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Films of chitosan and chitosan-oligosaccharide neutralized and thermally treated:  
Effects on its antibacterial and other activities

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1 FILMS OF CHITOSAN AND CHITOSAN-OLIGOSACCHARIDE NEUTRALIZED AND  
2 THERMALLY TREATED: EFFECTS ON ITS ANTIBACTERIAL AND OTHER  
3 ACTIVITIES

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15

16 ABSTRACT:

17 The present study focuses on the effects of heat and neutralization treatments on  
18 solubility, water vapour permeability and antimicrobial activity of chitosan (Ch) and  
19 chitosan/chitoooligosaccharide (ChO)-based films. ChO films showed stronger  
20 antimicrobial activity against *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*,  
21 *Serratia liquefaciens* and *Lactobacillus plantarum* than Ch films, indicating that this  
22 effect is attributed to the presence of chitoooligosaccharides (COS) in the films. Heat  
23 and neutralization treatments decreased significantly the solubility of chitosan films and  
24 gave rise to a sharp loss in their antimicrobial activity. The incorporation of COS in  
25 chitosan films increased the inhibitory effect against the studied microorganisms  
26 without affecting significantly the water vapour permeability of the films. Thus, it is  
27 possible to get a more insoluble chitosan film with high antimicrobial activity by means  
28 of incorporation of COS combined with heat or neutralization treatments.

29 KEYWORDS:

30 Chitosan, chitooligosaccharide, antimicrobial activity, heat treatment, neutralization

31

32 1. Introduction

33 Nowadays there is an increasing interest in biodegradable/compostable polymers from  
34 renewable sources due to environmental problems caused by conventional food  
35 packaging materials (Leceta, Guerrero & de la Caba, 2013). The problems in disposing  
36 of huge quantities of waste generated by non-biodegradable food packaging have led  
37 to the study of biopolymers as materials to be used as films and coatings in food  
38 packaging (Azeredo, Miranda, Ribeiro, Rosa & Nascimento, 2012).

39 Development of materials from biopolymers for different applications have been a hot  
40 topic for several years, due to increasing prices of petroleum, a non-renewable  
41 resource with diminishing quantities (Ruban, 2009; Souza, Cerqueira, Martins,  
42 Casariego, Teixeira & Vicente, 2010), and increasing environmental concerns. This  
43 approach will continue playing an important role in the food industry (Satyanarayana,  
44 Arizaga & Wypych, 2009).

45 Today, the use of polymers from renewable sources in food packaging is growing. The  
46 tendency is to use natural compounds to enlarge the shelf-life of all types of food  
47 increasing the preservation and protection from oxidation and microbial spoilage.

48 The natural polymers used in food packaging have the advantages to be available from  
49 replenishable resources, biocompatible, biodegradable, and all these characteristics  
50 lead to ecological safety (Prashanth & Tharanathan, 2007).

51 In this context, chitosan and its chitooligosaccharides (COS), which are known to  
52 possess multiple functional properties, have attracted considerable interest due to their  
53 biological activities and potential applications in the food, pharmaceutical, agricultural  
54 and environmental industries. Both have inherent antimicrobial activity owing to the fact  
55 that long positively charged chitosan molecules interact with negatively charged  
56 bacteria (Zivanovic, Chi & Draughon, 2005). Chitosan is a versatile material with

57 proved antimicrobial activity. Three antibacterial mechanisms have been proposed  
58 (Goy, de Britto & Assis, 2009): i) the ionic surface interaction resulting in wall cell  
59 leakage (Liu, Du, Wang & Sun, 2004). In this model, the interaction is mediated by the  
60 electrostatic forces between the protonated  $\text{NH}_3^+$  groups and the negative residues; ii)  
61 the inhibition of the mRNA and protein synthesis via the penetration of chitosan into the  
62 nuclei of the microorganisms (Sebti, Martial-Gros, Carnet-Pantiez, Grelier & Coma,  
63 2005). The chitosan molecules are assumed to be able to pass through the bacteria  
64 cell wall and reach the plasma membrane; and iii) the formation of an external barrier  
65 chelating metals and provoking the suppression of essential nutrients to microbial  
66 growth (Cuero, Osuji & Washington, 1991). It is well known that chitosan has excellent  
67 metal-binding capacities where amine groups are responsible for the uptake of metal  
68 cations by chelation. It is likely that all events occur simultaneously but at different  
69 intensities.

70 Besides, chitosan is a non-toxic compound and another fascinating advantage of this  
71 compound is the film-forming capacity that it presents, which allows its application  
72 directly as a film or as a coating without the necessity of a carrier matrix (Fernandez-  
73 Saiz, Soler, Lagaron & Ocio, 2010). Chitosan films are regarded as biofunctional  
74 material, well tolerated by living tissues, particularly applicable as edible films/coatings  
75 to prolong shelf-life and preserve quality of fresh foods.

76 Moreover, there is a growing interest to develop materials with antimicrobial properties  
77 in order to prevent alterations in food caused by microbial spoilage.

78 On the other hand, as food quality and safety are major concerns in the food industry,  
79 there is also a need for an efficient method for the delivery of preservatives into foods.

