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Modified Starch-Chitosan Edible Films: Physicochemical and Mechanical Characterization

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Abstract: Starch and chitosan are widely used for preparation of edible films that are of great interest in food preservation. This work was aimed to analyze the relationship between structural and physical properties of edible films based on a mixture of chitosan and modified starches. In addition, films were tested for antimicrobial activity against Listeria innocua. Films were prepared by the casting method using chitosan (CT), waxy (WS), oxidized (OS) and acetylated (AS) corn starches and their mixtures. The CT-starches films showed improved barrier and mechanical properties as compared with those made from individual components, CT-OS film presented the lowest thickness (74 ± 7 µm), water content (11.53% ± 0.85%, w/w), solubility (26.77% ± 1.40%, w/v) and water vapor permeability ((1.18 ± 0.48) × 10⁻⁹ g·s⁻¹·m⁻¹·Pa⁻¹). This film showed low hardness (2.30 ± 0.19 MPa), low surface roughness (Rq = 3.20 ± 0.41 nm) and was the most elastic (Young’s modulus = 0.11 ± 0.06 GPa). In addition, films made from CT-starches mixtures reduced CT antimicrobial activity against L. innocua, depending on the type of modified starch. This was attributed to interactions between acetyl groups of AS with the carbonyl and amino groups of CT, leaving CT with less positive charge. Interaction of the pyranose ring of OS with CT led to increased OH groups that upon interaction with amino groups, decreased the positive charge of CT, and this effect is responsible for the reduced antimicrobial activity. It was found that the type of starch modification influenced interactions with chitosan, leading to different films properties.

Keywords: edible films; chitosan; modified starch; Raman spectroscopy

1. Introduction

The development of plastic food packaging has allowed shelf life extension of food products; however, overuse of synthetic materials has led to serious environmental problems. Thus, there is increased interest of food and packaging industries to develop alternative solutions, such as the study of edible films [1]. The functionality of films based on starch depends on the ratio of amylose to amylopectin. High amylose content produces elastic films whereas high amylopectin starch leads to films exhibiting poor mechanical properties [2]. Chemical modifications of starch including acetylation
and oxidation may be used to improve edible films properties. Carbonyl and carboxyl groups of oxidized starch (OS) enhance hydrogen bonds among adjacent amylopectin molecules resulting in a more efficient association process [3]. On the other hand, waxy starch (WS) may be used as film forming material because of lower extent of retrogradation when compared to amylose [4].

Chitosan (CT) is a linear polysaccharide obtained by deacetylation of chitin, which comprises β-(1-4)-2-acetamido-D-glucose and β-(1-4)-2-amino-D-glucose, CT has many potential applications in the food industry because of its unique functional, nutritional and physicochemical properties besides, it enjoys the “generally recognized as safe” (GRAS) status. CT has been used for food coatings, as food additive due to its excellent emulsifying properties and source of dietary fiber [5]. It has been used to obtain elastic and transparent films, in addition to exerting antimicrobial activity [6]. CT antimicrobial activity is influenced by pH, molecular weight, degree of deacetylation, distribution of the deacetylated groups along the chain, ionic strength of the medium and the associated counterion in its salt [7]. CT films show low oxygen permeability and moisture content but high-water vapor permeability (WVP) [8]. The effect of CT on edible films made from mixtures with different types of starch and the relationship among structural and chemical interactions and physical properties have not been studied. The aim of this work was to analyze the effect of CT on physicochemical and structural properties of edible films based on WS, oxidized and acetylated starches.

2. Materials and Methods

2.1. Materials

Medium molecular weight chitosan from shrimp shells (375 kDa, deacetylation ≥ 75%), glycerol (≥99.5%), Tween 80 and hydroxylamine hydrochloride (99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA), lactic acid (85%) was obtained from Fermont (León, Mexico). Oxidized, acetylated and waxy starches were supplied by Ingredion (San Juan del Río, Mexico).

