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Design of an Active Edible Coating Based on Sodium Caseinate, Chitosan and Oregano Essential Oil Reinforced with Silica Particles and Its Application on Panela Cheese

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Abstract: Active edible films and coatings incorporating antimicrobial agents such as essential oils are studied to improve the shelf-life of fresh foods. The aim of this work was to study a mixture of sodium caseinate-chitosan (SC:CH), added with mesoporous silica nanoparticles filled with oregano essential oil (MSN-OEO), to produce an active edible coating for Panela cheese and to evaluate its properties during refrigerated storage for 15 days. The OEO was extracted by hydrodistillation and was incorporated into the MSN. Films based on SC:CH of 4:1 and 8:1 ratios with and without MSN-OEO were produced and the mechanical, barrier, physicochemical and microbiological properties of the films were evaluated. The SC:CH 8:1 ratio (*w/w*) with MSN-OEO showed reduced mean particle size (764.8 ± 23.3 nm), and a stable solution (zeta potential = 29.9 ± 1.1 mV). The thickness and solubility were influenced by the incorporation of MSN-OEO making it thinner and less soluble. Panela cheese samples were coated by the spray method using the SC:CH 8:1 containing MSN-OEO film forming solution. The final pH of the control cheese was 0.5 units lower than that of the coated cheese, whereas final moisture loss of the control cheese was 4.2 times that of the coated cheese. The mesophilic aerobic bacteria and of molds and yeasts populations achieved a reduction of about 2 Log₁₀ UFC/g after 15 days of storage. Due to microbial growth delay, and little moisture loss, this active coating may improve the quality and safety of Panela cheese.

Keywords: edible coating; sodium caseinate; chitosan; oregano essential oil; Panela cheese

1. Introduction

In the last few years, advances in the production of edible coatings and films using different compounds from renewable sources has been provided. In fact, it is well known that barrier, mechanical and optical properties of films and coatings depend on both the chemical-physical properties of utilized compounds and their specific interactions [1,2]. Chitosan, derived from chitin deacetylation is one of the most widely studied and used biopolymers for the preparation of edible films and coatings due to its excellent film forming ability, biodegradability and antimicrobial properties [3]. Sodium caseinate is soluble in sodium hydroxide [4]. Sodium caseinate shows good film-forming and thermoplastic properties due to its random coil structure, and its ability to form weak intermolecular interactions [5,6]. However, it does not confer antimicrobial properties.

Panela cheese is a type of fresh cheese made from pasteurized cow's milk very popular in Mexico, where its production accounted for 418,560 tons in 2018 [7]. In addition, it is the most popular Hispanic-style cheese consumed in the USA [8]. It is white, with a

porous, soft and fluffy texture with low fat content. The shelf life of fresh cheeses is about 15–18 days, due to its high moisture content and nutrients availability [9].

Cheese packaging is practiced to minimize weight loss due to moisture loss, and to prevent microbial contamination and spoilage without affecting the cheese composition [10,11]. Edible coatings are applied directly to the surface of the food, where a thin layer forms after drying, while the films dry separately to form a material that is then used to cover the food.

Essential oils (EOs) are concentrated oily liquids obtained from plant materials such as flowers, buds, leaves and bark, which contain a mixture of volatile aromatic compounds including terpenes, terpenoids (oxygenated terpenes) and phenols [12,13]. EOs have shown antimicrobial properties against foodborne pathogens, i.e., bacteria, molds and their associated toxins [14]. The antimicrobial activity of essential oils may be regulated by more than one mechanism of action, including changes in the fatty acid profile of the cell membrane, damage of the cytoplasmic membrane and disruption of the proton-motive force [13,15]. However, due to the high volatility and diffusivity of essential oils in foods, the development of strategies able to increase their retention and release control, are the main challenges that need to be solved. EOs encapsulation is a promising technique that offers numerous advantages, such as ease of handling, stability, protection against oxidation, improved distribution, solubility, controlled release, with little or no effect on the organoleptic properties of foods to which they are applied with improved bioavailability [1,16].

Mesoporous silica nanoparticles (MSNs) are a good system for encapsulating essential oils due to their porous structure, chemical stability, biocompatibility, adjustable pore size and porosity, simple and low-cost synthesis, and possible expansion for industrial use. Furthermore, silica is biologically inert, and can break down into relatively harmless silicic acid by-products [17,18].

The aim of this study was to evaluate the physicochemical, mechanical and barrier properties of sodium caseinate and chitosan films, added with mesoporous silica particles filled with oregano essential oil (OEO). In addition, this formulation was tested as coating of fresh Panela cheese and the microbiological and physicochemical changes were evaluated throughout 15-days storage period.

2. Materials and Methods

2.1. Materials

Sodium caseinate was purchased from Fonterra Group (Auckland, the Netherlands), Chitosan Powder (90% deacetylation; viscosity 50–800 mPa·s) was obtained from Chem-savers (Bluefield, VA, USA). Anhydrous glycerol was purchased from J.T. Baker (Radnor, PA, USA); lactic acid (85%), cetyltrimethylammonium bromide (CTAB) and tetraethyl orthosilicate (98%) (TeOS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fresh oregano leaves (*Lippia graveolens* Kunth) were collected in Cerrito Parado, Toluca (Queretaro, Mexico). A voucher specimen was authenticated and deposited in the Ethnobotanical Collection of the Herbarium of Querétaro “Dr. Jerzy Rzedowski” (QMEX), located at the Faculty of Natural Sciences, University of Querétaro, Mexico (voucher specimen: E. Hernández-Hernández No. 1). Potato dextrose agar, plate count agar and casein peptone were acquired from BD Bioxon (Estado de Mexico, Mexico).

All bacteria tested were obtained from the microbial collection of the Food Biotechnology Laboratory, DIPA, Universidad Autónoma de Querétaro (Mexico). Selected bacteria were those relevant in microbial contamination of fresh cheese, *Listeria innocua* and *Salmonella Saintpaul*. The strains were stored at -60°C in sterile glycerol. The bacteria were activated in nutrient broth (Bioxon) at 37°C for 24 h.

2.2. Extraction of Oregano Essential Oil (OEO)

The oregano leaves were air-dried at room temperature for 3 days in a dark chamber. The dry material was stored in black polyethylene bags until use [19]. Dry oregano leaves

(700 g) were suspended in 5 L of distilled water and subjected to hydrodistillation for 2 h, using a Clevenger-type apparatus (Cristalab, CDMX, México). The obtained OEO was dried with anhydrous sodium sulfate, sterilized by filtration using a Swinnex unit of 0.45 µm pore size polyvinylidene fluoride (PVDF) membrane (Millipore, Burlington, MA, USA), and then stored in sealed vials protected from light at 4 °C for later use [20].