80 Addition of compounds directly into food is an established practice with some  
81 disadvantages. Instant addition of antimicrobials in formulation often results in instant  
82 inhibition of non-desired microorganisms. However, the surviving microorganisms will  
83 continue growing, especially when the concentration of antimicrobials added to the  
84 formulation will get depleted. This may be due to complex interactions with the food

85 matrix, or by natural degradation over time causing short shelf-life. To overcome this  
86 issue, antimicrobial packaging can be considered a modern technology that could have  
87 a significant impact on shelf-life extension and food safety. Use of antimicrobial agents  
88 in food packaging can control the microbial population and targets specific  
89 microorganisms to provide higher safety and quality products. Many classes of  
90 antimicrobial compounds have been evaluated in film structures, synthetic polymers  
91 and edible films. Among them, COS have received much more interest because they  
92 are not only water-soluble, but also possess distinctive biological activity such as  
93 antifungal and antibacterial activity, immuno-enhancing effects, and antitumor effects.  
94 Studies on the biological activities of chitosan and its oligomers have been increasing,  
95 as no single type of chitosan or its oligomers exert all of the above mentioned activities.  
96 Moreover, different chitosan derivatives and enzymatic products have different  
97 structures and physicochemical properties, which may result in novel bioactivities or  
98 novel findings in known bioactive compounds (Xia, Liu, Zhang & Chen, 2011).

99 Several reports discuss the antimicrobial activity of chitosan, demonstrating different  
100 results depending on source of chitin, molecular weight, deacetylation degree, and the  
101 experimental methodologies used, but they all confirm that chitosan and its  
102 oligosaccharides have strong antimicrobial effects and are safe for human use. Hence,  
103 the antimicrobial characteristics of chitosan and its oligosaccharides present a  
104 profitable potential for developing natural food packaging materials and functional food-  
105 additives.

106 Chitosan is known to be a very hydrophilic material with very low water resistance. The  
107 biggest drawback in use of chitosan films is their hygroscopicity. In fact, this material  
108 may virtually dissolve in the presence of high moisture products. In food packaging, the  
109 dissolution of the biopolymer could compromise packaging structure, physical integrity  
110 and organoleptic or microbiological food quality aspects. Therefore, there are a number  
111 of strategies that have been used in literatures, such as crosslinking or blending with a  
112 more water resistant material, to reduce its water sensitivity (Fernandez-Saiz, Lagaron

113 & Ocio, 2009; Tang, Du & Fan, 2003). However, these alternatives to reduce the water  
114 effect on the polymer do also adversely alter its biocide properties, suggesting that both  
115 effects may well often be opposed. Therefore, further investigations on this issue are  
116 needed in order to develop formulations of chitosan with a proper balance of water  
117 resistance and antimicrobial properties.

118 Taking into account that there are not many reports about the effect of high  
119 temperatures and neutralization treatments on the functional properties of chitosan and  
120 its depolymerisation products (COS), we found interesting to study the effect of heat  
121 and neutralization treatments on antimicrobial activity of chitosan films alone, and with  
122 a COS incorporated in the formulation. Five representative bacteria, *Escherichia coli*  
123 and *Serratia liquefaciens* (Gram-negative), *Lactobacillus plantarum*, *Bacillus cereus*,  
124 and *Staphylococcus aureus* (Gram-positive), which are common spoilage bacteria for  
125 food contamination, have been tested.

126 Thus, the aim of this work is focused on analysing the addition of depolymerisation  
127 products, COS, and the effect of heat and neutralization treatments on functional  
128 properties and antimicrobial activity of chitosan-based films.

## 129 2. Materials and Methods

### 130 2.1. Materials

131 Commercial food-grade chitosan (PubChem CID: 21896651) with a molecular weight of  
132 169 kDa and a degree of deacetylation of 84% purchased from TRADES, S.A.  
133 (Barcelona, Spain) was utilized to obtain the films. Acetic acid (PubChem CID: 176,  
134 min. 99.8%, reagent grade, Scharlau, Spain) was used to fix the solution pH, and  
135 sodium hydroxide (PubChem CID: 14798, PA-ACS-ISO) used for neutralization, was  
136 provided by Panreac, Spain. All reagents were used as received.

137 Chitosan (DA 86%, *M<sub>w</sub>* 180 KDa) from fresh North Atlantic shrimp shells (*Pandalus*  
138 *borealis*) (Primex, Iceland) was purified and hydrolysed according to enzymatic  
139 depolymerisation (Mengibar, Mateos-Aparicio, Miralles & Heras, 2013) using  
140 chitosanase from *Streptomyces griseus* (EC 3.2.1.132) (Sigma-Aldrich, St. Louis, MO,

141 USA). COS (DA 83%,  $M_w$  8.6KDa) were separated by tangential ultrafiltration system  
142 Vivaflow 200 (Sartorius-Stedim Biotech, Goettingen, Germany) using polyetersulfone  
143 (PES) membranes with different molecular weight cut off size.

#### 144 2.2. Films preparation

145 Chitosan films (Ch) were prepared by casting, formed by solvent evaporation and the  
146 conversion of gelled solution rapidly to a solid film. A 10 g/L chitosan solution was  
147 prepared in a 10 g/L acetic acid aqueous solution. The chitosan solution was stirred at  
148 room temperature until it was completely dissolved, and then poured into multiwall  
149 plates and dried. The films used in the subsequent experiments were dried at 45 °C  
150 and 50% relative humidity and then peeled from the plates.