2.2. Methods

2.2.1. Starch Characterization

Acetyl Content

Acetylation was determined following Sánchez-Rivera et al. [9]. Acetyl percentage (w/w) was calculated using Equation (1), whereas degree of substitution (DS) was calculated using Equation (2).

\[
\text{% Acetyl} = \left( \frac{(V_{HCl} - V_{HCl \text{ b}}) \times M_{HCl} \times 0.043 \times 100}{m} \right)
\]

\[
\text{DS} = \frac{(162 \times \text{Acetyl} \%(\text{}))}{[4300 - (42 \times \text{Acetyl} \%(\text{))}]
\]

where \(V_{HCl}\) and \(V_{HCl \text{ b}}\) are the volumes of 0.5 M HCl used for sample and blank titrations respectively, \(m\) is sample weight (g), 0.043 is the meq of acetyl group, 4300 is the acetyl molecular weight times 100, whereas 162 is the molecular weight of anhydrous glucose in the starch molecule.

Oxidized Starch

Carboxyl and carbonyl content were evaluated according to Zhou et al. [10]. Carboxyl content (% w/w) was obtained using Equation (3).

\[
\text{% COOH} = \left( \frac{V_{NaOH} - V_{NaOH \text{ b}}}{M_{NaOH} \times 45 \times 100} \right)
\]

where 45 is the molecular weight of carboxyl group, \(M\) is the molarity of NaOH; \(V_{NaOH}, V_{NaOH \text{ b}}\) are the volumes of 0.05 M NaOH used for sample and blank titrations, respectively.
Carbonyl content (% w/v) was determined following the Sandhu et al. [11] method and was obtained from Equation (4).

\[
\text{% Carbonyl} = \frac{[V_{\text{HCl \ b}} - V_{\text{HCl}}] \times N_{\text{HCl}} \times 0.028 \times 100}{m}
\]  

(4)

where \(V_{\text{HCl \ b}}\) and \(V_{\text{HCl}}\) are the volumes of 0.1 M HCl used for blank and sample titration respectively, \(N_{\text{HCl}}\) is the normality of HCl and 0.028 is the meq of carbonyl group.

Amylose and Amylopectin Determination

The amylose/amyllopectin content of the starch samples used was determined using the amylose/amyllopectin kit (Megazyme, Wicklow, Ireland). Amylose (% w/w) was directly calculated following supplier instructions [12].

2.2.2. Chitosan Edible Film Formation

A suspension of chitosan (1% w/v) in lactic acid (0.5% v/v) was stirred for 60 min at 80 °C, then glycerol was added at 1:1 (w/w) ratio, followed by Tween 80 (0.2% w/v) and the mixture was stirred for 10 min at room temperature. Films were formed by the casting method and dried at 60 °C and 50% relative humidity (RH) in an environmental chamber (Binder, KBF 115, Tuttlingen, Germany), for 24 h [13].

2.2.3. Starch Edible Film

Three different starch suspensions (oxidized, acetylated and waxy) were prepared. Suspensions at 3.5% (w/v) in distilled water were stirred at 90 °C for 30 min, added with 1% (w/w) glycerol [14]. Casting and films drying were conducted as in Section 2.2.2.

2.2.4. Chitosan-Starch Edible Films

Three different starch suspensions (OS, AS, WS) were prepared as in Section 2.2.3, whereas a CT suspension was prepared as in Section 2.2.2. Mixtures of chitosan with each of the three starches were prepared, comprising a 3:1 (w/w) starch: chitosan ratio and stirred for 5 min at room temperature. Films were obtained by the casting method as in Section 2.2.2. To obtain similar thicknesses, the same volume of suspension (20 mL) was used to produce all films.