2.3. MSN-OEO Preparation

The MSN were synthesized, as previously reported [21], with modifications. In a solution of 240 mL of distilled water and 1.75 mL of 2 N NaOH, 0.5 g of CTAB was dispersed by using an ultrasonic bath at room temperature. The sample was heated up to 80 °C under constant stirring and then 2.5 mL of TeOS was added drop by drop during 5 min, and vigorously stirred for 2 h at 80 °C. The solid product was obtained by filtration, then washed with ethanol, and finally calcined at 500 °C for 5 h to remove the surfactant. The obtained MSNs (0.1 g) were dispersed into 2 mL of ethanol containing 0.1 g of the OEO. The final mixture was stirred overnight in a fume cupboard at room temperature to remove the ethanol.

The amount of OEO in the MSN-OEO particles was determined by suspending 5.0 mg of the samples in 10 mL of ethyl acetate, followed by centrifugation (IKA, Wilmington, NC, USA) at $10,000 \times g$ for 10 min. The supernatant containing the released OEO was quantified from a calibration curve constructed with different OEO concentrations in ethyl acetate, and measuring absorbance at 270 nm using a spectrophotometer (UV-1800 Shimadzu, Kyoto, Japan) [22]. The released OEO was subtracted from the total OEO to obtain the amount of OEO in the MSN.

2.4. Preparation of SC:CH Film Forming Solution (FFS)

The SC solution was prepared at 0.90% (*w/v*) in distilled water and stirred at 40 °C for 30 min, then the pH was adjusted to 5.3. The solution of chitosan 1% (*w/v*) in lactic acid (1% *v/v*) was stirred for 60 min at 80 °C, once dissolved, the pH was adjusted to 5.8; each solution was kept under stirring until complete dissolution. To produce the FFS, SC (360 mg) was added to the CH at 4:1 and 8:1 (SC:CH *w/w*) ratios, under stirring at 700 rpm. The MSN-OEO was suspended in distilled water (20 mg/mL) at pH = 10 and added to the FFS (3% relative to the total mass of SC plus CH, *w/w*). Glycerol was added as plasticizer at 30% of SC (*w/w*), and the mixture was adjusted to pH 5.3, followed by gently stirring for 20 min. Finally, the FFS was homogenized using an Ultra-Turrax (IKA T25, Staufen, Germany) at 9500 rpm for 2 min.

2.5. Characterization of the FFSs and Films

2.5.1. ζ-Potential and Particle Size Measurement

One ml of each FFS was analyzed for ζ-potential and particle size by using a Zetasizer Nano-ZSP (Malvern Instrument, Worcestershire, UK). ζ-potential was calculated through the electrophoretic mobility by applying a voltage of 200 mV and using the Helmholtz-Smoluchowski equation. Hydrodynamic size was obtained from dynamic light scattering by using a He–Ne laser (wavelength of 633 nm) and a detector angle of 173°.

2.5.2. Film Preparation

Films were prepared by the casting method. Each FFS (45 mL) was poured into 8 cm diameter polyester Petri dishes and dried at 38 °C and 50% relative humidity (RH) in an environmental chamber (Binder, KBF 115, Tuttlingen, Germany), for 18 h. Film thickness was measured with a digital micrometer (Mitutoyo, 293–185, Kawasaki, Japan) at five random points over the film, and the mean ± standard deviation (SD) of five replicates was reported.

2.5.3. Mechanical Properties

Film tensile strength (TS), elongation at break (EB) and Young's modulus (YM) were measured by using an Universal testing instrument (Instron Engineering, model No. 5543A, Norwood, MA, USA) as previously described [23]. Film samples were cut, using a sharp razor blade, into 10 mm wide and 40 mm length strips equilibrated overnight at $50\% \pm 5\%$ RH and 23 ± 2 °C in an environmental chamber. Five samples of each film type were tested.

2.5.4. Barrier Properties

Film permeabilities to gas (O₂ and CO₂) and water vapor (WV) were determined by using a MultiPerm apparatus (Extrasolution s.r.l., Pisa, Italy). Duplicate samples of each film were conditioned for 2 days at 50% RH before measurement. Aluminum masks were used to reduce the film test area to 5 cm², whereas the testing was performed at 25 °C under 50% RH.

2.5.5. Solubility

The solubility of films was determined according to Vahedikia et al. [24]. The initial dry matter of samples was determined by placing the films in a desiccator containing calcium sulfate at 25 °C and 0% relative humidity (RH) for 24 h. The films were then immersed in 50 mL distilled water and then placed in a shaker incubator at 25 °C and stirred for 24 h at 250 rpm. The film solubility (%) was calculated using Equation (1):

$$\text{Solubility (\%)} = \frac{\text{Initial dry weight} - \text{Final dry weight}}{\text{Initial dry weight}} \times 100 \quad (1)$$

2.5.6. Scanning Electron Microscopy (SEM)

Microstructural analysis of cross-sections of the films was carried out by using SEM, in a JEOL JSM-5410 (Tokyo, Japan) electron microscope. Pieces of 5 mm × 5 mm were cut from films and mounted on copper stubs. Films were fixed on the support using double side adhesive tape. Samples were gold coated and observed using an accelerating voltage of 10 kV.

2.5.7. Color and Transparency

The color of the films was evaluated according to the CIELAB method, using a Minolta CR400 colorimeter (Minolta, Osaka, Japan) with the parameters L* = lightness, a* = green to red color component and b* = blue to yellow color component. The color was standardized using a white reference plate.

The transparency of films was determined according to Lin et al. [6], with modifications. The edible film was cut into strips (1 cm × 4 cm), and the top and bottom edges of the specimen were fixed on the surface of a quartz cuvette with adhesive tape, and the transmittance at 600 nm was measured using a spectrophotometer. The transparency was calculated using Equation (2):

$$T = \frac{A_{600}}{\delta} \quad (2)$$

where: T = transparency; A = absorbance; δ = thickness.

2.5.8. Atomic Force Microscopy (AFM)

The surface roughness of the coating used on Panela cheese (SC:CH, 1:8 with and without SMN.OEO) was estimated by atomic force microscopy (AFM), according to Escamilla-García et al. [25], by means of an atomic force microscope (di Multimode V, Veeco, Plainview, NY, USA) in contact mode. Film pieces of 0.5 cm × 0.5 cm were used, and the resonance frequency of scanning was 286–362 kHz with a spring constant of 20–80 N/m, scanning speed of 1 Hz and resolution of 256 × 256 pixels. The results were analyzed using the Gwyddion Version 2.53 software (Okružní, Czech Republic). The roughness was obtained by evaluating the square root of the deviation from an average plane of the peaks and

surface valleys (R_q) (Equation (3)). The mean absolute value of surface height deviations from the middle plane (R_a), was estimated from Equation (4).

$$R_q = \sqrt{\frac{\sum Z_i^2}{N}} \quad (3)$$

$$R_a = \frac{1}{N} \sum_{j=1}^N Z_j \quad (4)$$

where R_a and R_q indicate the roughness (nm), Z_i and Z_j are the height difference of i and j relative to the heights average, and N is the number of points on the image.

2.5.9. Antimicrobial Activity

Antimicrobial activity was evaluated following Hernández-Hernández et al. [20]. Briefly, 10 mL of trypticase soy agar (0.8% w/v (Bioxon) was inoculated with 200 μ L of *L. innocua* solution (10^7 CFU/mL) or 100 μ L of *S. Saintpaul* (10^7 CFU/mL), subsequently poured onto plates containing solidified agar (1.5% w/v).