151 Chitosan-chitooligosaccharide films (ChO) were prepared in the same conditions but  
152 starting from two solutions: A 20 g/L chitosan solution in a 10 g/L acetic acid and a 20  
153 g/L COS solution in 10 g/L acetic acid, mixed at ratio 1:1 to get final solution of  
154 Chitosan 10 g/L-COS 10 g/L.

155 Two treatments were applied to the previous formed films: 1) heat treatment at 105 °C  
156 overnight and, 2) neutralization by spraying with  $13\mu\text{l}/\text{cm}^2$  of NaOH 0.25 mol/L.

#### 157 2.3. Total soluble matter (TSM)

158 Total soluble matter was measured by immersion in 25 mL of distilled water, with slight  
159 stirring at ambient temperature for 24 hours. After this time, samples were dried in an  
160 oven at 105 °C for 24 h. TSM was calculated in relation to the dry mass and it was  
161 expressed as the percentage of the film dry matter solubilized.

#### 162 2.4. Fourier transform infrared (FTIR) spectroscopy

163 Fourier transform infrared spectra of all films were carried out on a Performer  
164 SpectraTech spectrometer using ATR diamond crystal. A total of 64 scans were  
165 performed at  $4\text{ cm}^{-1}$  resolution. The measurements were recorded between 400 and  
166  $4000\text{ cm}^{-1}$ . The samples were measured at three points to check for film homogeneity  
167 and they yielded similar spectra.

#### 168 2.5. DSC measurements

169 Differential scanning calorimeter (DSC) measurements were performed using a TA  
170 Instruments (model DSC Q1000, New Castle, USA). The samples were scanned under  
171 a N<sub>2</sub> atmosphere from ambient temperature to 100 °C at a constant heating rate of 10  
172 °C /min. The weight film was 5-10 mg.

### 173 2.6. Scan Electron Microscopy (SEM)

174 The microstructure of films was observed by scanning electron microscopy (SEM). The  
175 samples were examined using a scanning electron microscope (JEOL JSM-6335,  
176 JEOL, Tokyo, Japan).

### 177 2.7. X-ray Diffraction (XRD)

178 X-ray diffraction patterns were obtained using an X-ray diffractometer (PHILIPS  
179 X'PERT SW) with a copper anode. The samples were scanned continuously from 0° to  
180 50° (2θ) at 45 kV and 40 mA.

### 181 2.8. Water Vapour Permeability (WVP)

182 WVP measurements were performed in a PERMATRAN W3/33 (Mocon), at 23 °C and  
183 50% relative humidity. The sample films were cut into a circle of 4 cm diameter and the  
184 test area was 5 cm<sup>2</sup>.

185 WVP was calculated as:

$$186 \quad WVP = WVTR \times \Delta P$$

187 Where WVTR is defined as:

$$188 \quad WVTR = \text{changing in weight (g)/time (h)} \times \text{test area (m}^2\text{)}$$

189 and ΔP is the partial pressure difference of the water vapour across the film.

### 190 2.9. Antimicrobial activity

191 The antimicrobial activity of all chitosan films was tested against the growth of five  
192 typical food spoilage bacteria; two Gram-negative: *E. coli* CECT45, *S. liquefaciens*  
193 CECT483, and three Gram-positive: *S. aureus* ATCC12599, *B. cereus* CECT148, and  
194 *L. plantarum* CECT220, obtained from the Spanish Type Culture Collection and the  
195 American Type Culture Collection. These strains were stored in nutritive broth with



196 20% glycerol at -80 °C until needed. For experimental use, the stock cultures were  
197 maintained by regular subculture to Plate Count Agar (PCA) or Man Rogosa and  
198 Sharpe (MRS) Agar at 4 °C and transferred monthly.

199 The antimicrobial effect of all Ch and ChO films was evaluated by determining the  
200 bacterial growth in nutrient broth. Mueller Hilton Broth (MHB) was used for *E. coli*, *B.*  
201 *cereus*, *S. aureus* and *S. liquefaciens* and Man Rogosa and Sharpe (MRS) Broth for *L.*  
202 *plantarum*. The assay was conducted in 2 mL of bacterial culture in exponential phase  
203 ( $10^5$  ufc/ml) with films pieces (sterilized with UV light) containing 0.01 g of chitosan in  
204 Ch films, and 0.01 g of chitosan and 0.01 g of COS in ChO films. Cell cultures were  
205 incubated for 24 hours at 37 °C for *E. coli*, *B. cereus*, *S. aureus*, and *L. plantarum* and  
206 at 26 °C for *S. liquefaciens*.

207 After incubation, serial decimal dilutions were prepared and spread onto fresh plates of  
208 PCA or MRS Agar. The number of colony-forming units (CFU) was assessed after  
209 plates had been incubated for 48 hours. Results were expressed as percentage of  
210 growth (Log (ufc/mL)) inhibition of bacteria respect to control without films.

## 211 2.10. Statistical analysis

212 Statistical analysis was performed with R program. Analysis of variance (ANOVA) and  
213 the Tukey's multiple range test were performed to detect significant differences in the  
214 film properties. The significance level used was 0.05.

