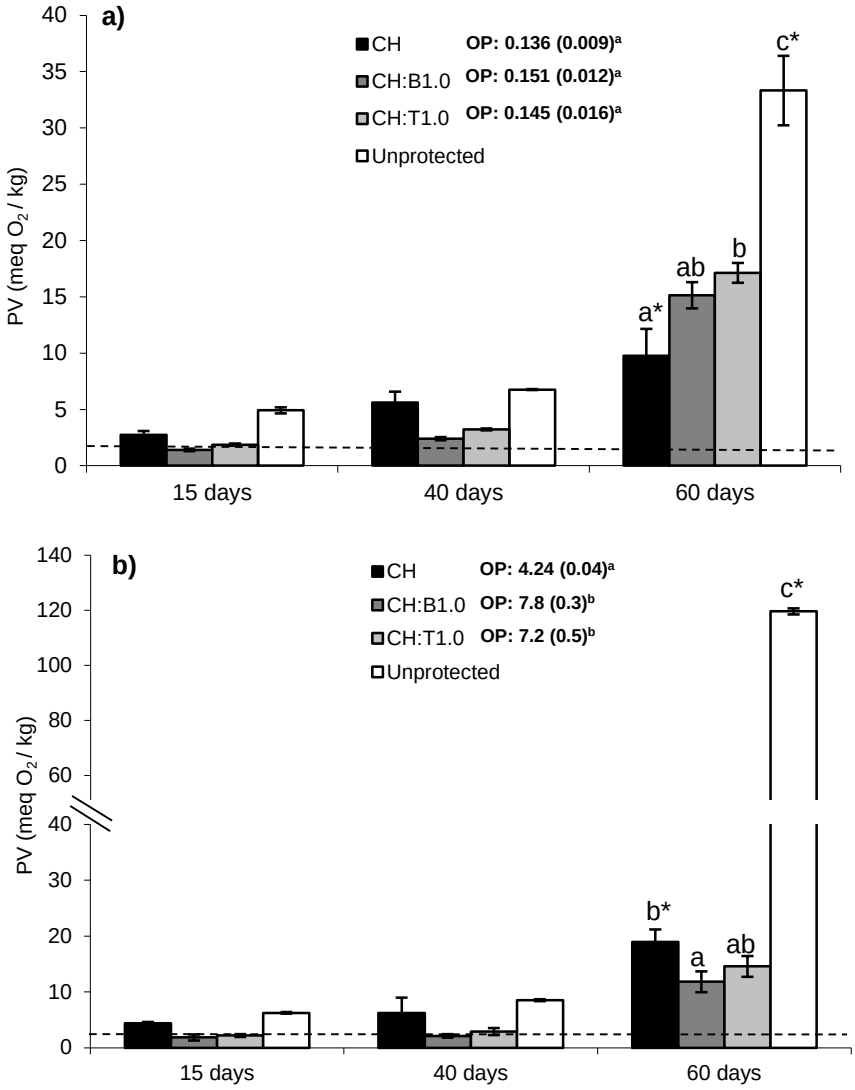

Figure 1. Progression of the peroxide value (PV) of fat samples, both uncoated and coated with films (CH = chitosan, CH:B1.0 = chitosan and basil essential oil 1:1 w/w, CH:T1.0 = chitosan and thyme essential oil 1:1 w/w) throughout storage at (a) 40ºC and 43% RH and (b) 40ºC and 83% RH. The dotted line represents the initial PV of the fat. Oxygen permeability (cm^3 mm m^{-2} atm^{-1} day^{-1}) of the films is indicated in the legend (average values and standard deviation in brackets). Different letters (a, b, c) indicate significant differences ($p<0.05$) due to film formulation. The asterisk indicates significant differences ($p<0.05$) due to RH.

