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Individual and Combined Coatings of Chitosan and Carnauba Wax with Oregano Essential Oil to Avoid Water Loss and Microbial Decay of Fresh Cucumber

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Abstract: The objective of the present study is to evaluate the effect of individual and combined coatings of chitosan (0.008 g·mL^{-1}) and carnauba wax (0.1 g·mL^{-1}) with oregano essential oil (OEO, 0.08 g·mL^{-1}) to reduce dehydration and microbial decay of fresh cucumbers stored at 10 °C. Chitosan-OEO-wax films showed the lowest water vapor transmission rate (0.141 g·m^{-2}·h^{-1}), compared to single chitosan films (0.257 g·m^{-2}·h^{-1}). While chitosan-OEO films completely inhibited the in vitro growth of *Alternaria alternata* and reduced the growth of *Salmonella* Typhimurium, *Escherichia coli* O157:H7, mesophilic bacteria, and fungi isolated from decayed cucumbers. Besides, the infrared analysis of chitosan-OEO-wax films showed shifts in O–H and N–H absorption bands, indicating possible hydrogen bonding between the components. Wax and wax-OEO were the most effective coatings to prevent weight loss in cucumbers during 15 days of storage at 10 °C, while the most effective antimicrobial treatments were chitosan and chitosan-OEO. Therefore, these results showed that carnauba wax and carnauba wax-OEO coatings were the most effective in weight loss, whereas chitosan and chitosan-OEO were the most effective to reduce the microbial load of the treated fresh cucumber.

Keywords: *Cucumis sativus* L.; dehydration; antimicrobial activity; bilayer coating; essential oils

1. Introduction

Cucumber (*Cucumis sativus* L.) is a low-calorie fruit belonging to the *Cucurbitaceae* family, non-climacteric, harvested, and consumed at an immature stage. It is a rich source of potassium, magnesium, iron, with a high water content—approximately 90% [1]. The quality of cucumber is based on its shape uniformity, dark green color, firmness, size, absence of defects, and rot [2]. However, during post-harvest, the cucumber is highly susceptible to physiological changes, microbial decay, and dehydration with the consequent loss of appearance, and nutritional quality [3]. Also, it is susceptible to the attack of bacteria (e.g., *Erwinia* spp. and *Xanthomonas* spp.) and fungi (e.g., *Alternaria* spp. and *Rhizopus* spp.) [4]. Commonly, some of these problems are solved with the use of low storage temperatures; however, cucumbers are sensitive to chilling injury below 10 °C; for this reason, they are stored at 10–12.5 °C, becoming more susceptible to quality loss after 14 days. These problems

justify the need for developing adequate water loss barrier and antimicrobial techniques to preserve the cucumbers' quality.

Edible coatings can act as barriers to reduce water loss and gas exchange, depending on their composition [5]. Different materials are used to formulate edible coatings, including proteins, lipids, and polysaccharides. Polysaccharides have excellent gas barrier properties; however, they are highly hydrophilic and show high water vapor permeability. On the other hand, lipids are hydrophobic compounds with water barrier properties, although, their nonpolymeric nature limits their ability to form films with good mechanical integrity [5]. Among polysaccharides, chitosan obtained from alkaline deacetylation of chitin has been widely used because of its antimicrobial properties [6]. Chiabrando et al. [7], reported that chitosan coatings significantly reduced microbial decay of minimally processed nectarines compared to control fruits. Similarly, chitosan coatings (2%) in broccoli florets stored at 5 °C resulted in a significant reduction of total mesophilic and psychrotrophic bacteria; also, a bactericidal effect was observed in the inoculated *E. coli* O157:H7 [8]. In addition, Pavinatto et al. [9] reported a reduction of gray fungus attack and an insignificant alteration in flavor, appearance, aroma, and texture of strawberries coated with 1% chitosan and stored for 7 days at 25 °C. Other polysaccharide-based coatings also have been applied to improve the quality parameters of fruits. *Aloe vera* coatings (3%) improved firmness and total soluble solids of tomato; however, no effect was observed in weight loss compared to control [10]. Similarly, alginate coatings showed a good gas permeability and maintained the firmness of fresh-cut melon; however, no antimicrobial activity and weight loss protection were observed. Besides their different benefits, polysaccharides-based coatings are considered a low water barrier [11].

On the contrary, natural waxes (carnauba, shellac, and beeswax) limit the water loss better than polysaccharides-based coatings. Carnauba wax is a lipid-based material obtained from *Copernicia cerifera* leaves, predominantly comprised of aliphatic esters and diesters of cinnamic acid with a high melting point and low solubility [12]. These characteristics grant the relatively inert and stable character to carnauba wax; in addition, it is generally recognized as safe by the Food and Drug Administration of the United States and normally used in edible coatings formulations [5,12]. Won and Min [13] reported a significant reduction in the weight loss of Satsuma mandarins treated with carnauba wax (18.1%) during storage at 4 and 25 °C for 28 days compared with control fruits. In addition, the weight loss was significantly reduced in Valencia oranges and avocados after the application of carnauba wax [14,15]. However, it is important to note that these authors did not evaluate the antimicrobial effect of these coatings, microbial decay being one of the main factors that compromise the postharvest life of these fruits, highlighting the need for an additional coating material as our study proposes. Therefore, it can be expected that a combination of constituents could obtain better results.

Edible coatings may carry antimicrobial additives protecting fresh produce of postharvest decay [16]. Oregano essential oil (OEO) possess antimicrobial activity, and it may be added to edible coatings as a food additive; its efficacy has been proved when added into several edible coatings [17,18]. The antibacterial activity of OEO is attributed to the constituents carvacrol and thymol; as a result of their lipophilic nature, these monoterpenes can become embedded in the bacteria or fungi membrane causing the alteration of enzymatic systems, cellular disruption, and loss of cellular constituents [19,20]. Some studies reported the efficacy of the combination of chitosan and carnauba wax with essential oils to maintain the postharvest quality of cucumber and other fruits. Mohammadi et al. [21] reported that nanochitosan-based coating loaded with *Zataria multiflora* essential oil improved physicochemical quality and significantly reduced total bacterial, yeasts, and molds counts of cucumber stored at 10 °C for 21 days. Won and Min [13] also reported that the addition of OEO provides an antimicrobial activity to carnauba wax coatings. However, these studies did not characterize the physicochemical properties of the coatings, which is important to compare the responses with those observed in vivo. Therefore, the present study evaluated the effect of individual and combined coatings of chitosan and carnauba wax with OEO to reduce dehydration and microbial decay of fresh cucumbers.