Disks were prepared from films of SC:CH at 8:1 ratio (w/w) with and without MSN-OEO of 25 mm in diameter. Additionally, PVDF membrane disks of 25 mm in diameter (Darmstadt, Germany) were impregnated with 8 mg of MSN-OEO or 75 μ L of OEO diluted at 25% (w/v) with Tween 80 at 10% (v/v). One disk of each was gently placed on top of the soft agar layer and OEO were allowed to diffuse for 2 h, at 4 °C and then incubated at 37 °C for 48 h. The growth inhibition zone, which included the disk diameter, was measured using Vernier callipers.

2.6. Application as a Coating on Panela Cheese

The coating was applied on samples of Panela cheese using a spray gun (Husky, Lincoln, NE, USA) the samples were sprayed 2 times at 2 min interval. After the coating process, all samples were drained on stainless steel screens and air-dried in a laminar flow cabinet for 20 min, and then were put into plastic “clam-shell” containers (Industry standard, 9756Z, Pactiv Corp., Zapopan, México) and stored at 4 °C. The cheese was subjected to physicochemical and microbiological analyses at 0 day, 5 days, 10 days and 15 days of storage. Uncoated cheeses were used as control, and were stored and analyzed at the same times.

2.6.1. Moisture Content, pH and Titratable Acidity

The moisture content (MC) in Panela cheese was analyzed by moisture analyzer XM50 (Precisa Gravimetrics AG, Dietikon, Switzerland) by using 1 g of homogenized cheese, and moisture was expressed as weight %. Titratable acidity and pH were evaluated following the Mexican standard NOM-243-SSA1 [26]. The pH was detected by using 1 g of cheese homogenized in 10 mL of distilled water, and from each sample, 3 pH measurements were taken using a calibrated potentiometer Orion (Star A211, Thermo Scientific, Waltham, MA, EUA). Titratable acidity was evaluated using 18 g of homogenized cheese in 36 mL of distilled water, followed by titration with 0.1 N NaOH until the appearance of a pink color for at least 30 s. The results are expressed as% lactic acid (w/w).

2.6.2. Microbiological Analysis

Molds and yeast, and mesophilic aerobic bacteria were quantified according to the Mexican standard [26]. Panela cheese with and without coating at 0 day, 5 days, 10 days and 15 days of storage were analyzed in triplicate.

Molds and Yeasts

For the population of molds and yeasts the cheese was homogenized followed by serial decimal dilutions; then 1 mL of each dilution was poured into potato dextrose agar

(Bioxon), previously adjusted to $\text{pH} = 3.5 \pm 0.1$ by using 10% tartaric acid (J.T. Baker), and incubated at 25 ± 1 °C. Colonies (CFU/g) were evaluated after 3 days of incubation, and results were reported as Log_{10} CFU/g.

Mesophilic Aerobic Bacteria

The population of mesophilic aerobic bacteria of the cheese samples was performed using plate count agar, pouring 1 mL of decimal dilutions and incubating for 48 h at 35 °C. Results were reported as Log_{10} CFU/g.

2.7. Statistical Analysis

Experiments were made in triplicate, and analysis of variance (ANOVA) was performed using the JMP software 13.0 (SAS Institute, Cary, NC, USA). Tukey's multiple range test was used, differences at $p < 0.05$ were considered significant and are indicated with different letters.

3. Results and Discussion

3.1. SEM of MSN and MSN-OEO

The MSNs before the addition of OEO exhibited large aggregates with size about 360 nm, possibly due to the interaction of microaggregates (Figure 1A). When OEO was added to the MSNs, a more homogeneous surface was observed indicating better particles dispersion, leading to a decreased particle size of about 250 nm (Figure 1B). This is probably associated to OEO encapsulation within the MSNs pores and over the particles surface counteracting their coalescence.

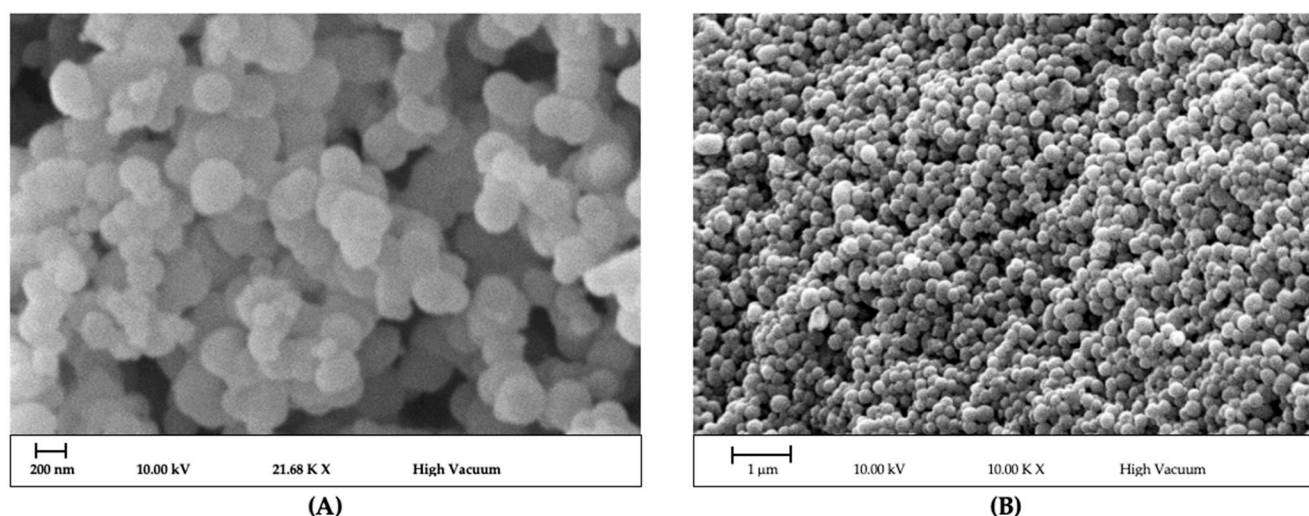


Figure 1. SEM micrographs of (A) MSN and (B) MSN-OEO. Magnification: $21,680\times$ (A) and $10,000\times$ (B).

3.2. FFSs Stability

It is well known that ζ -potential can be correlated with the stability of dispersions that can be classified according to their absolute values as follows: 0–10 mV highly unstable, 10–20 mV relatively stable, 20–30 mV moderately stable and >30 mV as highly stable [27].