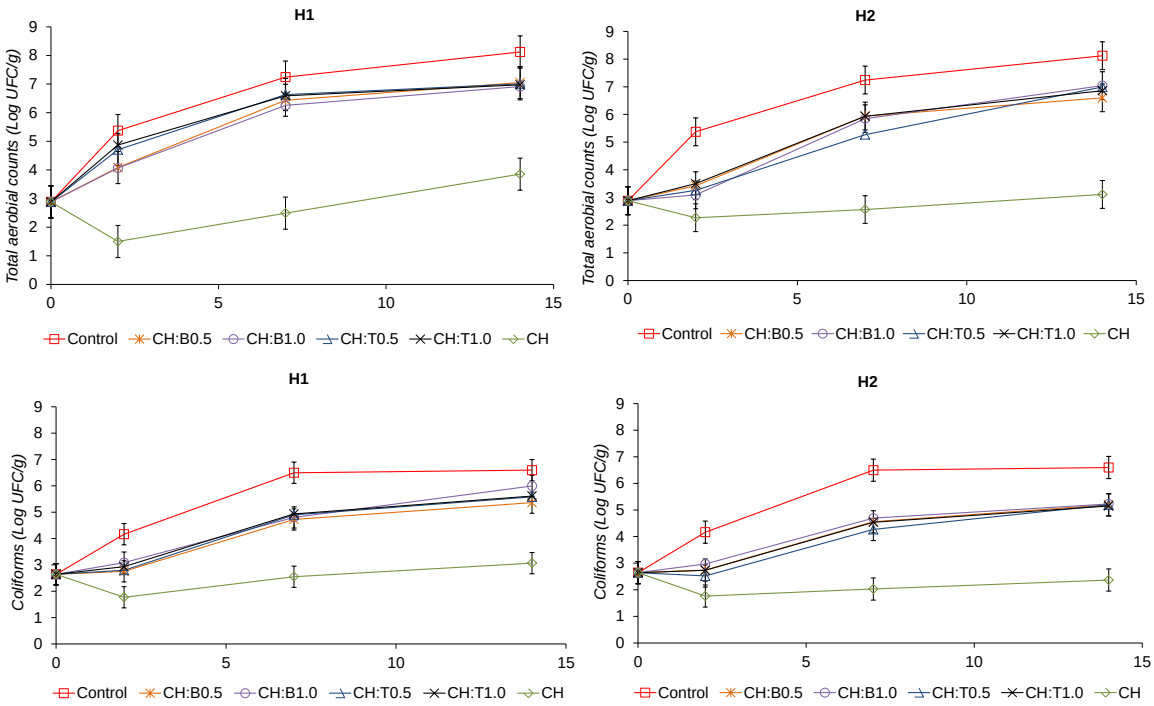

Figure 2. Microbial counts of minced pork meat samples coated with chitosan-based edible films as a function of storage time at 10ºC. Mean values and 95% LSD intervals. Control = non-coated samples.

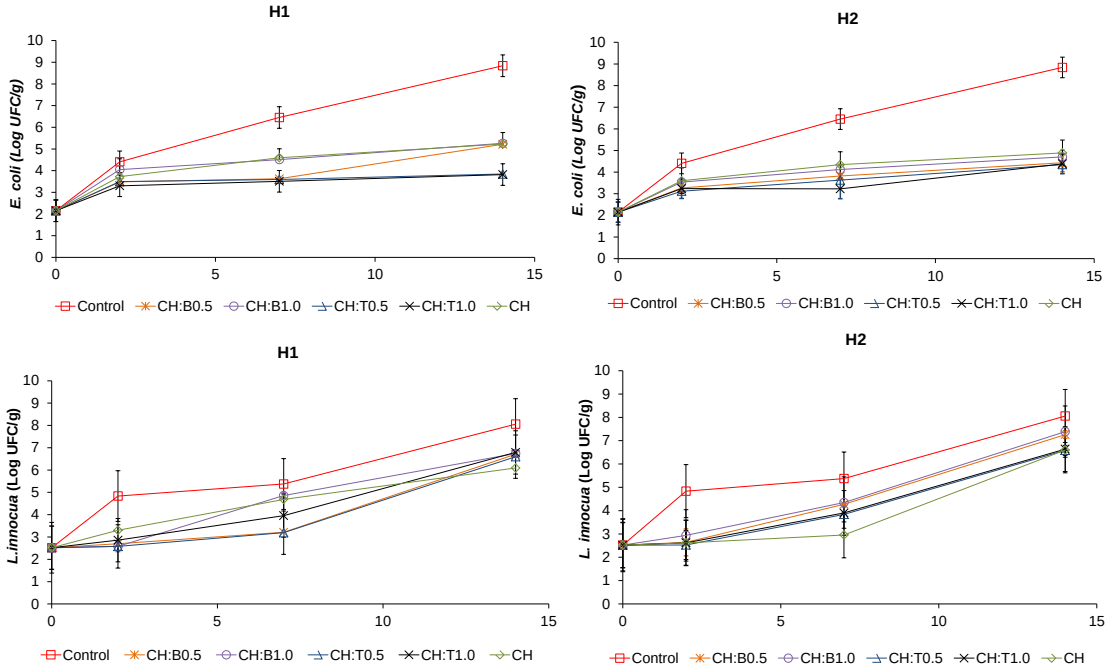

Figure 3. Microbial counts of minced pork meat samples inoculated with *L. innocua* or *E. coli* coated with chitosan-based edible films as a function of storage time at 10ºC. Mean values and 95% LSD intervals. Control = non-coated samples.