2. Materials and Methods

2.1. Chemicals

Medium molecular weight chitosan (75%–85% deacetylation), carnauba wax yellow No.1, glycerol, and anhydrous calcium chloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). Glacial acetic acid was obtained from JT Baker (Madrid, Spain), while the coconut oil was obtained from Oils by Nature Inc. (Solon, OH, USA).

2.2. Plant Materials

Cucumber (*Cucumis sativus* L.) fruits were harvested in a field in Hermosillo, Sonora, Mexico (29°17′20.4" N 110°54′35.0" W) after 57 days from flowering, at a slightly immature state, with a dark green color visually determined by using the color comparator for medium green CC-1 proposed by the USDA, uniform in shape, size, weight and free from growth defects and decay; in addition, firmness, CO_2 production and color of cucumbers were determined (Table 1). OEO (*Lippia graveolens* L.) was obtained from ORE Procesadora de Oregano Silvestre [22], Chihuahua, Mexico.

Table 1. Firmness, CO_2 production, and color of cucumbers.

Parameter	Mean ± Standard Deviation
Firmness (N)	48.80 ± 1.89
CO_2 production rate (mL CO_2/kg·h)	1.10 ± 0.65
Color	–
Lightness	29.21 ± 1.01
Chroma	59.50 ± 13.19
Hue	132.76 ± 0.54

2.3. Film Formulation

For chitosan films, 0.8 g of chitosan and 0.2 g of glycerol were dispersed in 100 mL of 1% glacial acetic acid solution and stirred at 25 °C for 24 h. The filmogenic mixture was filtered using cheesecloth to remove small impurities, sterilized at 121 °C for 15 min, and cooled at 25 °C. Subsequently, 20 mL of the chitosan dispersion was cast on Petri dishes with diameters of 8 cm and dried at 25 °C for 24 h. Then the films were peeled off from the plates and stored in a desiccator for further analysis. For carnauba wax films, a 10% carnauba wax solution was made by melting 10 g of wax in 90 mL of coconut oil heated at 70 °C with constant stirring. Bilayer films (chitosan-carnauba wax and chitosan-OEO-carnauba wax) were elaborated by brushing 0.5 mL of the carnauba wax on the preformed chitosan films. For the films added with OEO, 0.2 g of Tween 20 and 8 mg·mL^{-1} of OEO (this concentration was selected based on the minimal inhibitory concentration of OEO against microbiota of decayed cucumbers) were added directly into the chitosan and carnauba wax for their individual coatings, and in the chitosan-wax coating the oil was added into the carnauba wax. Each formulation was mixed at 13,500 rpm for 5 min in a Kinematica Polytron homogenizer PT 1200C (Cambridge Scientific Products, Watertown, MA, USA).

2.4. Characterization of the Formulated Films

2.4.1. Thickness and Water Vapor Transmission (WVT)

Film thickness was measured with a digital micrometer (E.J. Cady and Co., Wheeling, IL, USA); three measurements were carried out at different points of 5 films per treatment (chitosan, chitosan-wax, chitosan-OEO, and chitosan-OEO-wax) and results were expressed in millimeters (mm). WVT was determined gravimetrically based on the American Society for Testing and Materials method (ASTM) [23]; for this, each film was placed in the top of a moisture permeation cell (118.64 cm^3 volume) with 30 g of dried calcium chloride beads to ensure a relative humidity (RH) of 0% inside the cell. Subsequently, the cells were placed in a desiccator (volume 1500 cm^3) containing 70 mL of a saturated

solution of magnesium nitrate to ensure 90% RH and left at 25 °C for 8 h. The water mass transferred through the film and adsorbed by the calcium chloride was determined by the weight gained for each permeation cell. Cells were weighed each hour for eight hours, and the slope of weight gain vs. time was obtained by linear regression. The following formula determined the WVT of the films: WVT = m/a, where m is the slope of weight gain vs. time, and a is the film area where the mass transfer occurred. The analysis was performed by triplicate, and results were expressed as grams of water per square meter per hour (g $m^{-2} \cdot h^{-1}$). Because of the experimental complexity, the thickness and WVTR of the individual wax films were not evaluated.

2.4.2. In Vitro Antimicrobial Capacity of the Formulated Films

Cucumber microbiota (mesophilic bacteria and fungi) isolated from decayed fruits, *Salmonella enterica* subspecies *enterica* serovar Typhimurium (ATCC® 14028), *Escherichia coli* O157:H7 (ATCC® 43890), and *Alternaria alternata* (ATCC® 6663) were exposed to the formulated films. The antimicrobial assay was carried out based on the dilution method described by the National Committee for Clinical Laboratory Standards [24], with some modifications. For the antimicrobial assay, 20 mg of each film (2 mg·mL^{-1}) was introduced into tubes containing 10 mL of Mueller Hinton or potato dextrose broth for bacteria and fungi, respectively. After 15 min, tubes were inoculated with 1×10^6 colony-forming units per milliliter (CFU mL^{-1}) of each challenged bacteria or fungi. Subsequently, 1.0 mL from each tube was plated in Mueller Hinton or acidified potato dextrose agar and incubated at 37 ± 2 °C for 24 h and 25 ± 2 °C for five days for bacteria and fungi, respectively. The analysis was performed by triplicate, the microbial colonies were counted, and the results were expressed as Log CFU mL^{-1} and compared with a control without films.

2.4.3. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra (Instrument Corp. Madison, WI, USA) were obtained to evaluate physicochemical interactions among the film components (chitosan, wax, chitosan-wax, chitosan-OEO, and chitosan-OEO-wax). Data were recorded in the transmission mode using a spectrophotometer FTIR Nicolet Protegé 460 (Instrument Corp. Madison, WI, USA) under a wavenumber range of 4000–400 cm^{-1} with a resolution of 4 (cm^{-1}), taking 64 scans at a rate of 0.63 (s^{-1}). Solid samples were pressed within KBr pellets, and liquid samples (OEO and carnauba wax) were placed over preformed pellets, respectively.