Preliminary experiments on the mixing of SC ($\text{pI} = 4.5$) and CH ($\text{pKa} = 6.0$) demonstrated that to produce stable complexes, the pH of the stock solutions must be pH 5.3 for SC and pH 5.8 for CH, to counteract clotting and phase separation. These conditions are useful to keep the biopolymers weakly charged and with opposite charges to facilitate low electrostatic interactions aiming to stabilize the complexes formed, while avoiding phase separation. In fact, according to Anal et al. [28] the SC:CH complexes precipitated at pH range 4–4.5, and $\text{pH} > 6$, whereas the polymers tended to dissociate between pH 3 and 3.8 due to similar charges. On the other hand, stable complexes were formed at

pH 5–6, which was attributed to the interaction of positive charge density and linear structure of CH, in addition to the negative charge of SC [29]. Therefore, after preliminary experiments, the mixtures were made at pH 5.3 to produce stable complexes, in agreement with Volpe et al. [30]. By comparing the ζ -potential of the SC:CH dispersions obtained at different ratios and at pH 5.3, it is possible to observe a greater stability at 4:1 ratio (34.77 mV) compared to 8:1 (29.33 mV) even if the hydrodynamic radius is greater using the 4:1 ratio than 8:1 (Table 1). It is known that proteins have both positive and negative charges on the surface that can interact with each other, generating protein aggregates with a hydrodynamic radius of 1800.16 nm. At the higher chitosan concentration (4:1), the positive charge density and linear structure of the CH destabilizes the protein-protein interactions by generating protein-chitosan complexes. Furthermore, the excess of CH can produce different complexes through the residual negative charges on the proteins, producing aggregates of about 1010.64 nm. This effect increases the residual positive charges of both CH and proteins increasing the ζ -potential. Conversely, a lower CH Content (SC:CH 8:1) favors the formation of isolated protein-chitosan aggregates in which the CH envelops the proteins giving rise to smaller particles (781.33 nm). Furthermore, the negative surface charge of the protein aggregates partially counteracts the positive charge of the complexes leading to decreased average ζ -potential. Further experiments are needed to verify this hypothesis.

Table 1. ζ Potential, particle size and polydispersity index of the SC:CH FFSs at different ratios containing or not MSN-OEO.

SC:CH Ratio	pH	MSN-OEO	Particle Size (nm)	Z Potential (mV)	Polydispersity Index
0:1	5.8	-	1238.27 $\pm$ 25.9 ^d	31.07 $\pm$ 0.8 ^c	0.72 $\pm$ 0.1 ^c
1:0	5.3	-	1800.16 $\pm$ 10.2 ^e	27.77 $\pm$ 0.8 ^d	0.53 $\pm$ 0.1 ^a
4:1	5.3	-	1010.64 $\pm$ 8.1 ^a	34.77 $\pm$ 1.2 ^a	0.54 $\pm$ 0.0 ^a
4:1	5.3	+	1008.00 $\pm$ 4.5 ^a	36.93 $\pm$ 1.2 ^a	0.39 $\pm$ 0.1 ^b
8:1	5.3	-	781.33 $\pm$ 11.7 ^b	29.33 $\pm$ 1.3 ^b	0.53 $\pm$ 0.0 ^a
8:1	5.3	+	764.80 $\pm$ 23.3 ^c	29.93 $\pm$ 1.1 ^b	0.50 $\pm$ 0.0 ^a

Results are reported as the mean $\pm$ standard deviation ($n = 3$). Equal letters indicate that there is no significant difference ($p < 0.05$).

The stability of the FFSs (4:1 or 8:1 ratios) is not affected by the addition of MSN-OEO after the biopolymer complexation; in fact, no significant differences on the ζ -potential values were observed (Table 1). Fernandez-Bats et al. [21] reported that in film-forming solutions of bitter vetch protein, MSNs higher than 3% by weight destabilize the FFSs.

Similar to the effect on the particle size reduction observed by Navarro et al. [31], during the addition of different oil encapsulating agents in the film-forming solutions, the MSN-OEO significantly reduced the hydrodynamic size of the 8:1 SC:CH complex. Moreover, the samples showing PDI values < 0.6 indicate a Gaussian particle size distribution [32]. This result was attributed to decrease of the interfacial tension between the oil and the aqueous phases by the encapsulating agent, because it reduces the free energy required to deform and interrupt the drops, resulting in smaller particles.

3.3. Characteristics of the SC:CH Films

3.3.1. Microstructural Properties of Films

The final microstructure of the films resulted from the components interactions and the destabilizing phenomena taking place during the drying stage. The addition of MSN-OEO particles to films of 8:1 SC:CH ratio, resulted in different surface patterns (Figure 2). The film without MSN-OEO showed a smooth and homogeneous microstructure (Figure 2A), whereas the surface of films containing the MSN-OEO (Figure 2B) reveal a less continuous structure with the particles already embedded on the surface of the film. The cross section of the SC:CH 8:1 MSN-OEO film shows that the OEO was incorporated with partly uneven distribution in the polymers matrix, displaying some micro-sized cracks and holes (Figure 2C2). Magnification of the cross section revealed that the dispersion of the MSN-

OEO phase was well distributed throughout the films, showing layers of the MSN-OEO phase (Figure 2C1). A similar behavior was reported by Fabra et al. [33], with SC films containing mixtures of oleic acid and beeswax. Here, addition of the hydrophobic material changed the films microstructure from homogeneous and smooth surface to one showing the lipid mass embedded in the matrix of the film.