2.2.5. Physical Characterization

Films thickness, color, WVP, solubility and roughness were determined. Film thickness was measured using a digital micrometer (Mitutoyo, 293-185, Kawasaki, Japan), whereas color was evaluated according to the CIELAB method [15], using the parameters \(L^*\) = lightness, \(\pm a^*\) = green to red color component and \(\pm b^*\) = blue to yellow color component. A Minolta CR400 colorimeter (Minolta, Osaka, Japan) was used, with illumination source D65 and 10° angle, color was standardized using a white reference plate. Color differences were calculated using Equation (5).

\[
\Delta E = \sqrt{\Delta a^2 + \Delta b^2 + \Delta L^2}
\]  

(5)

where \(\Delta E\) is the color difference, \(\Delta L\) is brightness difference; \(\Delta a\) is the red-green chromaticity difference and \(\Delta b\) is the yellow-blue chromaticity difference [16].

The gravimetric method ASTM E 96-80 [17], was employed to calculate WVP, using permeability cells of 3.2 cm in diameter. A saturated solution of KNO\(_3\) (92.5% RH, \(T = 25 \degree C\)) was added into the cells and films circles of 4 cm in diameter placed on top of them. Permeability cells were placed in a desiccator containing a saturated NaCl solution (75% RH, \(T = 25 \degree C\)) and weight variations were recorded until constant weight. WVP was obtained from Equation (6) [18].
\[ WVP = \frac{\Delta W}{tA} \times \frac{L}{\Delta P} \]  

where $\Delta W$ is the change in weight of the permeation cell (g), $t$ is the duration of the test (s), $A$ is the contact area of the edible film ($m^2$), $L$ is the thickness of the film (m) and $\Delta P$ is the pressure difference (Pa).

Films were cut into squares of 2 cm, placed in petri dishes, dried at 103 $\pm$ 2 $^\circ$C for 24 h and weighed, followed by immersion in 15 mL of distilled water for 6 h with constant agitation on a rocker plate (Ultra Rocker, Bio Rad, Carlsbad, CA, USA). Then, samples were passed through Whatman No. 1 filter paper (Whatman, Maidstone, UK) and the remaining pieces of film were recovered, dried for 24 h at 103 $\pm$ 2 $^\circ$C and the final dry weight was determined. Solubility ($\% w/w$) was obtained using Equation (7) [19].

\[
\% \text{ Solubility} = \frac{\text{initial weight} - \text{final weight}}{\text{initial weight}} \times 100
\]  

The edible films surface roughness was evaluated using atomic force microscopy (AFM) (di Multimode V, Veeco, Plainview, NY, USA). This method produces a 3D profile of the sample surface by measuring changes in force between a cantilever and the sample [20]. Roughness was calculated from the images of the edible film surface, calculating the square root of the deviation from a mean plane of the surface peaks and valleys ($R_q$) (Equation (8)). The average of the absolute values of the surface height deviations measured from the mean plane ($R_a$), was calculated using Equation (9), from the Nano Scope Analysis 1.2 software (Veeco, Plainview, NY, USA). The method of contact and using silicon tips (Bruker RTESP Cantilever, Karlsruhe, Germany) was applied with a resonant frequency of 286–362 kHz and a spring constant of 20–80 N/m and a scan speed of 1 Hz with a resolution of 256 $\times$ 256 pixels.

\[
R_q = \sqrt{\frac{\sum Z_i^2}{N}}
\]  

\[
R_a = \frac{1}{N} \sum_{j=1}^{N} Z_j
\]

where $R_q$ and $R_a$ are the values of roughness (nm), $Z_{ij}$ is the deviation from the average height relative to a mean plane and $N$ is the number of points in the image. For each film (CT, AS, WS, OS, CT–AS, CT–OS and CT–WS), three different samples were produced, of which three different areas were analyzed (5 $\mu$m, 1 $\mu$m and 0.5 $\mu$m) and the average value was calculated.

Mechanical Properties

Young’s modulus and hardness of films were obtained using a nanoindentation tester (TTX-NHT, Peseux, Switzerland). Indentation was performed by applying a maximum load of 5 mN, loading and unloading rate of 7.5 mN/min and a pause of 35 s. A Berkovich diamond tip of pyramidal geometry and triangular base, with tip radius of 100 nm was used to obtain a loading and unloading curve (Figure 1).