2.5. Effect of the Formulated Coatings on Water Loss and Microbial Decay of Fresh Cucumbers

2.5.1. Coating Application

A total of 252 cucumbers (whole and unpeeled) were used in this experiment; 216 fruits were divided into six groups and coated with each treatment, and 36 fruits were left uncoated as controls. Cucumbers were washed with chlorinated water (200 ppm) for 3 min and air-dried at 25 °C, and coated manually with brushes (1.5-inch-wide, Maxtool®, Mexico City, Mexico), adding uniformly 0.5 mL of each treatment per cucumber. For applying bilayer coatings, cucumbers were first coated with chitosan and dried with forced air at 25 °C for 10 min; afterward, wax (with and without OEO) was applied, then cucumbers were stored on polypropylene trays at 10 °C and 90% RH. The effects of edible coatings on the weight loss and microbial spoilage of the fruit were assessed in three trays with two cucumbers per treatment at 0, 3, 6, 9, 12 and 15 days.

2.5.2. Fruit Weight Loss

The fruit was weighed at 0, 3, 6, 9, 12 and 15 days of storage at 10 °C, and the slope of weight loss vs. time was obtained by linear regression. Weight loss was measured using the equation: $(A - B)/A$, where A was the initial weight (g) at t_0, while B was the weight (g) at a given storage time (t_n). The analysis was performed by triplicate, and results were expressed as weight loss (%).

2.5.3. Changes of Microbial Load of the Coated Fruit

Total mesophilic bacteria, molds and yeasts were counted on 0, 3, 6, 9, 12 and 15 days of storage at 10 °C. Cucumbers were sampled (10 g) and homogenized for 1 min in 90 mL of peptone water. Subsequently, decimal dilutions were made, and 1 mL of each sample was poured on plate count agar or potato dextrose acidified agar, and incubated at 37 ± 2 °C for 24 h or 25 ± 2 °C for five days, for mesophilic bacteria and for molds and yeasts, respectively [25]. The analysis was performed with four replicates, and results were expressed as Log CFU·g^{-1}.

2.6. Statistical Analysis

The effect of the films composition (chitosan, chitosan-wax, chitosan-OEO, wax-OEO, and chitosan-OEO-wax) on the WVT, thickness, and in vitro antimicrobial capacity was evaluated with a completely randomized design. The effect of the edible coatings over dehydration and microbial spoilage was a completely randomized experimental design with a factorial arrangement (5 × 6), where the factors were the coating treatments (chitosan, wax, chitosan-wax, chitosan-OEO, wax-OEO, and chitosan-OEO-wax) and the storage time (0, 3, 6, 9, 12, 15 days), and the analyzed responses were weight loss and microbial growth. All experiments were done by triplicate, expressing the results as means ± standard deviation. An analysis of variance (ANOVA) was performed, and means were compared by the Tukey Kramer test ($p \leq 0.05$) using the statistical software NCSS 2007 (NCSS, LLC, Kaysville, UT, USA).

3. Results

3.1. Characterization of the Formulated Films

3.1.1. FTIR Spectra

Figure 1 shows the infrared spectra of OEO (a), chitosan (b), wax (c), chitosan-OEO (d), and wax-OEO (e) in the wavelength range of 4000–400 cm^{-1}. OEO spectrum showed at 3361 and 2958 cm^{-1} the characteristic peaks of O–H and C–H stretching, respectively; these signals could be attributed to the presence of carvacrol and thymol. On the other hand, the peaks of the C=O stretch (amide I) and flexion for the N–H group (amide II) of the chitosan were identified at a wavenumber around 1645 and 1554 cm^{-1}, respectively (Figure 1b). These peaks represent the N-acetylglucosamine structure, which could be found in chitosan with a low acetylation degree [26]. Also, at 3334 cm^{-1}, the corresponding absorption band of the O–H stretching was observed. On the other hand, a shift in the O–H stretching peak of the chitosan was observed (Δ_{OH} = 11) when combined with OEO (Figure 1c), which could be attributed to the interactions between the terpenoids and chitosan. Figure 1d showed the spectrum of carnauba wax with the C–H and C=O stretching signals observed at 2930 and 1743 cm^{-1}, respectively. In contrast, no changes were observed in the wax spectrum after the addition of OEO, which indicated the lack of interaction between these components (Figure 1e).