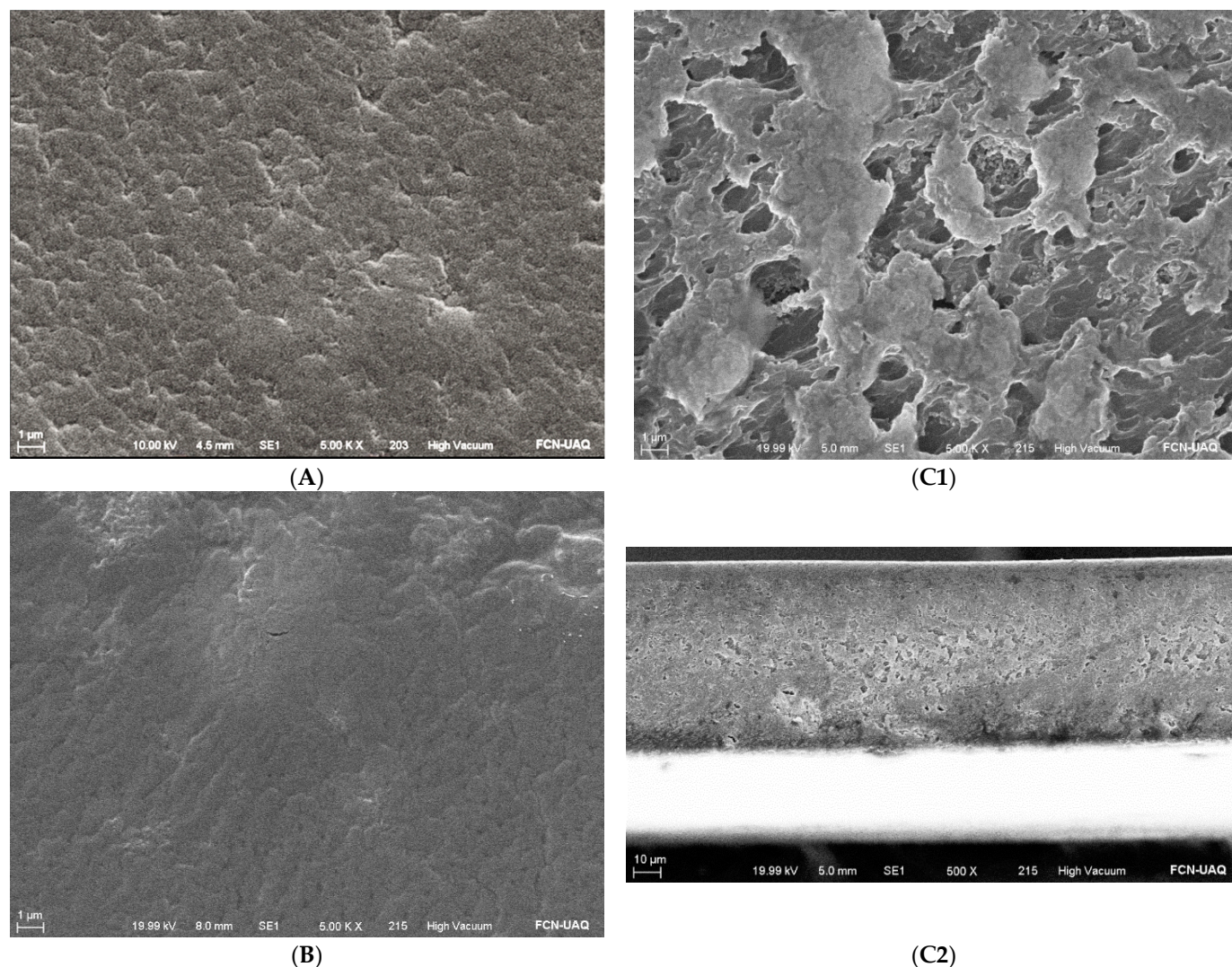


Figure 2. SEM micrographs of films made from 50 mL of FFS; magnification: 5000 $\times$. (A) SC:CH 8:1. (B) SC:CH 8:1 MSN-OEO. (C1) Cross section SC:CH-8:1 MSN-OEO. (C2) Cross section SC:CH-8:1-MSN-OEO (Magnification: 500 $\times$).

In conclusion, the structural differences are clearly visible due to the addition of MSN-OEO, probably due to different drying behavior, which resulted in the exposure of the MSN-OEO particles between layers of the complex SC:CH.

3.3.2. Thickness

The produced edible films were transparent and flexible, and their thickness is an important factor in creating a modified atmosphere able to delay food spoilage. Films thickness varied between 57.5 ± 5.1 and 69.2 ± 4.7 μm (Figure 3). Pereda et al. [34], obtained similar results for films made with ratio 8:1 (SC:CH) ratio with thickness ranging 50–90 μm . Addition of MSN-OEO to the FFS produced slightly decreased films thickness, with lower value for the SC:CH (8:1 ratio) attributed to high protein concentration, producing increased interactions with the MSN-OEO, and avoiding particles agglomeration. However, we could not find any significant thickness difference among the different films.

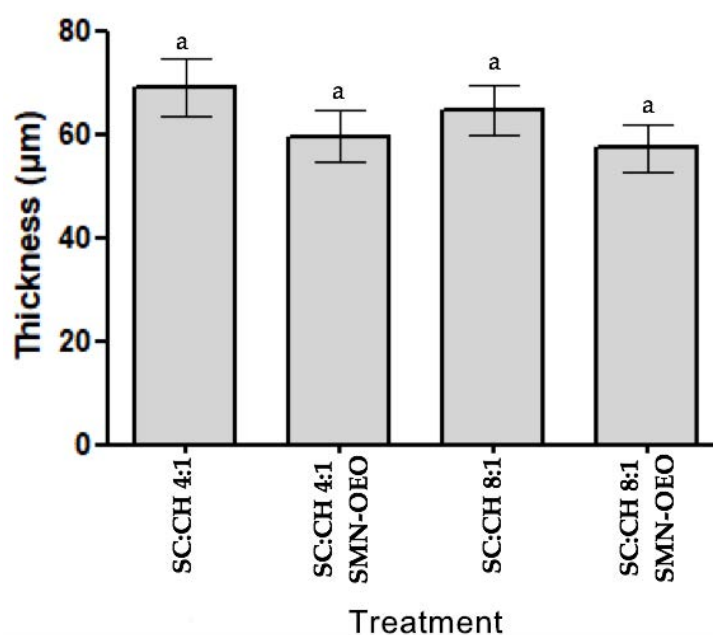


Figure 3. Thickness of SC:CH films with and without the presence of MSN-OEO.

3.3.3. Solubility of the Films

The solubility of the films produced in this work ranged from $34.6\% \pm 1.3\%$ to $36.8\% \pm 1.2\%$. (*w/v*). Films containing MSN-OEO showed slightly decreased solubility, whereas films having more SC content were more water soluble. The solubility in edible films and coatings is an important parameter for fresh cheese due to syneresis or liquid leakage, causing packaging biopolymers to dissolve. Vahedikia et al. [24], reported zein protein films solubility of 40.1%, which slightly decreased by addition of chitosan-cinnamom EO to 39.1%. The addition of nanocomposites shows a more hydrophobic pattern, mainly due to the interaction of the EO components with side chain groups of the amino acids of the protein matrix.

3.3.4. Optical Properties of the Films

The addition of the MSN into the films did not exhibit significant difference in the L^* parameter (Table 2). The films presented high L^* values, whereas a^* and b^* values are close to the L^* axis, indicating low saturation (i.e., dull color), essentially gray. The SC:CH films prepared with MSN-OEO revealed a transparency of 0.82 ± 0.0 , whereas addition of MSN-OEO to the FFS resulted in significantly improved films transparency, which may permit consumers a clearer appreciation of the food surface. Transparency may affect appearance, merchandising and applications, and greater values in edible films and coatings can confer better food visibility. The optical properties of edible packaging depend on the characteristics of the compounds and the film preparation conditions: pH, homogenization and drying. Lin et al. [6], presented good transparency for SC films (0.97 ± 0.3), but addition of 1.5 mg of the cross-linking agent genipin, it increased to 3.69 ± 0.4 . The transparency values obtained in this work compared favorably to those of synthetic films, such as low-density polyethylene, directional polypropylene and polyvinyl chloride that were 3.05, 1.67 and 4.58, respectively [35]. The transparency of the edible SC:CH films significantly increased by adding the MSN-OEO.

Table 2. Color analysis and transparency of SC:CH films with MSN-OEO.

SC:CH Ratio	MSN-OEO	L*	a*	b*	Transparency
4:1	+	91.62 ± 0.2 ^a	0.33 ± 0.0 ^{a,b}	−1.16 ± 0.2 ^a	0.89 ± 0.0 ^a
4:1	−	92.00 ± 0.1 ^a	0.34 ± 0.0 ^{a,b}	−1.39 ± 0.1 ^a	1.14 ± 0.1 ^{b,c}
8:1	+	92.01 ± 0.3 ^a	0.29 ± 0.0 ^b	−1.18 ± 0.4 ^a	0.82 ± 0.0 ^c
8:1	−	92.19 ± 0.1 ^a	0.39 ± 0.0 ^a	−1.79 ± 0.0 ^a	1.03 ± 0.0 ^{a,b}

SC: sodium caseinate, CH: chitosan, MSN-OEO: mesoporous silica nanoparticles filled with oregano essential oil. Results are reported as the mean ± standard deviation ($n = 3$). Equal letters indicate that there is no significant difference ($p < 0.05$).