![Figure 1. Curve of loading and unloading used to measure films mechanical properties.](image-url)
From this figure, the maximum load \( P_{\text{max}} \), the total penetration at maximum load \( h_{\text{max}} \), stiffness of the contact discharge start \( S \), contact area (under the curve, \( A_c \)), residual displacement after indenter unloading \( h_f \), real contact depth \( h_c \) and the elastic subsidence around the contacting surface \( h_s \) were obtained [21].

Hardness was calculated using Equation (10), whereas Young’s modulus was calculated from Equations (11) and (12):

\[
H = \frac{P_{\text{max}}}{A \times h_c}
\]

\[
E_r = \frac{S \sqrt{\pi}}{2 \sqrt{A_c}}
\]

\[
E_m = \frac{1 - v^2}{\frac{1}{E_i} - \frac{1 - v^2}{E_i}}
\]

where \( H \) (MPa) is the film’s hardness, \( E_m \) is the elastic modulus (GPa), \( E_r \) is the reduced modulus (GPa), \( S \) is the initial unloading stiffness in the load curve, \( v \) is the Poisson’s ratio for polymeric samples estimated as 0.35, \( E_i \) is the elastic modulus of 1141 GPa for the indentation [22]. Tests were conducted in five different spots of three different films and average values were calculated.

### 2.2.6. Chemical Properties

Chemical interaction of CT with the three different types of starch was analyzed by Raman spectroscopy (Yvon Horiba, Edison, NJ, USA) coupled to a microscope (Olympus BX 41, Olympus Corporation, Shinjuku-ku, Tokyo, Japan). Samples were irradiated using a 735 nm laser with a 50× objective, a numerical range of 0.55 and a yield of 702 nm in diffraction; spectral resolution of 0.16 cm\(^{-1}\) was used, using a detector charge coupled (CCD) device with a spectral range of 450–950 nm. The confocal aperture and the entrance slit of the monochromator were kept constant at 400 µm. Readings were taken in a spectral range of 200–4000 cm\(^{-1}\) [18]. The program Spekwin 32 (Oberstdorf, Germany) was used for data treatment.

### 2.2.7. Antimicrobial Activity

Antimicrobial activity was evaluated following the methodology of Hernández-Hernández [23]. Briefly, 10 mL of trypticase soy agar (0.8% w/v, Bioxon, Cuautitlan, México) was inoculated with 500 µL of \( L. \) innocua solution (10\(^7\) CFU/mL), subsequently poured onto plates containing solidified agar (1.5% w/v). Discs from each edible film (25 mm in diameter) were placed on top of the soft agar layer, incubated at 37 °C for 48 h, the growth inhibition zone was measured using Vernier calipers.

### 2.2.8. Statistical Analysis

For each test three edible films samples were analyzed, conducting measurements at five different points (ends and center). Data were evaluated by one-way analysis of variance (ANOVA) and significant differences were analyzed by the Tukey test \((p < 0.05)\).

### 3. Results and Discussion

#### 3.1. Starch Characterization

The amylose content for WS, AS and OS were 7.1% ± 0.9%, 27.5% ± 1.6%, 15.7% ± 0.9%, respectively. According to Li et al. [24] and Cano et al. [25] retrogradation depends on ordered helical aggregation of amylose and amylopectin chains and increasing crystalline state and the higher the percentage of amylose the greater the extent of retrogradation, resulting in films with poor mechanical and barrier properties. Thus, starches with increased amylopectin content may minimize these effects. Due to the hydrophilic nature of starch, its resulting films show high WVP and to improve this property modified starches were used [26]. Modification involves introduction of functional groups into the
starch molecule, AS showed 6.46% ± 0.13% of acetylation, equivalent to a DS = 0.14 ± 0.01, whereas OS contained 0.073% ± 0.005% of carboxyl groups and 0.074% ± 0.006% of carbonyl groups. The presence of carboxyl groups within the starch molecule interrupts amylose and amylopectin linear sections, producing a reduced tendency to intermolecular association, promoting hydration of the granule [27]. Modified starches functional groups may interact with other components of edible films and hence alter their mechanical and barrier properties.