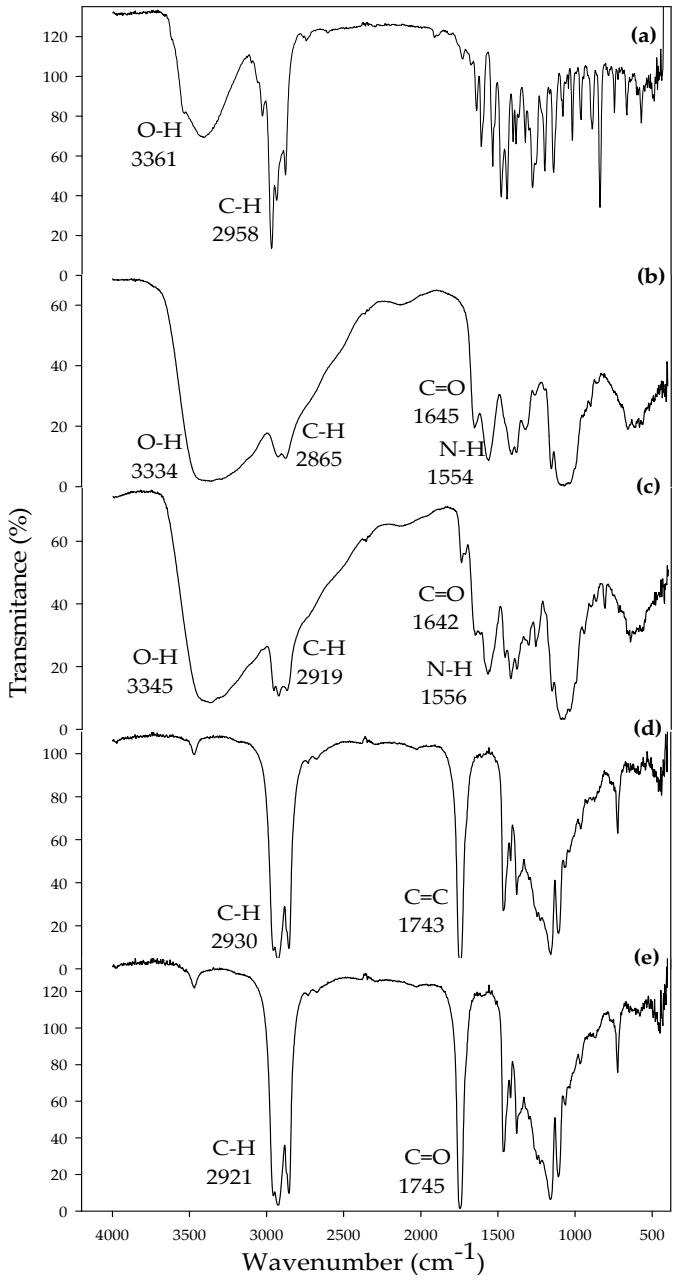

Figure 1. Fourier transform infrared spectroscopy (FTIR) spectra of oregano essential oil (OEO) (**a**), chitosan (**b**), chitosan-OEO (**c**), wax (**d**), and wax-OEO (**e**) films.

Moreover, the addition of wax on the chitosan film (chitosan-wax film) caused a shift toward higher frequencies in the absorption bands of the O–H (Δ_{OH} = 50) and C=O stretch of amide I (Δ_{CO} = 48.2), and the presence of the characteristic C=O peak of the fatty acid esters (Figure 2a). The same behavior was observed in the chitosan-OEO-wax film (Figure 2b), where the maxima absorption of the O–H (stretching), C=O (stretching), and N–H (flexion) peaks were shifted toward higher frequencies (Δ_{OH} = 7.7, Δ_{CO} = 44.85, and Δ_{NH} = 14). This behavior reflected that the incorporation of wax or OEO on the chitosan films could affect the maxima absorption peaks of the groups responsible for the antibacterial activity. Similar results were reported by Kaya et al. [27], who observed a shift of the N–H and O–H absorption peaks of chitosan after the addition of *Berberis crataegina* fruit extract, indicating hydrogen bond formation between the NH_2 group of the chitosan molecule and the O–H group of the fruit phenolic compounds. It is well-known that changes in the absorption spectra (in frequency and

shape) can be correlated with physicochemical interactions among the components [27]. In this sense, special attention must be given to O–H and N–H groups, considering that their electronic changes could cause variations in the properties of the film [28]. Based on this, an additional NMR analysis could be made to confirm the interactions between these components. Also, it is well-known that the antimicrobial activity of the OEO is related to the active hydroxyl groups of carvacrol and thymol [29]; therefore, any interaction in these sites could lead to a reduction of this property.

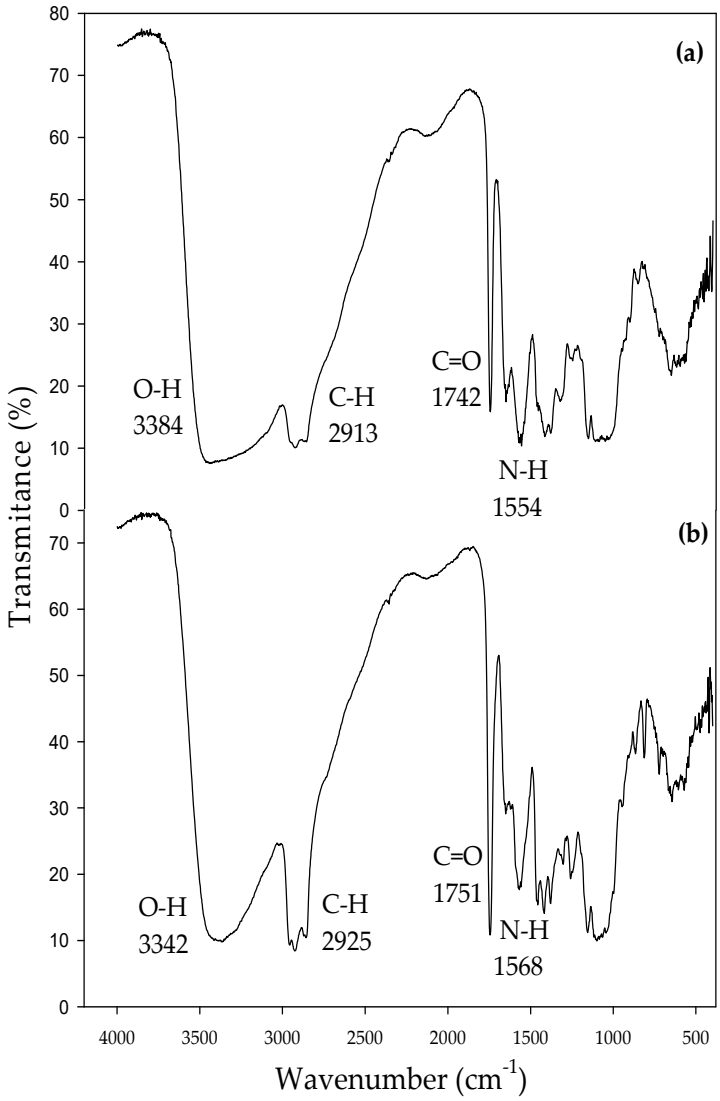

Figure 2. FTIR spectra of chitosan-wax (**a**) and chitosan-OEO-wax (**b**) films.

Several studies have tested the effect of the formulation on the film properties [30,31]; however, few studies have analyzed the physicochemical interactions among the constituents as the present study did. Considering that the presence and availability of certain functional groups grant the specific properties to the formulated materials, it becomes crucial to study their interactions and impact on the films functionality.

3.1.2. Thickness and WVT

Table 2 shows the results of thickness and WVT of the formulated films; the thickness was similar ($p \geq 0.05$) for chitosan, chitosan-wax, and chitosan-OEO, being the thickness of the chitosan-OEO-wax film the highest ($p \leq 0.05$) of all treatments. Chitosan films showed the highest WVT, followed by chitosan-OEO, with no significant differences between them ($p < 0.05$). Moreover, chitosan-wax and

chitosan-OEO-wax films reduced the WVT by 35% and 45%, respectively, compared to chitosan films. The WVT and FTIR spectra results could support the discussed interaction of chitosan O–H groups with the wax functional groups (Figure 2a,b). In this sense, the decrease in WVT after the wax incorporation could be attributed to the limited availability of free O–H groups, which could cause a decrease in the absorption of water molecules and thus lower its permeability.