3.4. Surface Roughness of the Films

From the three-dimensional image of the films surfaces (Figure 4), those based on SC:CH (4:1) show significantly larger surface roughness than those using the 8:1 ratio (Table 3), probably associated to the protein super-aggregation due to higher protein content. However, addition of MSN-OEO causes an increase in surface roughness, especially for the SC:CH (8:1) film (Figure 4). This film also shows some isolated microparticles that in the presence of MSN-OEO are less visible, but with more evident peaks and valleys leading to significantly higher R_a and R_q values (Table 3); additionally, the presence of MSN-OEO produces films showing similar roughness for both SC:CH ratios.

Figure 4. AFM micrographs of SC:CH films with and without MSN-OEO. (A) SC:CH 8:1 (w/w); (B) SC:CH 8:1 (w/w) MSN-OEO; (C) SC:CH 4:1 (w/w); (D) SC:CH 4:1 (w/w) MSN-OEO.**Table 3.** Roughness parameters obtained from AFM analysis of films based on SC:CH, with and without MSN-OEO.

Treatment	R_q (nm)	R_a (nm)
SC:CH 8:1	3.20 ± 1.0 ^a	1.05 ± 0.2 ^a
SC:CH 8:1 MSN-OEO	5.04 ± 0.6 ^b	3.89 ± 0.5 ^b
SC:CH 4:1	3.76 ± 0.9 ^b	2.88 ± 0.6 ^b
SC:CH 4:1 MSN-OEO	4.50 ± 0.3 ^b	3.44 ± 0.2 ^b

R_q : square root of the deviation from an average plane of the peaks and surface valleys, R_a : mean absolute value of surface height deviations from the middle plane. Results are mean values ± standard deviation ($n = 3$). Different letters indicate significant difference ($p < 0.05$).

In the SC-CH films micrographs, the observed agglomerations are attributed to reduction of electrostatic repulsions of the protein, due to close proximity to its isoelectric point. The agglomerations are larger in the 8:1 ratio than in the 4:1 ratio, in agreement with Anal et al. [28], who observed that increased CH concentration in the SC:CH complexes produced a slight increase in particle size, attributed to fewer aggregations. The SC:CH 4:1 MSN-OEO film surface presented better dispersion of the MSN, due to decreased -OH groups. According to Lu et al. [36], as the amount of MSN:OEO increases the pores diameter decrease and become denser, thus favoring their dispersion.

3.5. Mechanical Properties of the Films

Mechanical properties expressed as tensile strength (TS), elongation at break (EB) and Young's modulus (YM) of the SC:CH films are shown in Table 4. The samples containing MSN-OEO presented significantly higher TS ($p > 0.05$), and slightly decreased EB values compared to those without MSN-OEO. According to Giosafatto et al. [37], the mechanical properties can be associated to a great extent with the distribution and density of biopolymer molecular interactions that determine the films network. In addition, the mechanical properties of packaging materials arise from the intramolecular and intermolecular interactions of the biopolymers, and this depends entirely on the nature of their components. Lin et al. [6], reported similar results in relation to TS and EB for SC films crosslinked with genipin.

Table 4. Mechanical properties of SC:CH films with MSN-OEO.

SC:CH Ratio	MSN-OEO	Tensile Stress at Break (MPa)	Elongation at Break (%)	Young's Modulus (MPa)
4:1	-	7.06 ± 0.9 ^{a,b}	103.24 ± 2.4 ^a	12.48 ± 1.4 ^a
4:1	+	8.71 ± 1.6 ^a	99.52 ± 1.3 ^{a,b}	15.71 ± 1.4 ^a
8:1	-	4.59 ± 0.6 ^b	97.81 ± 0.3 ^b	7.60 ± 3.0 ^b
8:1	+	9.11 ± 1.9 ^a	94.12 ± 2.2 ^c	13.70 ± 2.3 ^a

Results are reported as the mean ± standard deviation ($n = 3$). Equal letters indicate that there is no significant difference ($p < 0.05$). Tensile strength (TS) significantly increased when MSN-OEO was incorporated for both 4:1 and 8:1 SC:CH ratios ($p > 0.05$), but the latter increased twice the value without MSN-OEO. This could be primarily explained by the reinforcement effect of the nanoparticles on the network structure, and to a strong interaction between the biopolymers and the MSN-OEO surface through the remaining free side chain groups of SC and amino groups of CH that are not involved in electrostatic interactions during complexation. According to Ojagh et al. [38] this effect leads to decreased free volume and molecular mobility of the biopolymers with a consequent increase in TS and decrease in EB. This effect is observed significantly ($p > 0.05$) in the SC:CH 8:1 films values, indicating a greater number of these interactions within the biopolymers network.

Several studies have shown that the SC-CH films structure depends on their proportion and concentration. The mechanical properties of SC-CH films are affected by the number of cross-links formed between the CH and SC, and increasing the SC proportion stimulates the crosslinking, forming a three-dimensional network that allows a structure with greater resistance [30].

Finally, a significant increase in YM was observed for the SC:CH 8:1 when adding MSN-OEO, whereas the 4:1 SC:CH ratio showed a moderate increase only. It is well known that the increase of interactions among polymer chains decreases their mobility increasing the stiffness, leading to increased YM values. Thus, this effect is in line with previous reports on the presence of higher number of crosslinks in the SC:CH 8:1 ratio films, containing MSN-OEO (Table 4)

3.6. Barrier Properties

The samples without MSN-OEO showed larger permeability to CO₂, especially significant for the SC:CH (4:1) film (Table 5). Upon the incorporation of MSN-OEO, the films demonstrated a positive effect because the permeability to CO₂ and WV decreased.

Table 5. Permeability to gases of the SC:CH films with and without MSN-OEO.

Material	O ₂	CO ₂	WVP
	cm ³ · mm/(m ² · 24 h)		
4:1 SC:CH	0.260 ± 0.0 ^{a,b}	3.659 ± 0.3 ^a	0.0806 ± 0.0 ^a
4:1 SC:CH MSN-OEO	0.218 ± 0.0 ^b	0.214 ± 0.0 ^b	0.0144 ± 0.0 ^b
8:1 SC:CH	0.217 ± 0.0 ^b	0.514 ± 0.0 ^b	0.0735 ± 0.0 ^a
8:1 SC:CH MSN-OEO	0.328 ± 0.0 ^a	0.340 ± 0.0 ^b	0.0321 ± 0.0 ^b
Mater-BI *	22.69 ± 0.1	8.35 ± 0.2	15.68 ± 0.1
Low-density polyethylene **	7	1	0.2
High-density polyethylene **	1	0.03	0.04

WVP = WV permeability. Results are reported as the mean ± standard deviation ($n = 3$). Equal letters indicate that there is no significant difference ($p < 0.05$). * (Di Pierro et al. [39]). ** Low- and high-density polyethylene (Di Pierro et al. [40]).