3.2. Physical Characterization

Films containing the mixtures CT–WS and CT–OS showed decreased luminosity (L value) whereas those made with CT–AS mixture increased the L value (Table 1). Color is important to assess the application of the films as this will affect the appearance of the product over which films are applied.

<table>
<thead>
<tr>
<th>Edible Film</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
<th>ΔE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>91.37</td>
<td>-1.15</td>
<td>4.38</td>
<td>4.58</td>
</tr>
<tr>
<td>OS</td>
<td>91.06</td>
<td>-0.24</td>
<td>0.92</td>
<td>2.39</td>
</tr>
<tr>
<td>AS</td>
<td>90.42</td>
<td>-0.24</td>
<td>0.43</td>
<td>1.64</td>
</tr>
<tr>
<td>WS</td>
<td>93.06</td>
<td>-0.23</td>
<td>0.47</td>
<td>2.51</td>
</tr>
<tr>
<td>CT–OS</td>
<td>88.87</td>
<td>-1.64</td>
<td>7.13</td>
<td>7.70</td>
</tr>
<tr>
<td>CT–AS</td>
<td>91.14</td>
<td>-1.37</td>
<td>6.70</td>
<td>6.66</td>
</tr>
<tr>
<td>CT–WS</td>
<td>90.26</td>
<td>-1.32</td>
<td>4.83</td>
<td>5.55</td>
</tr>
</tbody>
</table>

Notes: CT, Chitosan; OS, Oxidized starch; AS, Acetylated starch; WS, Waxy starch. a–d: used next to reported values, indicate that if the same letter appears in the same column, the values compared are not significantly different (p > 0.05).

Films prepared from WS, OS and AS did not exhibit significant difference (p < 0.05) in L value. This is consistent with a report by Levien-Vanier et al. [28], who found that starch subjected to an oxidation process does not change color parameters. The observed a* value indicates a reddish color of the starch-only films but significant difference (p < 0.05) was found when compared to CT-only films. Similar to the L value, the presence of CT mixed with any of the starches tested, affected films appearance, causing increased yellowish color. This result agrees with Kurek et al. [29], where CT incorporated to whey protein isolate films provided yellow tint, while increasing the value of ΔE*.

Luminosity, chromatic parameters (a*, b*) and ΔE* for CT-starches films (Table 1) are similar those previously reported [30].

Thickness, WVP and solubility are shown in Table 2. Thickness can affect barrier properties, particularly WVP due to differences between the water vapor pressure below the film and that of the moisture build-up above the film [16]. To obtain films with similar thickness, same suspension volumes were used. However, films prepared with mixtures CH-starches exhibited lower thickness that those prepared with starches only (Table 2).

AS films showed the highest thickness (112 ± 11 μm) due to the introduction of acetyl groups that promote spacing between starch chains, reduction of retrogradation and syneresis and thus, more water retention within the film after drying [31]. Moreover, high swelling of starch granules leads to higher film thicknesses [32]. Thus, AS was expected to show the best barrier properties but instead it showed the highest WVP (Table 2). High WVP and solubility were attributed to the degree of substitution (0.142 ± 0.005), where the introduction of acetyl groups into the starch molecule prevented inter-chains association, facilitating water penetration inside AS films, which may explain its low retrogradation [33]. CT films presented the smallest thickness and solubility associated to its deacetylation extent (≥75%), since it is known that CT solubility and WVP decrease with the number of amino groups [34].
Table 2. Physical properties of edible films made from CH-only, starches-only and mixtures CH-starches (1:3 w/w) ratio, respectively.