Table 2. Thickness and WVT of edible films constituted by chitosan, OEO, and carnauba wax at 25 °C.

Film	Thickness (mm) *	WVT (g/m^2 h) **
Chitosan	0.025 ± 0.007 ^{a***}	0.257 ± 0.013 ^{a}
Chitosan-OEO	0.022 ± 0.002 ^{a}	0.241 ± 0.019 ^{a}
Chitosan-Wax	0.027 ± 0.005 ^{a}	0.167 ± 0.012 ^{b}
Chitosan-OEO-Wax	0.037 ± 0.004 ^{b}	0.141 ± 0.008 ^{b}

* n = 15; ** n = 3; *** Different letters in the same column indicate significant differences ($p \leq 0.05$).

Several studies have shown a decrease in the water vapor permeability of polysaccharide films with the incorporation of lipidic compounds [30]. Ochoa, Almendárez, Reyes, Pastrana, López, Belloso and Regalado-González [30] developed composite films with low WVT and antimicrobial activity based on corn starch, beeswax, lauric alginate, and natamycin. However, they did not study the contribution of each component to the efficacy of the whole film, as the present study did. In the same study, an increment of thickness from 0.050 to 0.064 mm after beeswax incorporation was recorded. Similarly, Santos et al. [32] observed an improvement in the water barrier properties of chitosan films after the addition of beeswax. Haq et al. [33] reported a 50% lower water vapor permeability of gum Cordia films added with beeswax. Agar/maltodextrin films showed better barrier properties when beeswax was added as a bilayer [34]. Therefore, hydrophilic-based films with a coat of hydrophobic material could diminish the WVT. This lower WVT could be a good alternative to decreased weight loss in fresh produce caused by dehydration; this approach is shown in Section 3.2.1.

3.1.3. In Vitro Antimicrobial Activity of Films

Figure 3 shows the antimicrobial activity of edible films against bacteria (a) and fungi (b) isolated from the decayed cucumber. Figure 3a showed that after incubation at 37 °C for 24 h, chitosan-OEO films showed the greatest in vitro reduction ($p \leq 0.05$) of bacteria isolated from cucumber (3.23 Log CFU·mL^{-1}), while the wax-OEO films reduced by about 1 Log CFU·mL^{-1}. Also, chitosan and chitosan-wax films showed a reduction ($p \leq 0.05$) of 0.4–0.6 Log CFU·mL^{-1}; however, chitosan, wax, chitosan-wax, and chitosan-OEO-wax showed bacterial counts similar to the control with no differences among them ($p \geq 0.05$). A similar pattern can be observed in Figure 3b, where chitosan-OEO showed the highest reduction (1.12 Log CFU·mL^{-1}) of fungal counts compared to the control ($p \leq 0.05$). In addition, chitosan, wax, chitosan-wax, and chitosan-OEO-wax showed no differences among them ($p \geq 0.05$) against fungal counts; it has to be mentioned that these treatments showed fungal counts significantly lower than the control (0.47–0.63 Log CFU·mL^{-1} reduction).

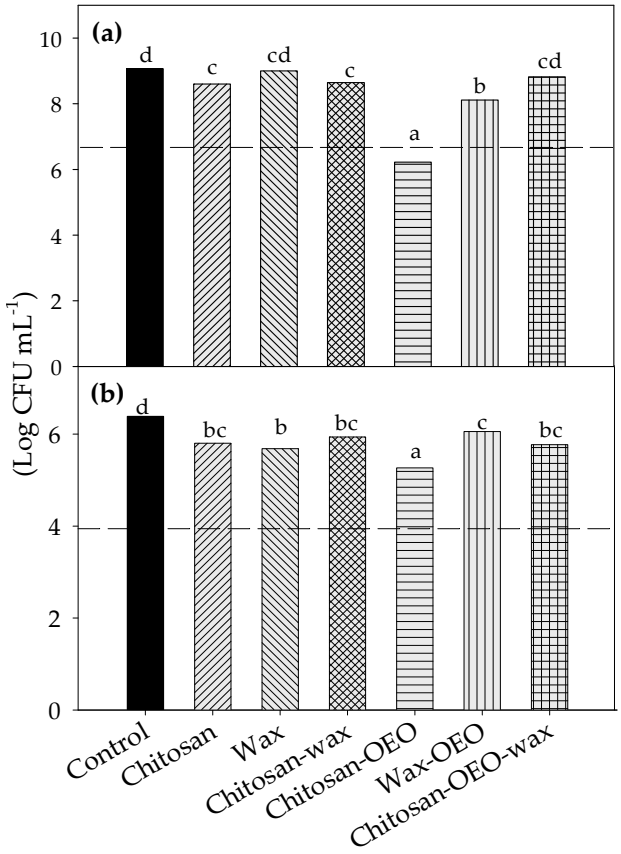

Figure 3. Antimicrobial activity of chitosan, carnauba wax, and oregano essential oil (OEO) films against mesophilic bacteria (**a**) and fungi (**b**) isolated from the decayed cucumber. Dotted lines signify the added inocula, and different letters among bars indicate significant differences ($p \leq 0.05$).

Figure 4 shows the in vitro antibacterial effect of edible films against *S.* Typhimurium (a) and *E. coli* O157:H7 (b). It was observed that the addition of OEO into chitosan films improved ($p \leq 0.05$) the antibacterial activity against *S.* Typhimurium (3.12 Log CFU·mL^{-1} reduction) compared to the control after 24 h of incubation at 37 °C (Figure 4a). On the other hand, chitosan and wax-OEO films significantly reduced *Salmonella* counts by 1.25 and 0.79 Log CFU·mL^{-1}, respectively. However, wax, chitosan-wax, and chitosan-OEO-wax films did not inhibit the growth of *S.* Typhimurium, showing counts similar ($p \geq 0.05$) to the control. On the other hand, for *E. coli* O157:H7, chitosan-OEO films reduced 3.45 Log CFU·mL^{-1} (Figure 4b), while chitosan and chitosan-wax reduced 1.58 and 0.43 Log CFU·mL^{-1}, respectively. Wax, wax-OEO, and chitosan-OEO-wax films showed no effect against *E. coli* O157:H7.