The hydrophilic nature of proteins and polysaccharides-based films or coatings makes them sensitive to moisture transfer, affecting their WV barrier properties. However, the OEO incorporated within the films provides a hydrophobic character resulting in lower WV permeability. Fernandez-Bats et al. [21], reported a reduction in permeability to

O₂ and CO₂ when adding NSM in films made with pea protein and pea protein with chitosan, respectively. The low O₂ permeability of the films with and without MSN-OEO is remarkable, being 70 times lower than Mater BI, a commercially available film; and 20 times less than low-density polyethylene. Oxygen permeability is of vital importance since its presence in certain foods causes microbial growth and oxidation reactions that affect the taste, quality and acceptability of food. Similarly, the low WV permeability of films with and without MSN-OEO is significantly less than Mater BI and low-density polyethylene, and slightly less than that of high-density polyethylene. WV permeability is important in coatings because they help to reduce water transfer between the food and its environment, causing less deterioration of the food and extending its storage life.

3.7. Antimicrobial Activity

The inhibition halos exhibited by OEO, MSN-OEO, SC:CH and these films incorporated with MSN-OEO indicated antimicrobial activity (Table 6). The greatest effect was observed against *L. innocua* (Gram +), whereas *S. Saintpaul* (Gram −) was not inhibited by the plain SC:CH films. Carmagnini et al. [41], showed inhibition zone of 5 mm for 25% OEO concentration against *L. monocytogenes* and *S. enteritidis*. Iturriaga et al. [42], showed antimicrobial effect of different EO against *L. innocua*, and observed higher inhibition at lower temperature (4 °C). The decreased antimicrobial activity of MSN-OEO was attributed to the screening effect of other compounds present in the FFS that reduces their diffusion [43].

Table 6. Inhibition zone of oregano essential oil (OEO) in the SC:CH films.

Microorganisms	Inhibition Zone Diameter (mm)			
	OEO	MSN-OEO	8:1 SC:CH	8:1 SC:CH MSN-OEO
	25%	8 mg	0 mg	8 mg
<i>L. innocua</i>	24.8 ± 2.4 ^a	6.0 ± 0.2 ^a	0.2 ± 0.0 ^a	3.5 ± 0.5 ^a
<i>S. Saintpaul</i>	8.3 ± 2.5 ^b	2.7 ± 1.5 ^b	ND	2.2 ± 1.0 ^a

Results are reported as the mean ± standard deviation ($n = 3$). Equal letters indicate that there is no significant difference ($p < 0.05$).

3.8. Application of FFS on Panela Cheese

3.8.1. Moisture Content

The FFS of SC:CH 8:1 with and without MSN-OEO was chosen to coat Panela cheese because of better physicochemical and antimicrobial properties. The effect of the different edible coatings used here on the evolution of Panela cheese moisture content, during the 15 days of refrigerated storage is shown in Figure 5. Control samples showed the highest moisture losses ($54.5\% \pm 2\%$), whereas those coated with MSN-OEO ($13\% \pm 1\%$) and without MSN-OEO ($22\% \pm 1\%$) showed lower moisture loss after 15 days of storage.

Visual evaluation confirmed that uncoated cheeses suffered severe water loss compared to coated cheeses after 15 days of storage (results not shown). In addition, the uncoated Panela cheese showed cracks attributed to water migration to the surface, which was not observed in coated cheeses with and without MSN-OEO. Moisture content changes are expected, because of whey losses due to syneresis occurring throughout the storage of fresh cheeses.

Cheese fat causes a yellowish color appearing when the surface dries out together with a leathery crust, unpleasant for most consumers [44]. In a study by Zhong et al. [45], Mozzarella cheese moisture loss was higher in coated (about 25%) than in uncoated cheese, but it must be considered that the initial moisture of the coated cheeses was significantly higher than that of the uncoated cheeses, essentially due to the water present in the coating itself.

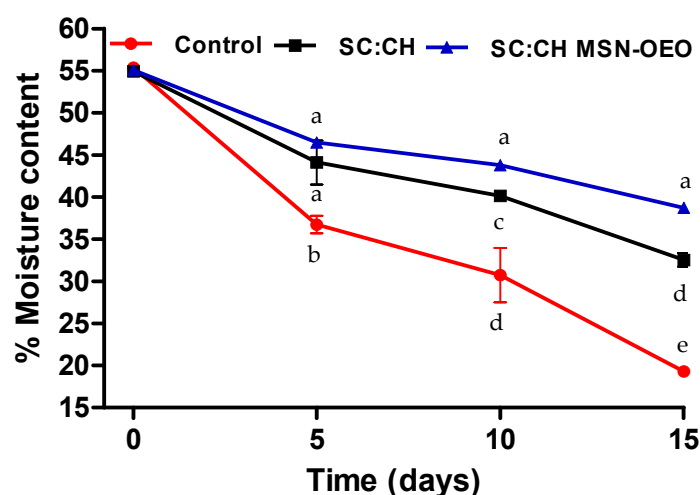


Figure 5. Moisture content of Panela cheese uncoated (control) and coated with SC:CH (8:1), and SC:CH (8:1) MSN-OEO. Different letters indicate significant differences ($p < 0.05$) for moisture content of each day.

3.8.2. pH and Titratable Acidity (TA)

Along the storage period of Panela cheese, the TA increased until day 5, then moderately increased between days 5 and 10. Afterwards, a steep increase occurred from day 10 to the end of the storage time, achieving about 0.85% lactic acid for coated cheese with and without MSN-OEO, whereas the control cheese showed about 60% higher lactic acid content. Coated Panela cheese exhibited significantly ($p < 0.05$) lower TA than the uncoated cheese (Figure 6) during the whole storage period. A similar behavior was reported for Ricotta cheese covered with a film of milk protein and chitosan observing that the TA of the samples varied between 1.10% and 2.82% (w/w) [46]. The increase in TA is attributed to the production of organic acids, mainly lactic acid produced by the bacterial population that increased during storage. The coated cheeses, irrespective of the EO used, showed a moderate decrease in TA, probably due to the partial inhibition of lactic acid bacteria.

