Characterization, microstructure, and spectroscopic study of optimized sodium caseinate-sorbitol active biofilms with citral microencapsulate

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Abstract

There is increasing interest in the development of biodegradable active packaging, as well as in the wide availability of resources and methods for its production. This study aimed to formulate and characterize biofilms with sodium caseinate (SC) and sorbitol (Sb), incorporating citral microparticles (CM) as antimicrobial. The active biofilms were prepared by the casting method and optimized by response surface methodology, minimizing transparency, opacity, and elastic modulus concerning the SC: Sb (between 1:0.5 and 1:1.5) and SC:CM (between 1:0.5 and 1:1.5) ratios. The thickness, color, mechanical, and microstructural properties of the biofilms made with the optimal conditions (the SC:Sb and SC:CM ratios of 1:0.91 and 1:0.95, respectively) were then characterized. In addition, Fourier transformed infrared (FT-IR) and mid-infrared (MID) spectroscopy were performed for qualitative purposes to assess the molecular interactions in the supplies and mixtures used in the formulation of the active biofilms. The optical, physical, and mechanical properties were experimentally evaluated at this optimum point. The FT-IR data indicate intermolecular interactions between the biofilm components. The active biofilms obtained under optimal conditions show a thickness of 150.0 μm, with acceptable optical and mechanical properties. The active biofilm obtained may be a promising material for the packaging of perishable fresh foods.

Keywords: FT-IR, active packaging, atomic force microscopy, microencapsulation, scanning electron microscopy.

Introduction

The packaging industry is among the most dynamic enterprises in the world, considering its importance in various value chains. For this reason, in 2018, this sector recorded a global production worth $975 billion [1]. Containers and packaging systems are widely used in different industrial sectors, such as the food industry. In this sector, the most utilized manufacturing materials include paper, cardboard, plastics, metal, and glass [2]. In recent years, plastic packaging systems have increased their share, accounting for 45% of the total market value, due to their lightweight, versatility, and low cost [3]. However, plastic packaging (the main being polyethylene, polystyrene, and polypropylene) originates from petroleum derivatives, which are non-renewable; furthermore, they are non-biodegradable, since it takes more than a hundred years to degrade and considering that it is a major cause of environmental pollution [2].

This is why biopolymers are considered biotechnological resources with unique properties, such as the absence of toxicity, degradability, and biological compatibility [2]. The main biopolymers used for the manufacture of biofilms are obtained from starch, cellulose, seaweed, chitosan, fish scales, proteins, and fruit seeds, among others that are often enriched with other materials, such as lipids, plasticizers, active agents, and solvents [1,3].

Among the biopolymers obtained from protein sources, those obtained from dairy products, such as casein, stand out [4,5]. The plasticizer that gives better results with sodium caseinate is sorbitol, since it is miscible with this biopolymer and is considered a good crosslinking agent. In addition, biofilms made with sorbitol possess good physical and mechanical properties [6].

Casein biofilms are transparent, biodegradable, and have good oxygen barrier properties; they can be used as a support for antimicrobial or antioxidant compounds (biocomposites), giving the biofilm and packaging system a functional property that makes

them active biofilms or active packaging [7,8]. The effect of these biocomposites is enhanced when they are incorporated in a protected form, such as microencapsulation [9]. These active biofilms are very useful for the packaging perishable foods, such as dairy products, meat, fruit, and vegetables, as well as in prepared foods, since they reduce water loss and oxygen permeability, delay lipid oxidation, improve texture and taste, decrease microbial count, and generally improve the shelf-life of foods. These shelf-life-promoting characteristics of active biofilms are due to the combined effects of biocomposites and containers [3].

The production of active biofilms with antimicrobial and/or antioxidant properties can be formulated with the incorporation of natural substances, such as extracts or essential oils (EOs), either in the free or microencapsulated form [9,10]. Among the EOs, menthol, geraniol, thymol, eugenol, carvacrol, and citral stand out [11,12]. Citral (lemonal or 3,7-dimethyl-2,6-octadienal) is an acyclic monoterpene aldehyde composed of two geometric isomers: geranial (citral A in its *cis* form) and neral (citral B in its *trans* form) [13]. This EO mainly has an antimicrobial activity [14] and is extracted from lemongrass (lemon verbena or *Cymbopogon citratus*) and other vegetables, such as *Litsea cubeba*, *Citrus aurantiifolia*, and *Citrus limon* var. *pompia* [15].