Figure 5 shows the fungicidal effect of the chitosan-OEO film against *A. alternata*. On the other hand, it was observed that chitosan-OEO-wax reduced 3 Log CFU·mL^{-1}, compared to the untreated fungus after five days at 25 °C. On the other hand, no differences were observed among chitosan, wax-OEO, and control, showing similar fungal counts. Furthermore, wax films did not inhibit the growth, showing counts 2.3–5 Log CFU·mL^{-1} higher ($p \leq 0.05$) than other treatments and control. The antimicrobial potential of chitosan-OEO films could be attributed to the action of each component. The antimicrobial activity of chitosan could be exerted by the interaction of its amino groups with the phospholipids in the bacterial membrane, leading to loss of functionality [28]. On the other hand, different studies suggest that carvacrol and thymol, major OEO components, may disintegrate the outer membranes of microorganisms because of their physicochemical interactions with lipids and proteins, causing a release of the cellular content and affecting viability [20]. It is important to mention that the reduced activity of wax-OEO and chitosan-OEO-wax films (versus the effect observed in chitosan-OEO films) could be attributed to a low diffusion of OEO compounds due to the different

components of coating's structure or their affinity to the hydrophobic phase of wax, reducing the oils migration from the film. In this sense, more studies are needed to characterize the diffusion of OEO compounds throughout these coating systems.

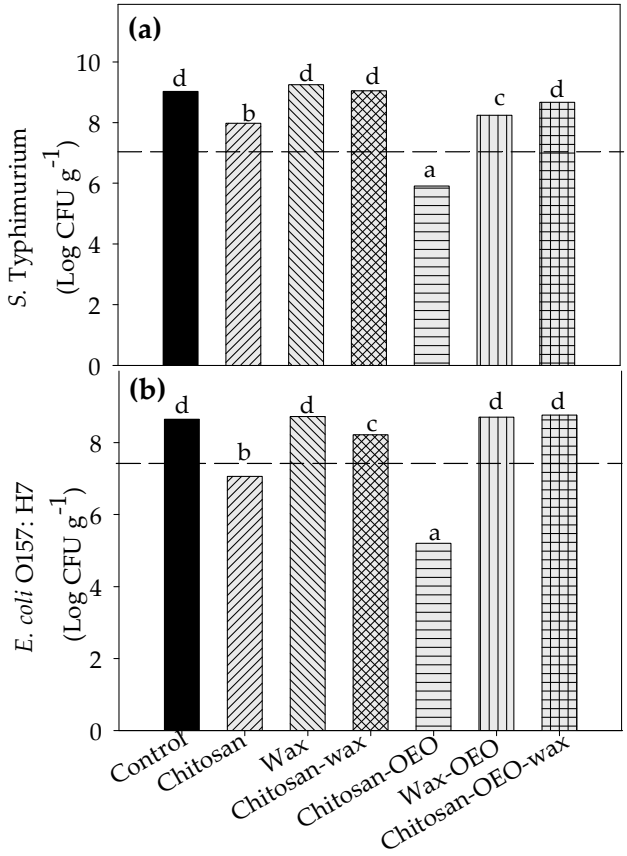

Figure 4. Antibacterial activity of chitosan, oregano essential oil (OEO), and carnauba wax films against *S.* Typhimurium (**a**) and *E. coli* O157:H7 (**b**) Dotted lines indicate the initial bacterial load, and different letters among bars indicate significant differences ($p \leq 0.05$).

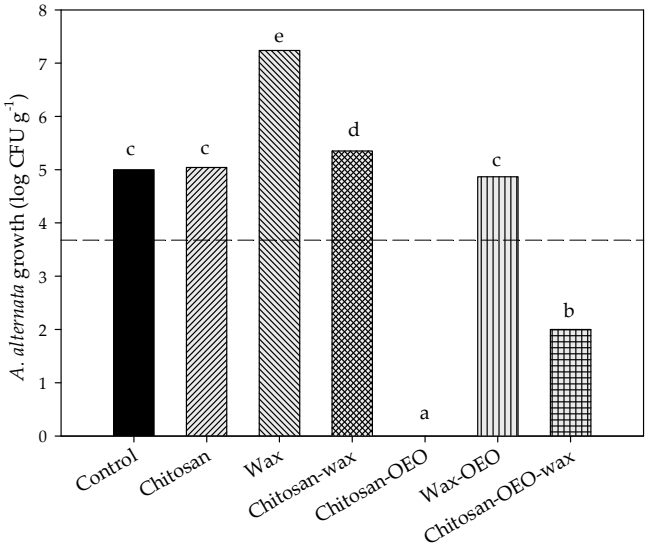

Figure 5. Antifungal activity of chitosan, oregano essential oil (OEO), and carnauba wax films against *A. alternata*. The dotted line indicates the initial fungal load, and the different letters indicate significant differences ($p \leq 0.05$).

Several studies have shown the ability of chitosan in carrying plant compounds to inhibit the growth of pathogenic bacteria [35]. Fernández-Pan et al. [36] reported that chitosan films with 20% carvacrol (the main terpene of OEO) reduced the growth of *Pseudomonas fragi*, *Shewanella putrefasciens*, and *Aeromonas hydrophila*. Similarly, Yuan et al. [37] observed an increase in the antibacterial activity of chitosan films against *Staphylococcus aureus* and *E. coli* after the addition of 10 mg·mL^{-1} of carvacrol. On the other hand, chitosan films incorporated with *Thymus piperella* essential oil reduced the growth of *Serratia marcesce*ns and *Listeria innocua* [38]. Chitosan alone or combined with OEO has also shown fungicidal activity against *Botrytis cinerea*, *Penicillium sp.*, *Rhizopus stolonifer*, and *A. alternata*. As mentioned above, the polycationic structure of this compound is responsible for its antifungal properties. Chitosan could induce morphological and structural changes in fungal cells by causing molecular disorganization [28]. Finally, the incorporation of OEO within chitosan films improved their antimicrobial activity, which was reflected by the significant reduction of bacterial and fungal growth.