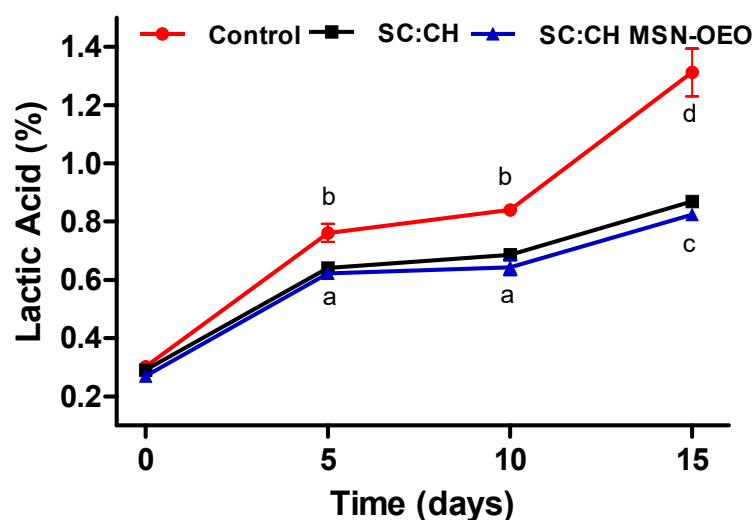


Figure 6. Changes in acidity in coated and uncoated Panela cheese, expressed as lactic acid. Different letters indicate significant differences ($p < 0.05$) for lactic acid content of each day.

The pH of the stored cheese showed a decreasing trend over time for coated and uncoated cheeses (Figure 7). The final pH of the cheeses was 5.70 ± 0.1 for the control cheese, 6.0 ± 0.0 for coated cheese, and 6.2 ± 0.1 for that coated with SC:CH MSN-OEO. All values recorded for each day are significantly different ($p < 0.05$).

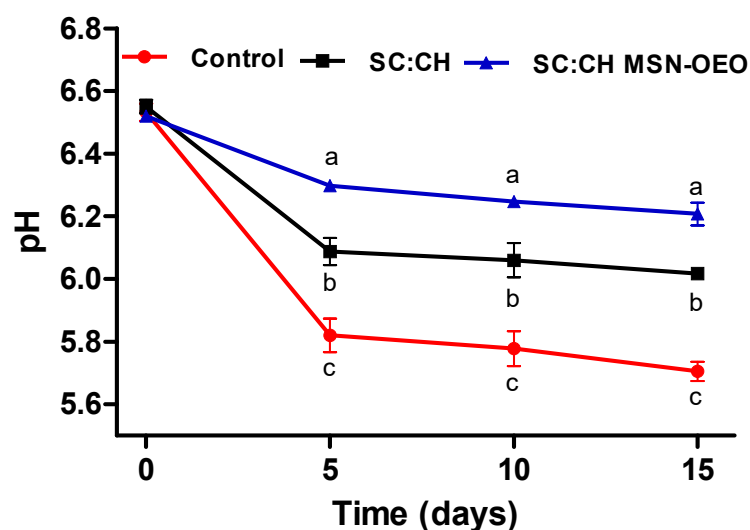


Figure 7. Values of pH of the coated and uncoated Panela cheese. Different letters indicate significant differences ($p < 0.05$) for pH of each day.

3.8.3. Microbiological Analysis

The application of the SC:CH coating to control microbial populations was effective. The total count of mesophilic aerobic bacteria, and yeasts and molds were lower in coated than in uncoated cheeses on days 5 and 15 (Figure 8). The population of mesophilic aerobic bacteria and yeasts increased over time as a result of decreased pH and increased TA probably due to microbial growth.

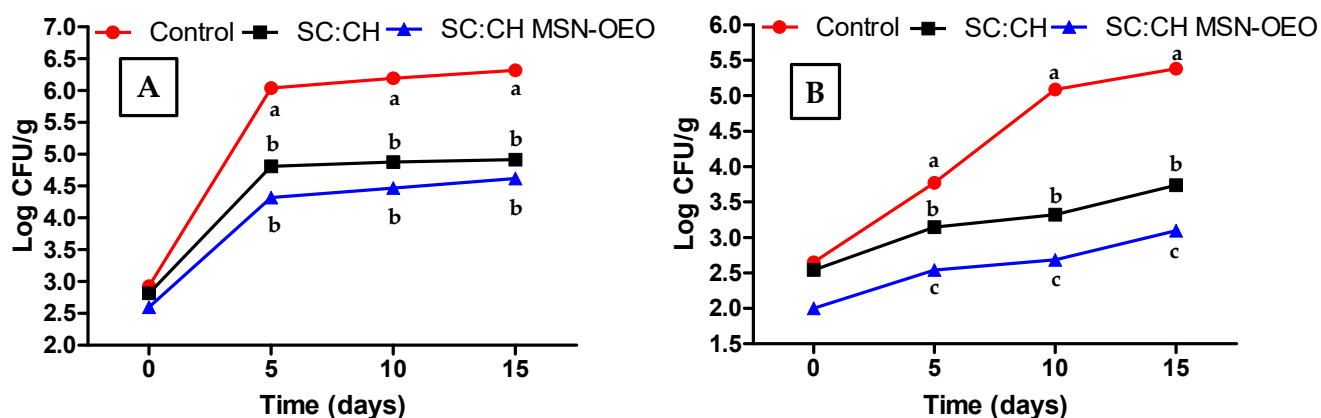


Figure 8. Antimicrobial activity of Panela cheese with and without edible coating. (A) mesophilic aerobic bacteria. (B) molds and yeasts. Different letters indicate significant differences ($p < 0.05$) for microbial population of each day.

Initially, all cheeses showed a total count of molds and yeasts below the specified Mexican standard limit (500 CFU/g), but the population of uncoated cheese showed a quick growth to reach 5 Log₁₀ CFU/g after 5 d. The coating with MSN-OEO showed the lowest population, since after 15 days it exhibited a reduction of 2 Log₁₀ CFU/g compared to the control. On the other hand, the aerobic mesophilic bacterial population showed significant difference between the control and the coated cheeses over the storage time. Mei et al. [47], working with coated Mongolian cheese with a mixture of starch-chitosan, reported that after 15 days coated cheese revealed lower total count of aerobic mesophilic microorganisms and fungi compared to uncoated cheese.

The cheese coated with the SC:CH MSN-OEO showed a decrease of 1.75 Log₁₀ CFU/g of mesophilic aerobic bacteria compared to the uncoated one after 15 days of storage. It must be noted that coating protects the microbial contamination from the cheese surface,

whereas microbial growth is also occurring inside the cheese. It may be possible to delay microbial growth by storing the cheeses under vacuum, to avoid contact with the air from the environment.

It was decided to store the cheese samples in clamshell containers, because many fresh cheeses are stored in Mexico in this way. Artiga-Artigas et al. [48] used a coating of sodium alginate and mandarin fiber with 2% (*w/w*) OEO on low-fat cheese bars, placed in polyethylene trays heat sealed using a film made of polyamide and polyethylene. This procedure effectively protected the cheese from fungus and yeast after 13 days of storage.

4. Conclusions

SC:CH has good film-forming properties and coating capabilities, with high stability of the film forming solution. Tensile strength, elongation and barrier properties were positively influenced by the addition MSN-OEO. In addition, the thickness and reduction of water solubility of SC:CH films were improved. The films showed inhibition of Gram (+) and Gram (−) bacteria. The coating on Panela cheese delayed acidification and moisture loss. Microbial population over the 15 days storage period was reduced in coated Panela cheese compared to the uncoated one. Therefore, this SC:CH coating added with MSN-OEO may be successfully used to increase Panela cheese shelf life.

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