## 215 3. Results and Discussion

### 216 3.1. Total soluble matter

217 Chitosan and COS formed homogeneous solutions separately. When both solutions  
218 were mixed, the resulting liquid was completely clear. It would seem that chitosan  
219 solution is not affected by the incorporation of the mixture of COS. As Chitosan and its  
220 oligosaccharides are similar molecules with different molecular weights, the mix would  
221 only increase the polydispersity of the sample minimally, which may lead to a  
222 reorganization of the matrix network in solution. DSC thermograms (Figure 1) showed  
223 similar structures for both Ch and ChO films. Similar endothermic peaks attributed to

224 water loss represented the energy required to vaporize water present in both films.  
225 have not provided any relevant information about miscibility. Table 1 shows the total  
226 soluble matter (TSM) of Ch and ChO films, heat treated and neutralized. It can be seen  
227 that the films without any treatment were totally soluble.

228 However, when the films were heat-treated or neutralized, TSM values decreased  
229 significantly, indicating a change in the chemical structure of the film. Temperature  
230 promotes Maillard reaction which brings the browning of compounds due to the  
231 interactions between carbonyl groups and amino compounds. The increase of insoluble  
232 matter was related to the decrease of free amino groups (Umemura and Kawai, 2007),  
233 as it was observed by FTIR results shown below. On the other hand, heat can change  
234 the physical properties of chitosan (Leceta, Guerrero, Ibaburu, Dueñas & de la Caba,  
235 2013; Leceta, Guerrero & de la Caba, 2013) affecting its aqueous solubility, rheology,  
236 and appearance. The formation of chitosan films reduced the crystallization of chitosan,  
237 showing amorphous behaviour as it can be observed in Figure 2, where a wide  
238 diffraction peak at  $2\theta=20.2^\circ$  for heat treated and non-treated chitosan films can be  
239 seen. According to Rivero et al. (Rivero, Garcia & Pinotti, 2012), heat curing of chitosan  
240 films led to a structural change characterized by peaks located at  $2\theta = 15^\circ$  and  $20^\circ$ ,  
241 but in this study, heat treatment had not effect on the crystallinity of the films. For  
242 neutralized films, the insolubility was lower compared with the heat-treated films.  
243 Because of the treatment with NaOH, some of the protonated amine groups ( $-\text{NH}_3^+$ )  
244 were neutralized causing partial insolubility of the films (Fernandez-Saiz, Lagaron &  
245 Ocio, 2009).

### 246 3.2. FTIR spectroscopy

247 In order to study the interactions between functional groups in Ch and ChO films, FTIR  
248 analysis was carried out. Chitosan's typical structure shows in FTIR spectra  
249 characteristic absorption bands at  $1594$  and  $1650\text{ cm}^{-1}$ , attributed to amide II (N-H) and  
250 amide I (C=O) respectively, at  $1380\text{ cm}^{-1}$  due to the distorting vibration of C-CH<sub>3</sub>, and at  
251  $3441\text{ cm}^{-1}$  which indicates the -OH stretching vibration and the intramolecular

252 hydrogen bonding of chitosan molecules. Absorption bands of chitosan powder at 1594  
253 and  $1650\text{ cm}^{-1}$  usually shift to a lower wavenumber, at  $1634$  and  $1546\text{ cm}^{-1}$   
254 approximately, in the chitosan films, due to the relaxation of the chains. These are the  
255 wavenumbers where the bands can be approximately observed in Figure 3 for the films  
256 of this study. The two dominant bands centred at  $1546$  and  $1405\text{ cm}^{-1}$  are associated to  
257 carboxylate ions ( $-\text{NH}_3^+ \text{ } ^-\text{OOC}^-$ ) (Lagaron, Fernandez-Saiz & Ocio, 2007). The amine  
258 groups in this chemical form are referred to as “activated” or protonated amine groups,  
259 and are responsible for the biocide character of chitosan. It can be observed that the  
260 intensity of the band at  $1634\text{ cm}^{-1}$  (amide I) was always lower than the intensity of the  
261 band between  $1546\text{-}1558\text{ cm}^{-1}$  (amide II) for the films without treatment, as  
262 consequence of the presence of available protonated amine groups ( $-\text{NH}_3^+$ ) produced  
263 in the evaporation of solvent to form the films (Fernandez-Saiz, Ocio & Lagaron, 2006).  
264 However, when the films were submitted to treatment the difference in the intensity of  
265 those two bands became smaller for both studied processes (Figure 3). This result  
266 could indicate that crosslinking and Maillard reaction between carbonyl and amine  
267 group in the same chitosan chain could be promoted by temperature in the case of  
268 heat treated films, and also that there has been a decrease of the number of biocide  
269 groups ( $-\text{NH}_3^+$ ) as a consequence of neutralization treatment. This is in agreement with  
270 the decrease of solubility observed for treated films. According to Leceta et al. (Leceta,  
271 Guerrero, Ibaburu, Dueñas & de la Caba, 2013), temperature and relative humidity  
272 promote crosslinking and chemical reactions, such as the Maillard reaction. The early  
273 stage of Maillard reaction involves the formation of conjugates between the carbonyl  
274 group of the carbohydrate ends with the amine group in chitosan, producing a Schiff  
275 base, which subsequently cyclizes to produce the Amadori compound and insoluble  
276 polymeric compounds, referred as melanoidins. In the treatment with NaOH, the  
277 neutralization effect is joined to the formation of sodium salt of acetic acid.  
278 When the COS were introduced in the films (Figure 3), it was observed that in the heat  
279 treated films, the decrease in relatively intensity bands centred at  $1546$  and  $1405\text{ cm}^{-1}$

280 were less marked with respect to the neutralized ChO films, indicating the minor  
281 capacity to establish crosslinking in presence of short chains of polymer. However, the  
282 presence of COS in the films increases the number of protonated amine groups and  
283 carboxylate ions which are neutralized and form salts, reducing the intensity of these  
284 bands.