3.2. Postharvest Changes of Coated Cucumbers

3.2.1. Coating Influence on Postharvest Weight Loss

Table 3. shows the weight loss of coated cucumbers stored at 10 °C for 15 days. Wax and wax-OEO treatments significantly reduced cucumber weight loss by 0.18%–1.63% compared to control fruits, with wax coatings the most effective ($p \leq 0.05$) compared to the other treatments, which showed similar losses to those of control fruits. On the other hand, chitosan coatings caused no decrement of cucumber weight loss, which was expected given their hydrophilic characteristics. Based on the WVT results described in Section 3.1.2, it was expected that there would be a better barrier property when carnauba wax was added to chitosan film; however, no differences ($p \geq 0.05$) were observed between chitosan, chitosan-OEO, chitosan-wax, wax-OEO, and chitosan-OEO-wax. WVT was measured at 25 °C, while cucumbers were stored at 10 °C. This condition was corroborated by microphotographs from fruit coated with wax and stored at 25 and 10 °C. The carnauba wax coated cucumber stored at 25 °C showed a smooth and homogenous surface (Figure 6a), but the fruit with the same treatment and stored at 10 °C showed a rough surface (Figure 6b), with the appearance of crystals (Figure 6c). Meanwhile, the chitosan and chitosan-OEO coated cucumbers showed a homogeneous coated surface at both temperatures (data not shown). Some factors influencing these phenomena could be the interaction between coating components and the temperature and cooling rate. Natural waxes in coating formulations tend to crystallize at low temperatures, and at low crystallization rates, bigger crystals can be formed, acquiring an amorphous and porous structure. All these structural changes could alter the wax stability, becoming more fragile and permeable to water. For this reason, it is possible that the low efficiency of this coating could be attributed to this phenomenon [39–41].

Table 3. Weight loss of cucumbers coated with chitosan, OEO, and carnauba wax and stored at 10 °C for 15 days.

Treatment	Weight Loss at Day 15 (%)
Control	7.11 ± 0.20 ^{b*}
Chitosan	7.18 ± 0.50 ^{b}
Wax	5.48 ± 0.50 ^{a}
Chitosan-wax	7.42 ± 0.21 ^{b}
Chitosan-OEO	7.66 ± 0.29 ^{b}
Wax-OEO	6.93 ± 0.10 ^{a}
Chitosan-OEO-wax	8.03 ± 2.01 ^{b}

Means ± standard deviation, $n = 9$. * Different letters in the same column indicate significant differences ($p \leq 0.05$).

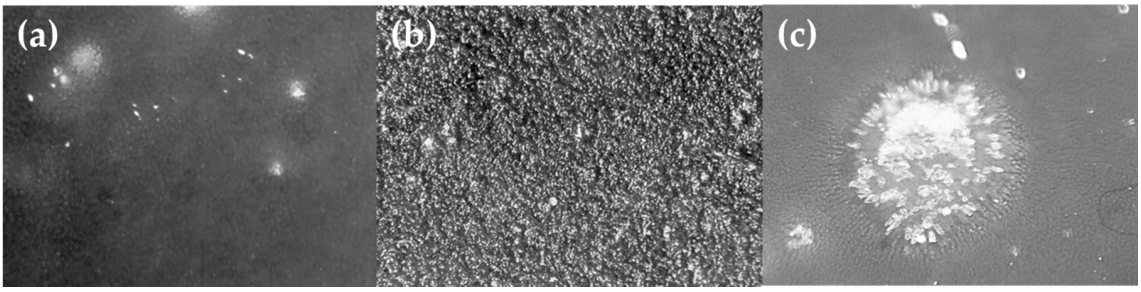

Figure 6. Stereoscopic microphotographs of the wax coated cucumbers stored at (**a**) 25 °C and (**b**,**c**) 10 °C; these images were acquired at a magnitude of 10×.

Won and Min [13] reported that carnauba wax coating significantly reduced weight loss (18.1%) of Satsuma mandarins treated with carnauba wax during storage at 4 and 25 °C for 28 days, compared with control fruit. Also, weight loss was significantly reduced in Valencia oranges after the application of carnauba wax for eight weeks at 4 and 20 °C [14]. Moreover, Miranda et al. [42] observed a similar trend when applying carnauba wax (18%) to papaya fruit, obtaining a 70.6% reduction of weight loss compared to uncoated fruits after 6 days of storage at 20 °C. Therefore, the use of these coatings during storage at higher temperatures can be suggested to avoid crystallization and maintain the response observed in the WVT analysis at 25 °C.

3.2.2. The Microbial Load of Coated Cucumbers

Table 4 shows the bacterial counts of cucumbers stored at 10 °C for 15 days. A decrease of bacterial counts due to the coating application was observed on day 0, and there was a slight increase on day 3, showing all treatments a bacterial growth lower than control. At day 6, wax, wax-OEO, and the bilayer coatings showed an increment of 0.1–1.62 Log CFU·g^{-1} of bacterial load. Moreover, chitosan and chitosan-OEO coatings maintained the cucumber bacterial loads significantly lower than control. However, at day 9 and 12 of storage, bacterial counts of chitosan-OEO increased. During the storage period, the chitosan coating maintained the lowest bacterial growth.

Table 4. Antibacterial activity of chitosan, OEO, and carnauba wax coatings on the mesophilic bacteria load of cucumbers stored at 10 °C for 15 days.

Storage (Days)	Log CFU·g^{-1} *						
	Control	Chitosan	Wax	Chitosan-Carnauba Wax	Chitosan-OEO	Wax-OEO	Chitosan-OEO-Wax
0	6.70 ^{a*}	3.81 ^{b}	4.44 ^{b}	4.62 ^{b}	3.76 ^{b}	4.94 ^{b}	4.80 ^{b}
3	6.0 ^{c}	4.80 ^{a}	5.45 ^{b}	4.90 ^{ab}	5.20 ^{ab}	5.36 ^{ab}	4.96 ^{ab}
6	5.90 ^{b}	4.80 ^{a}	6.97 ^{c}	5.0 ^{a}	4.85 ^{a}	6.98 ^{c}	5.09 ^{a}
9	5.23 ^{bc}	4.70 ^{a}	5.54 ^{c}	4.85 ^{ab}	5.51 ^{c}	5.34 ^{c}	5.11 ^{abc}
12	6.19 ^{c}	5.22 ^{a}	5.39 ^{ab}	5.99 ^{c}	5.73 ^{bc}	6.30 ^{c}	5.80 ^{bc}
15	7.37 ^{d}	5.78 ^{a}	6.90 ^{c}	6.68 ^{bc}	6.39 ^{b}	6.89 ^{c}	6.56 ^{b}

The initial cellular load of mesophilic bacteria in cucumbers before coating was 6.7 Logs CFU·g^{-1}. * Different letters amongst rows indicate significant differences amongst treatments per day ($p \leq 0.05$), $n = 4$.