<table>
<thead>
<tr>
<th>Edible Film</th>
<th>Thickness (µm)</th>
<th>Solubility (%)</th>
<th>Water Vapor Permeability × 10^9 (g mm/(s m Pa))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>66 ± 6 a</td>
<td>17.07 ± 1.38 a</td>
<td>1.65 ± 0.47 a</td>
</tr>
<tr>
<td>OS</td>
<td>105 ± 26 b</td>
<td>80.06 ± 2.37 b</td>
<td>1.00 ± 0.39 a</td>
</tr>
<tr>
<td>AS</td>
<td>128 ± 14 b</td>
<td>39.57 ± 1.68 c</td>
<td>2.06 ± 0.61 b</td>
</tr>
<tr>
<td>WS</td>
<td>112 ± 11 b</td>
<td>40.97 ± 3.41 c</td>
<td>1.15 ± 0.33 b</td>
</tr>
<tr>
<td>CT-OS</td>
<td>74 ± 7 a</td>
<td>26.77 ± 1.40 d</td>
<td>1.18 ± 0.48 a</td>
</tr>
<tr>
<td>CT-AS</td>
<td>93 ± 13 b</td>
<td>32.02 ± 2.2 c</td>
<td>1.11 ± 0.03 a</td>
</tr>
<tr>
<td>CT-WS</td>
<td>81 ± 5 b</td>
<td>27.71 ± 1.56 d</td>
<td>1.32 ± 0.54 a</td>
</tr>
</tbody>
</table>

Notes: CT, Chitosan; OS, Oxidized starch; AS, Acetylated starch; WS, Waxy starch. a–d: used next to reported values, indicate that if the same letter appears in the same column, the values compared are not significantly different (p > 0.05).

Starch films changed their properties by CT incorporation, decreasing thickness, WVP and solubility. WS–CT and AS–CT films showed high thickness values, associated to high swelling of starch granules produced by the high amylopectin content of WS and the presence of acetyl groups in AS [27,35]. When CT was used in mixture with AS, the films revealed low WVP, improving this barrier property, while OS–CT highly affected solubility due to the carboxyl groups in its structure leading to increased hydrophilicity. According to Liu et al. [36], starch oxidation results in partial depolymerization and this could cause structural weakening of OS granules increasing molecular mobility [36]. AS and WS with CT Raman spectrogram shows interactions of AS and WS with CT amino groups leading to more soluble films, while OS interacted with amino groups and with hydroxyl groups at a higher extent than the other starches, making the films less soluble. These phenomena are associated to CT interactions with each type of starch (larger amount and type of bonds formed between CT and OS than those formed using WS) [37]. According to Alves et al. [38], CT interactions with starch depend on the functional groups that characterize each of the starches used.

3.3. Mechanical Properties

Nanoindentation can provide detailed information from a high local deformation, which is of great importance for systems with limited dimensionality, such as thin films and coatings. This technique permits identification of transition zones between phases in heterogeneous materials, specifically on their surface or interface at very low sensitivity ranges [39]. Thus, mechanical properties of samples showing small local deformation such as thin coatings can be successfully characterized by analyzing their surface [40]. CT–OS films exhibited the lowest surface hardness, whereas AS-film was the hardest because of its higher amylose content (Table 3). This effect agrees with Lopez et al. [26], who found that linear amylose chains show high tendency to interact via hydrogen bonds, resulting in stiffer and stronger films than those presenting high amylopectin content. From Table 3, it is noted that CT addition produced films with reduced surface hardness, attributed to increased molecular mobility among polysaccharide chains [40]. A study established correlations between mechanical properties and polymers crystallinity, showing that vitreous materials exhibit low hardness, whereas semicrystalline samples hardness tend to rise with increasing crystallinity [41].

WS edible film surface revealed the highest hardness value, attributed to its amylopectin composition, since the higher content the more rigid structure is presented due to its degree of crystallinity. OS and AS edible films contain lower amylopectin proportion than WS and this was reflected in less rigid surface structures and therefore low hardness [42]. CT exhibits low crystallinity (7.3% ± 0.5%) [18] that is reduced in mixtures with starch films by interruption of amylopectin chains, preventing structural rearrangement of starch during film drying process. The addition of CT in the starch films resulted decreased Young’s modulus of films surface. Surface elastic modulus was similar for mixtures CT–OS and CT–AS but lower than that of starch films and these changes may be attributed to cross links of polysaccharides network. This phenomenon is similar to that observed by Alvarado-González et al. [43], when producing edible films based on Aloe Vera gel/gellan gum,
observing surface increased hardness and decreased surface elastic modulus, as compared to their individual components.