There are reports on some studies that incorporate citral microencapsulated natural substances in the formulation of active biofilms. Following their addition to a sodium alginate matrix, stable biofilms with microbial reducing properties were obtained *in vitro* [9]. On the other hand, there are studies on the utilization of sodium caseinate in the preparation of active biofilms with the incorporation of EO in a free form, such as carvacrol [7,8], corn germ [16], and tung [17]. The majority of these studies suggest that the addition of biopolymers, plasticizers, and/or active agents significantly influences the physical, optical, and mechanical properties of biofilms. However, there is no evidence of the use of

91 microencapsulated citral with sodium caseinate and sorbitol for the production of active
92 biofilms.

93 Therefore, this study aimed to optimize the formulation of sodium caseinate-sorbitol
94 active biofilms with the incorporation of microencapsulated citral using the response surface
95 methodology. This study also evaluates the optical, mechanical, and microstructural
96 properties of the active biofilms obtained under optimal conditions.

97

98 # Materials and methods

99 ## Materials

100 Sodium caseinate, sorbitol (Sigma-Aldrich, Germany), and citral microparticles (made with
101 dextrin, soy lecithin, and citral), obtained as reported in Yoplac *et al*. [18, 19], were used for
102 the elaboration of the active biofilms.

103

104 ## Active biofilms preparation

105 The biofilms were prepared according to the methodology proposed by Arrieta *et al*. [7],
106 with modifications. The solutions were prepared in distilled water with 5% by weight of
107 sodium caseinate (SC). Sorbitol (Sb) was added obtaining SC:Sb ratios ranging from 1:0.5
108 to 1:1.5, homogenized at 50 °C for 10 minutes under continuous stirring at 1000 rpm on an
109 SP131015 Cimarec magnetic stirrer (Thermo Scientific, USA), and then cooled to room
110 temperature. The SC-Sb solutions had an average pH of 6.48 ± 0.01. The citral microparticles
111 (CM) were then added at an SC:CM ratio that varied between 1:0.5 and 1:1.5 and
112 homogenized at 35 °C for 5 minutes at 1000 rpm. The resulting solutions had an average pH
113 of 6.39 ± 0.04. Finally, to eliminate foams and air bubbles, all solutions underwent ultrasonic
114 degassing (Q55, QSonica, USA) at 35% digital intensity for 10 minutes at room temperature.

To achieve optimization and characterization, biofilms were manufactured by the casting method, which involved pouring 9 mL of these solutions into glass Petri dishes of 9 cm diameter (EULab, Germany). A total of four petri dishes per solution. They were conditioned for 24 hours at 25 ± 0.5 °C and 55 ± 2.0% relative humidity (RH) in a Venticell-VC222 forced air drying chamber (MMM Group, Germany) with a Traceable® hygrometer (Thermo Scientific, USA).

The resulting active biofilms had an expected thickness of 150 ± 25 µm and were stored in properly ruptured polyethylene bags, at room temperature. This was to aid subsequent evaluation within the first 48 hours; the transparency was subsequently evaluated after 14 days in the optimization trials, and after 3, 7 and 14 days in the optimal active biofilm testing.

Optimization of active biofilms preparation

The face-centered composite design (FCD) approach of response surface methodology (RSM) was used to assess the effect of sorbitol and citral microparticles in sodium caseinate biofilms, based on characteristics, such as opacity (Y_1), transparency value (Y_2), and elastic modulus (Y_3). SC:Sb (X_1: 1:0.5–1:1.5) and SC:CM (X_2: 1:0.5–1:1.5) ratios served as the independent variables. The dependent variables for the optimization of active biofilms (opacity, transparency value, and elastic modulus) were chosen based on similar studies [20–22]. The experiments were conducted in a single block. Within this block, the order of assays was randomized. Table 1 displays the experimental design matrix. Each analysis was performed three times.

The experimental data obtained were fitted into a polynomial response surface function. The second-order response function was predicted by the equation 1:

$$Y_n = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \quad (1)$$

where β_0 is the value of the fitted response at the center point of the design, β_1 and β_2 are the linear, β_{12} the interaction effect, and β_{11} and β_{22} the quadratic coefficients regression terms.

All dependent variables were minimized during the optimization process. The Box-Cox algorithm revealed that data transformation was necessary only for opacity, however, its inverse transformation did not improve the model performance, or change the optimization conditions.