### 285 3.3. Water Vapour Permeability

286 Water vapour permeability is a key property for the films intended to be used as food  
287 packaging. The WVP values are shown in Table 1.

288 WVP was not affected by heat treatment, as it was also stated by Leceta et al. (Leceta,  
289 Guerrero, Ibaburu, Dueñas & de la Caba, 2013), who also reported changes in TSM  
290 but not in WVP for heat-treated chitosan films. Although heat treatment can reduce  
291 hygroscopicity, through crosslinking and Maillard reaction, and subsequently water  
292 vapour permeability, both the temperature and duration of heat treatment influenced  
293 the degree of heat induced changes in the films. As it was shown in X-ray Diffraction  
294 analysis (Figure 2), no changes in the microstructure of the films were observed. SEM  
295 analysis (Figure 4) also revealed that the internal microstructure of Ch and ChO films  
296 was homogenous, smooth and with relatively roughness and, in the heat-treated films,  
297 no irregularities caused by the crosslinking were observed.

298 On the contrary, for chitosan films, WVP was significantly increased due to  
299 neutralization, but in all cases, the values remained in the same range as the values  
300 reported by Park et al. (Park, Marsh & Rhim, 2002) at the same test conditions (25°C  
301 and 50% RH). Higher values reported by other authors (Leceta, Guerrero & de la  
302 Caba, 2013) can be attributed to the higher temperature and relative humidity of the  
303 measures (38°C and 90% RH).

304 Incorporation of COS in the film composition increased notably the WVP value, which  
305 can be attributed to the hydroxyl groups, which are hydrophilic and less resistant to  
306 water vapour transmission, since the polar groups attract migrating water molecules  
307 and thereby facilitate water transport, and also due to the shorter length of the COS

308 chains, which would facilitate the diffusion of water vapour through the film (Sun,  
309 Wang, Kadouh & Zhou, 2014).

### 310 3.4. Antimicrobial activity

311 Antimicrobial activities of Ch and ChO films in liquid culture medium are shown in Table  
312 2.

313 As it can be seen, the presence of Ch films affected the cell viability of the tested  
314 microorganisms inhibiting their growth. However, the inhibitory effect differed  
315 depending on the type of bacterium although the present study showed that there was  
316 not a clear difference between the Gram-positive and Gram-negative bacteria studied.  
317 The activity of chitosan and its derivatives or oligomers against different bacteria and  
318 fungi has already been widely reported. The mode of antibacterial activity is a complex  
319 process that differs among bacteria due to different cell surface characteristics. In  
320 several studies, stronger antibacterial activity was apparent against Gram-negative  
321 bacteria than Gram-positive, while in another study Gram-positive bacteria were more  
322 susceptible, perhaps as a consequence of the Gram-negative outer membrane barrier.  
323 Many works have already demonstrated that there were no significant differences  
324 between the antibacterial activities against the bacterium. Various initial reaction  
325 materials and conditions could contribute to the diverse results (Kong, Chen, Xing &  
326 Park, 2010).

327 The heat treated and neutralized Ch films presented lower antimicrobial activity than  
328 the non-treated ones. These results are in good agreement with FTIR results, which  
329 showed a reduction of the absorption bands at  $1546$  and  $1405\text{cm}^{-1}$ , attributed to the  
330 carboxylate groups, which in the literature (Leceta, Guerrero, Ibaburu, Dueñas & de la  
331 Caba, 2013; Lagaron, Fernandez-Saiz & Ocio, 2007) have been related to the  
332 antimicrobial character of chitosan. Chitosan shows optimum performance only in  
333 gelled or viscous acid solution form, when the amine groups are allegedly protonated  
334 or “activated” (Fernandez-Saiz, Ocio & Lagaron, 2006).

335 ChO films showed stronger antimicrobial activity than Ch films, indicating that this  
336 effect is caused by the presence of the COS into the films, which denotes the  
337 reinforcing effect of more active groups that produce inhibition. Since such  
338 antimicrobial mechanism is supposed to be based on electrostatic interaction, it  
339 suggests that the greater the number of cationized amines, the higher will be the  
340 antimicrobial activity. Therefore, COS increased the number of cationized amines and  
341 improved the antimicrobial activity of ChO films. As it has been reported by other  
342 authors (Goy, de Britto & Assis, 2009; Kong, Chen, Xing & Park, 2010), this would not  
343 be possible by increasing the concentration of chitosan, because the amount of  
344 chitosan available to bind to a charged bacterial surface is apparently reduced as the  
345 concentration of chitosan increases. A possible explanation is that in the presence of a  
346 larger number of charged sites, the chains tend to form clusters by molecules  
347 aggregation. Observations have confirmed that, at higher concentrations, chitosan, due  
348 to its filmogenic character, tends to form a coating over the bacteria, no necessarily  
349 attached to the surface, and independently of the bacteria type. Other possible  
350 mechanism reported is the penetration of low molecular weight chitosan in the cell,  
351 blocking the transcription of RNA from DNA due to adsorption (Fernandez-Saiz, Ocio &  
352 Lagaron, 2006). In this sense, the addition of COS to the film could favour also this  
353 mechanism, as it can be observed in the results of inhibition.