Table 3. Hardness and Young’s modulus of edible films based on starch, CH and CH-starches mixtures (1:3 ratio, w/w).

<table>
<thead>
<tr>
<th>Edible Film</th>
<th>Hardness (MPa)</th>
<th>Young Modulus (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>5.87 ± 0.46 a</td>
<td>0.07 ± 0.01 a</td>
</tr>
<tr>
<td>OS</td>
<td>168.90 ± 3.00 b</td>
<td>2.76 ± 0.15 b</td>
</tr>
<tr>
<td>AS</td>
<td>198.77 ± 22.55 c</td>
<td>4.31 ± 0.35 c</td>
</tr>
<tr>
<td>WS</td>
<td>180.99 ± 4.31 c</td>
<td>3.36 ± 0.12 c</td>
</tr>
<tr>
<td>CT-OS</td>
<td>2.30 ± 0.19 a</td>
<td>0.11 ± 0.06 a</td>
</tr>
<tr>
<td>CT-AS</td>
<td>3.97 ± 0.73 a</td>
<td>0.09 ± 0.01 a</td>
</tr>
<tr>
<td>CT-WS</td>
<td>9.54 ± 1.42 a</td>
<td>0.44 ± 0.03 d</td>
</tr>
</tbody>
</table>

Notes: CT, Chitosan; OS, Oxidized starch; AS, Acetylated starch; WS, Waxy starch. *d*: used next to reported values, indicate that if the same letter appears in the same column, the values compared are not significantly different (p > 0.05).

3.4. Topography Properties

From AFM, it was observed that CT-films exhibited the lowest surface roughness (Figure 2D) and considering that $R_q$ or $R_s < 10$ for all films, they are regarded as exhibiting a smooth surface [44]. CT addition promoted films displaying smoother surface (Figure 2A–C), whereas OS–CT-films showed a mild roughness increase (9.7% for $R_q$ and 20.7% for $R_s$) compared to CT films (Figure 2B,D). Bonilla et al. [45] reported that CT addition to starch produced smoother film surfaces because of high compatibility between the two polymers. AS films (Figure 2G) showed the highest roughness values, associated to structural rearrangement due to acetylation, resulting in greater space occupation by the starch molecules [46].

![Figure 2. Cont.](image-url)
This suggests a possible reaction of a methyl group of CT with the C6 OH of WS, leaving a free C=O group in CT–WS mixture. The CT signal at 542 cm\(^{-1}\) disappeared, which represents vibrations out of the flexion plane of the NH and C=O groups [46], that interacted with AS, possibly associated to hydrogen bonds formation. The acetyl groups of AS may also interact with the NH\(_2\) groups of CT to form new links. In addition, the oxygen of the carbonyl group of CT bearing partial negative charge can interact with OH groups of starch [18].
Signals at 890 and 1220 cm\(^{-1}\) (Figure 3c), characteristic of glucopyranose ring and vibrations in the plane of bending \(\gamma(\text{OH}...\text{O})\) of CT [50] are not observed. This effect is associated to a decreased signal of AS at 653 and 1202 cm\(^{-1}\), attributed to the C–C and C–O. bonds of the pyranose ring [49], suggesting that the ring breaks and loses its structure. More studies are needed to confirm this assumption. In the CT-OS spectrogram (Figure 3d) signals at 542 and 778 cm\(^{-1}\), which were observed in the OS film were not detected in the mixture and according to Almeida [48] these signals are attributed to C–C–O bonds and a stretch of the pyranose ring of starch, respectively, suggesting chemical interaction. Another signal lost in the CT–OS spectrogram was that at 896 cm\(^{-1}\) attributed to CH\(_2\) \(\gamma(\Phi) + \rho(\text{CH}_2)\) of CT [47]. In addition, a new signal appeared at 1730 cm\(^{-1}\), attributed to OH groups [48]. It must be bore in mind that starch-CT mixtures were heated at 90 °C for 10 min at pH about 3.5, leading to partial hydrolysis of starch glycosidic bonds, as reported by Hong et al. [51]. These authors stated that terminal linkages are more susceptible than those within the chains and thus glycosidic bond rupture (starch hydrolysis) increases the number of –OH groups in free C1, C4 and C6 atoms and this may explain our findings. This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.