As explained before, the antibacterial effect of chitosan is attributed to their amino groups; however, it is known that in multicomponent systems some interactions may occur among the functional groups of the components and the coated surface, causing a blockage of active sites and, hence, a reduction of antibacterial activity [28]. It is important to mention that chitosan only affects microorganisms that are in direct contact with its active sites [35]. Based on this, it can be supposed that carnauba wax led to a steric hindrance between the active sites of chitosan and bacteria. On the other hand, OEO components are volatile, and their presence could decrease during storage. Similar to this study, Moreira, Roura and Ponce [8] reported a significant bactericidal effect of chitosan coatings

(with reductions of 1.5–2.5 Log CFU·g^{-1}) on aerobic mesophilic bacteria of treated broccoli, compared to uncoated samples. Chiabrando and Giacalone [43] reported that chitosan films caused higher reductions of yeasts (<2.71 Log CFU·g^{-1}) and molds (<1.05 Log CFU·g^{-1}) in fresh-cut nectarines stored at 4 °C, compared to chitosan added with alginate. Similar to our study, Tokatlı and Demirdöven [44] demonstrated that chitosan caused a reduction of 2.71 Log CFU·g^{-1} in aerobic mesophilic bacteria of sweet cherries. Also, Alvarez et al. [45] reported that chitosan coatings (10 and 20 mg·mL^{-1}) reduced aerobic mesophilic counts (2.5–3 Log CFU·g^{-1}) of broccoli florets stored at 7 °C, compared to uncoated florets.

Table 5 shows the mold and yeast counts of coated cucumbers stored at 10 °C for 15 days. It was observed that all treatments showed similar initial counts ($p \geq 0.05$), being different from the higher values of uncoated fruit. However, chitosan-OEO-coated cucumbers showed the lowest counts (the half of the uncoated cucumbers counts). At day 6, no counts of molds and yeasts were detected on chitosan-OEO and chitosan-wax coated cucumbers, while uncoated fruits continue to increase to the end of the storage time. At the end of storage, the most effective treatment against molds and yeasts was chitosan-wax films, followed by chitosan-OEO, wax-OEO, wax, and chitosan-OEO-wax ($p \leq 0.05$).

Table 5. Molds and yeast counts of fresh cucumber coated with chitosan, OEO, and carnauba wax and stored 15 days at 10 °C.

Storage (Days)	Log CFU·g^{-1} *						
	Control	Chitosan	Wax	Chitosan-Wax	Chitosan-OEO	Wax-OEO	Chitosan–OEO-Wax
0	3.58 ^{c*}	2.14 ^{ab}	2.30 ^{ab}	2.18 ^{ab}	1.82 ^{ab}	2.72 ^{bc}	1.87 ^{a}
3	5.07 ^{c}	3.72 ^{a}	5.22 ^{d}	4.12 ^{ab}	4.10 ^{ab}	4.69 ^{bcd}	4.27 ^{abc}
6	4.30 ^{b}	3.0 ^{a}	3.53 ^{a}	–	–	3.37 ^{a}	3 ^{a}
9	4.25 ^{b}	3.20 ^{a}	–	–	4.13 ^{b}	3.07 ^{a}	–
12	4.98 ^{c}	4.12 ^{b}	3 ^{a}	3.0 ^{a}	3 ^{a}	4.05 ^{b}	–
15	5.02 ^{c}	3.94 ^{b}	3.55 ^{ab}	–	3 ^{a}	3.30 ^{ab}	3.69 ^{ab}

The initial load of molds and yeasts in cucumbers before coating was 3.58 Log CFU·g^{-1}. - below 250 CFU·g^{-1}.
* Different letters in rows indicate significant differences amongst treatments per day ($p \leq 0.05$), $n = 4$.

The observed effect of wax coating could be attributed to the oxygen barrier limiting the growth of aerobic microorganisms. Except for specific rumen-inhabiting species, fungi are strict aerobes or can grow only as microaerophiles [46]. Velickova et al. [47] reported that chitosan-beeswax coatings reduced the fungal infection of strawberries stored at 20 °C for 7 days. On the other hand, chitosan coatings (1%) reduced the disease incidence of *B. cinerea* in grape berries by 16.9% and 28.4% after 12 and 24 days post-infection at 10 °C, respectively [48]. In addition, it has been reported that a reduction of fungi infections in plants treated with chitosan, and this effect was attributed to the antifungal properties of chitosan and its ability to stimulate defense mechanisms, such as chitinase and phytoalexins [49]. It is important to mention that no major changes on the firmness of the coated cucumbers were observed during the experiment (Figure S1). Whereas the coated cucumbers showed higher °hue values than the uncoated fruit (Figure S2), no significant changes on L^* were observed among the treatments on control. Finally, the chitosan-wax and wax-OEO showed lower Chroma values than the uncoated fruit and the rest of the treatments.

4. Conclusions

The addition of carnauba wax helped to decrease the WVT of the formulated chitosan films and coatings. Furthermore, the addition of OEO to chitosan films increased the in vitro antimicrobial activity. Also, it can be concluded that the occurrence of physicochemical interactions among the components altered their individual properties. Finally, carnauba wax and carnauba wax-OEO coatings were the most effective in reducing weight loss, while chitosan and chitosan-wax were the most effective to reduce the microbial load of the treated fresh cucumber.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-6412/10/7/614/s1, Figure S1: Global effect of chitosan, wax, chitosan-wax, chitosan-OEO, wax-OEO, and chitosan-OEO-wax coatings on the firmness of the cucumbers stored at 10 °C for 15 days. Values are means of $n = 3$. Different literals among treatments indicate significant differences ($p \leq 0.05$), Figure S2: Global effect of chitosan, wax, chitosan-wax, chitosan-OEO, wax-OEO, and chitosan-OEO-wax coatings on the surface color of the cucumbers stored at 10 °C for 15 days. Values are means of $n = 3$. Different literals among treatments in the same parameter indicate significant differences ($p \leq 0.05$).

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