354 The effect of COS incorporation produced more differences in antimicrobial activity in  
355 the case of neutralized films than in heat treated ones. Heat treated ChO films showed  
356 higher antimicrobial activity (except for *L. plantarum*) with respect to Ch films treated at  
357 the same temperature. As it was shown, the solubility of these films was lower than that  
358 of the neutralized films, due to the strong interactions produced by the crosslinking and  
359 the Maillard reaction. However, the conditions of antimicrobial growth medium when  
360 the COS are present in the film, would make that the small molecules could be  
361 redissolved, being presented in the medium low molecular weight chitosan and more

362 amine groups activated available to give the possible actuation mechanism to inhibit  
363 the different bacteria tested.

364 The percentage of inhibition of all the tested bacteria was significantly higher for ChO  
365 neutralized films with respect to Ch ones. In this case, the results does not seem to be  
366 in agreement with the intensity of the bands at 1546 and 1405  $\text{cm}^{-1}$  associated to  
367 carboxylate ions with biocide action, which showed less intensity in presence of COS  
368 according the ATR\_FTIR spectroscopy experiments. The process of neutralization  
369 would be higher in presence of higher number of amine groups causing a decrease in  
370 the percentage of these protonated groups and therefore, in the bands corresponding  
371 to carboxylate ions, due to the formation of sodium salt of acetic acid. In the same way,  
372 the conditions of the antimicrobial experiments in which a liquid culture medium with pH  
373 6 is used, the amine groups would start to be protonated, and redissolution of small size  
374 chain of COS could be favoured, which was demonstrated by the higher solubility of  
375 these films observed by TSM experiments.

376 The lack of biocide properties of neutralized chitosan films showed by Ouattara et al.  
377 (Ouattara, Simard, Piette, Bégin & Holey, 2000) when they were applied onto the  
378 surface of processed meat, was attributed to the inactivation of amine groups by  
379 titration with an alkaline component. But, in apparent opposition to these results, Tang  
380 et al. (Tang, Du & Fan, 2003) also obtained bactericidal effect toward *S. aureus* and  
381 *E.coli* when suspensions of both neutralized chitosan films and neutralized cross-linked  
382 chitosan films were measured. Among some feasible reasons for this behaviour could  
383 be the incomplete neutralization process of the tested films (Fernandez-Saiz, Lagaron  
384 & Ocio, 2009).

385 These results show that it is possible to reduce the solubility of chitosan films while  
386 their antimicrobial properties are maintained, by incorporation of antimicrobial COS.

#### 387 4. Conclusions

388 The results of this work showed that the incorporation of chitooligosaccharides (COS)  
389 in neutralized or heat treated chitosan films led to obtain films with reduced solubility

390 and with stronger antibacterial activity due to the reinforcement of more functional  
391 active groups.

392 When the chitosan films were neutralized or heat treated, they became more water  
393 insoluble but lost their antibacterial properties. Heat treatment decreased further the  
394 solubility due to the crosslinking and the Maillard reaction. Besides, in the neutralization  
395 treatment, some of the neutralized protonated amine groups with the corresponding  
396 formation of sodium salt of acetic acid caused partial insolubility of the films. The  
397 incorporation of COS to chitosan films increased the inhibitory effect against the  
398 studied microorganism.

399 In view of these results, it can be concluded that incorporation of COS combined with  
400 heat or neutralization treatments has been proved to be a viable method to get a final  
401 chitosan film with decreased solubility, without affecting their water vapour  
402 permeability, and antibacterial activity with higher efficiency depending on the  
403 microorganism.

404 Further experiments are necessary to know about the behaviour of the film in different  
405 food matrices but, according to the results ChO, films seem to be a promising material  
406 which can be used for food packaging applications.

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1 Table 1. Total soluble matter (TSM) and water vapour permeability (WVP) average  
 2 values and standard deviations of non-treated chitosan (Ch) and chitosan-  
 3 oligosaccharide (ChO) films, heat-treated (Ch105, ChO105) and neutralized (ChNaOH,  
 4 ChONaOH).

FILM SAMPLE	TSM (%)	WVP. $10^8$ (g·mm/m <sup>2</sup> ·s·Pa)
Ch	100 <sup>a</sup>	2.573±0.111 <sup>a</sup>
ChO	100 <sup>a</sup>	4.037±0.117 <sup>b</sup>
Ch105	31.3±1.0 <sup>b</sup>	2.330±0.086 <sup>a</sup>
ChO105	41.6±3.6 <sup>b</sup>	3.934±0.202 <sup>b</sup>
ChNaOH	57.4±8.6 <sup>c</sup>	4.421±0.084 <sup>b</sup>
ChONaOH	78.6±1.5 <sup>d</sup>	3.879±0.820 <sup>b</sup>

5 <sup>a-d</sup> Two values followed by the same letter are not significant ( $p>0,05$ ) different thought  
 6 the Tukey's multiple range test. Measurements were made in triplicate and  
 7 quadruplicate, respectively