![Figure 3. Cont.](image-url)
3.6. Antimicrobial Activity

The presence of CT in edible films allowed them to show antimicrobial activity against *L. innocua*, whereas films made with only starch did not show inhibition (Table 4). CT films showed the highest bactericidal activity (Figure 4) and its mechanism was attributed to the amino groups in its molecular structure being attached to the cell membrane through electrostatic interactions [52]. Other studies found that after 6 h of exposure to CT, it caused cells morphological alterations, where most cells showed enlarged and spongy walls with remarkable signs of lysis [53].

**Table 4. Inhibitory effect of EF against *Listeria innocua*.**

<table>
<thead>
<tr>
<th>Edible Films</th>
<th>Average Diameter (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS</td>
<td>0</td>
</tr>
<tr>
<td>AS</td>
<td>0</td>
</tr>
<tr>
<td>WS</td>
<td>0</td>
</tr>
<tr>
<td>CT</td>
<td>5.4 ± 0.3 a</td>
</tr>
<tr>
<td>CT-OS</td>
<td>3.7 ± 0.5 b</td>
</tr>
<tr>
<td>CT-AS</td>
<td>4.2 ± 0.4 b</td>
</tr>
<tr>
<td>CT-WS</td>
<td>3.9 ± 0.4 b</td>
</tr>
</tbody>
</table>

Notes: CT, Chitosan; OS, Oxidized starch; AS, Acetylated starch; WS, Waxy starch. a,b: used next to reported values, indicates that if the same letter appears in the same column, the values compared are not significantly different (p > 0.05).
were altered due to chemical interactions and structural changes of starch-CT edible films. Additionally, AS and WS interacted with CT amino groups producing more soluble films. The mixture CT–OS showed partial hydrolysis of starch associated to low pH and high temperature treatment, whereas films made from CT–AS mixture exhibited chemical interaction between NH2 and OH groups. CT–AS mixture showed good barrier and mechanical properties as well as the highest antimicrobial effect against Listeria.

4. Conclusions

The effect of CT addition on edible films based on native and modified starches has not been studied. This study demonstrated that CT addition on starch affected surface edible films mechanical and barrier properties, depending on the type of starch and its chemical modification. These properties were altered due to chemical interactions and structural changes of starch-CT edible films. Additionally, AS and WS interacted with CT amino groups producing more soluble films. The mixture CT–OS showed partial hydrolysis of starch associated to low pH and high temperature treatment, whereas films made from CT–AS mixture exhibited chemical interaction between NH2 and OH groups. CT–AS mixture showed good barrier and mechanical properties as well as the highest antimicrobial effect against Listeria, which suggests its further application in food products to maintain their safety.

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Author Contributions: Monserrat Escamilla-García carried out the experimental part of the research project and manuscript writing. Andrea Reyes-Basurto collaborated in the experimental part of this project and Georgina Calderón-Domínguez conducted Raman spectroscopy analyses and their interpretation. Elvia Hernández-Henández collaborated with the AFM measurements and their interpretation. Blanca E. García-Almendárez developed microbiological experiments and their interpretation. Giovanna Rossi-Márquez performed mechanical properties of films. Carlos Regalado-González advised on planning of experiments and manuscript writing.

Conflicts of Interest: The authors declare no conflict of interest.

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