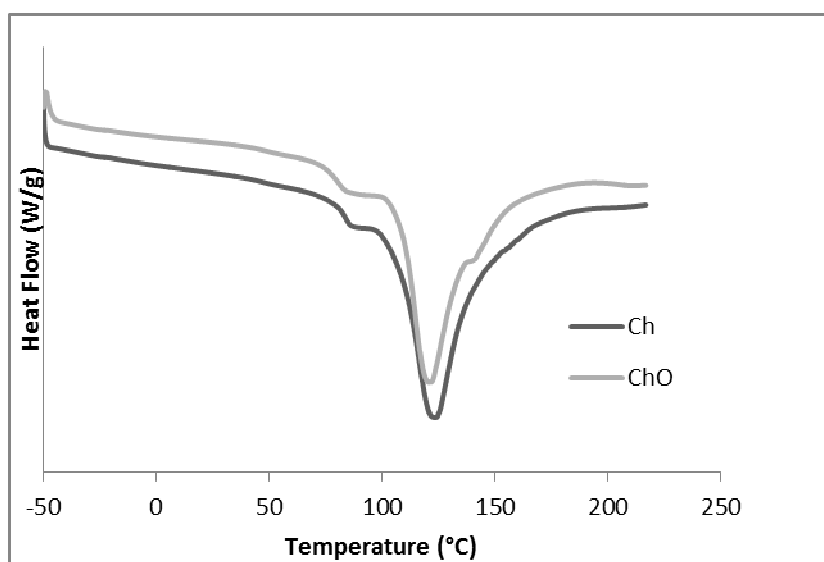
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9 Table 2. Average % inhibition values and standard deviations of non-treated chitosan  
 10 (Ch) and chitosan-oligosaccharide (ChO) films, heat-treated (Ch105, ChO105) and  
 11 neutralized (ChNaOH, ChONaOH) on the viable count of *E. coli*, *S. liquefaciens*, *B.*  
 12 *cereus*, *S.aureus* and *L.plantarum*. Data are expressed as % inhibition with respect to  
 13 control.

	% Inhibition				
	<i>E. Coli</i>	<i>S. liquefaciens</i>	<i>B. cereus</i>	<i>S. aureus</i>	<i>L. plantarum</i>
Ch	>88.2 ± 0.0 <sup>a</sup>	48.9 ± 4.3 <sup>a</sup>	82.8 ± 6.3 <sup>a</sup>	72.48 ± 7.0 <sup>a</sup>	>89.6 ± 0.0 <sup>a</sup>
Ch105	63.4 ± 4.3 <sup>b</sup>	9.5 ± 1.4 <sup>b</sup>	56.6 ± 4.1 <sup>b</sup>	17,4 ± 2.4 <sup>b</sup>	75.7 ± 0.3 <sup>b</sup>
ChNaOH	38.5 ± 7,2 <sup>c</sup>	3.7 ± 0.2 <sup>c</sup>	11.9 ± 0.9 <sup>c</sup>	2.2 ± 0.3 <sup>c</sup>	64.3 ± 1.5 <sup>c</sup>
ChO	>88.2 ± 0.0 <sup>a</sup>	61.9 ± 1.3 <sup>d</sup>	>87.2 ± 0.0 <sup>a</sup>	>85.3 ± 0.0 <sup>d</sup>	>89.6 ± 0.0 <sup>a</sup>
ChO105	>87.7 ± 0.0 <sup>a</sup>	27.0 ± 1.2 <sup>e</sup>	75.7 ± 3.1 <sup>a</sup>	30.1 ± 2.7 <sup>e</sup>	61.2 ± 1.0 <sup>c</sup>
ChONaOH	>89.9 ± 0.0 <sup>a</sup>	54.7 ± 2.4 <sup>f</sup>	>86,8 ± 0.0 <sup>a</sup>	>88,6 ± 0.0 <sup>d</sup>	>89.6 ± 0.0 <sup>a</sup>

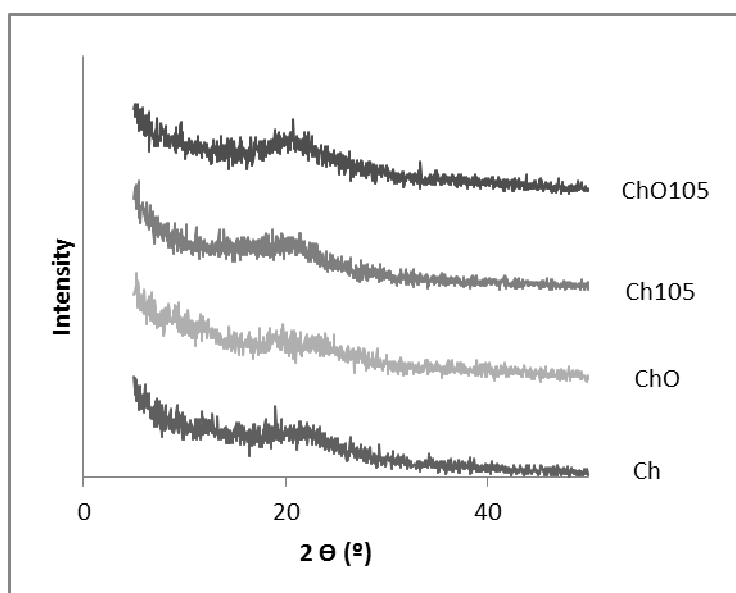
14 <sup>a-f</sup> Two values followed by the same letter for each microorganism are not significant  
 15 ( $p>0,05$ ) different thought the Tukey's multiple range test. Experiments were made in  
 16 triplicate.

- 1 Figure 1. DSC of non-treated Ch and ChO films.
- 2 Figure 2. X-ray diffractograms of heat treated and non-treated chitosan films (Ch) and
- 3 Chitosan-oligosaccharide films (ChO).
- 4 Figure 3. FTIR spectra of treated and non-treated chitosan films (Ch) (top) and Chitosan-
- 5 oligosaccharide films (ChO) (bottom).
- 6 Figure 4. SEM spectra of heat treated and non-treated chitosan films: a) Ch, b) Ch105, c) ChO
- 7 and d) ChO105.



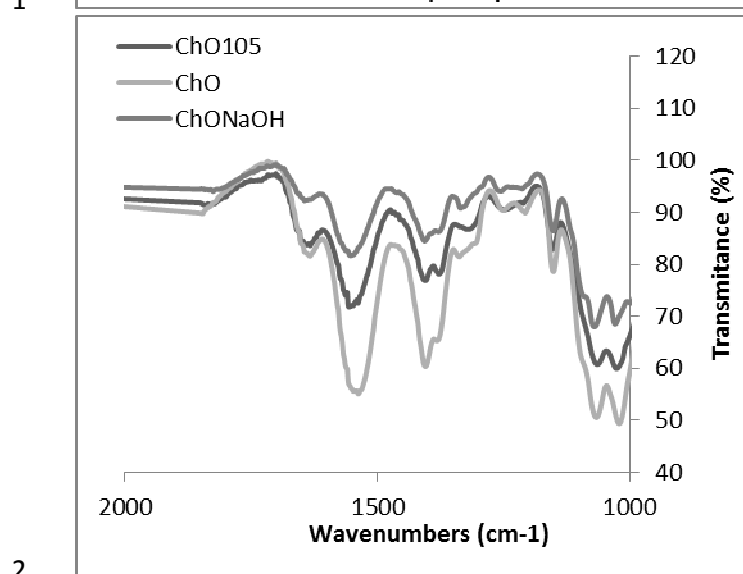
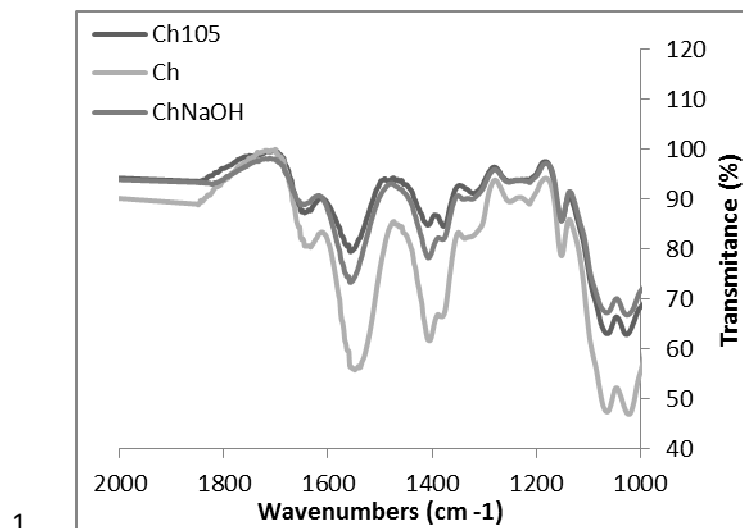
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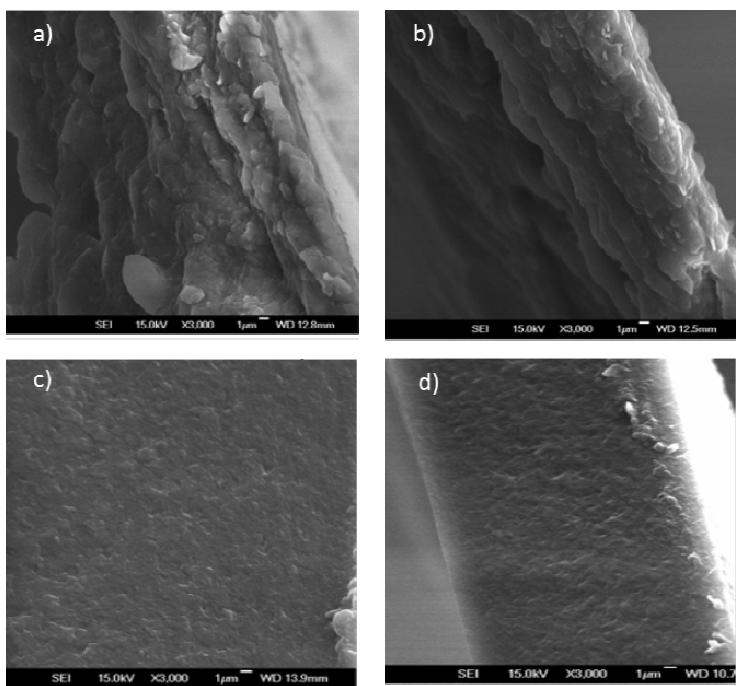


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- 1 • A new chitosan-based insoluble film with antimicrobial activity has been
- 2 developed
- 3 • Heat or neutralization treatments decreased the solubility of chitosan films but
- 4 caused a lost in their antimicrobial activity
- 5 • Incorporation of COS in chitosan films increased their antimicrobial activity

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