Characterization of active biofilms

The active biofilms were optimized based on their opacity, transparency value, and elastic modulus (mechanical property). The new biofilms obtained under optimal conditions were characterized by assessing their thickness, opacity, transparency value, color, and mechanical properties. Microscopy and spectroscopy tests were also performed.

Morphological characterization

The average thickness of the films was measured with a Digimatic Micrometer IP-65 Series 293–240 (Mitutoyo, Japan) ± 0.001 mm in five random positions on the surface of four biofilms per repetition, from a total of three repetitions.

Optical characterization

The opacity (Op) was measured following the indications of the HunterLab Method [23] and the methodology proposed by Pires *et al*. [24], using a CR-400 colorimeter (Konica Minolta Co., Japan). The opacity percentage of the samples was calculated with equation 2 from the reflectance measurements for the optimization trials with a black background ($Y_{black\ background}$, L^* = 21.84, a^* = 0.29, b^* = 1.70) and a white background ($Y_{white\ background}$, L^* = 93.12; a^* = -

164 0.65, b^* = 3.99), as well as for characterization of the optimal biofilm with a black
165 background ($Y_{black\ background}$, L^* = 26.12, a^* = 2.13, b^* = -1.03) and a white background (Y_{white}
166 $_{background}$, L^* = 93.11; a^* = -0.63, b^* = 3.82).

167 $$Op = (Y_{black\ background}/Y_{white\ background}) \text{ x } 100 \quad (2)$$

168 where *Y* is the tristimulus value *Y*. Opacity tests were performed at four different positions
169 of three biofilms per experiment.

170 Transparency value (TV) was calculated using equation 3 as indicated by Pires *et al*.
171 [24], using a spectrophotometer Genesys 10S UV-VIS (Thermo Fisher Scientific, USA).
172 Strips of 10x80 mm (width x length) were cut and placed in quartz cuvettes for measurement.
173 Empty cuvettes were measured as blank.

174 $$TV = A_{600}/x \quad (3)$$

175 where A is the absorbance at 600 nm and x is the thickness of the film (mm). According to
176 this equation, higher transparency values indicate less transparency. The transparency was
177 measured on three different biofilms per experiment. The results are reported as A_{600}/mm.

178 Color parameters (*L**, luminosity; *a**, red-green; *b**, yellow-blue) were measured with
179 a CR-400 colorimeter (Konica Minolta Co., Japan) using the standard-white reflector plate
180 (*L** = 93.11; *a* * = -0.63, *b** = 3.82) and illuminant C. The values of Chroma (C *) and hue
181 (h *) of the biofilms were obtained directly from the colorimeter; the whiteness (W) was
182 calculated using equation 4:

183 $$W = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}} \quad (4)$$

184 The color of the films was expressed as the total color difference (ΔE^*), which is the
185 numerical comparison of the color values of a sample compared to a known standard,
186 calculated with equation 5 [24].

187 $$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (5)$$

where ΔL^*, Δa^*, and Δb^* are the differentials between the color parameter of the samples and the color parameter of a standard white card used as a film background. Color tests were performed at four different positions of four different biofilms per repetition (n=3).

Mechanical characterization

The mechanical properties were tested following the methodology proposed by Arrieta *et al*. [7]. Average elastic modulus (*E*), percentage deformation at break (ε_B%), and tensile strength (TS) was calculated from the resulting stress-strain curves as the average of the measurements from three films of each composition. Strips of 10x80 mm (width x length) were cut. Measurements were made at room temperature and 50% RH using an Instron Instrument 3365 (Farnham, Hants, UK).

Microscopy analysis

The surface and internal morphology of the active biofilms generated under ideal conditions were evaluated using scanning electron microscopy (SEM) [25,26,27]. SEM analyses of the surface and cross-section were performed using a Thermo-Scientific microscope (Q250 Analytical SEM, Czechoslovakia). The samples were cut into 10x10 mm squares for superficial evaluation; for cross-section evaluations, the cuts were placed on a glass surface and examined using an SZ51 stereoscope microscope (Olympus, USA). The samples were placed on top of a setup, consisting of a carbon tape with double adhesives placed on aluminum support. Afterward, they were taken to the microscope camera to be observed. Images were captured at 1200x magnification.

Atomic force microscope (AFM) analysis was performed using a NaioAFM Easyscan 2 (Nanosurf AG, Switzerland) microscope in the dynamic mode. A cantilever with a nominal

spring constant of 42 Nm^{-1}, a resonance frequency of 179 kHz, and a tip radius of less than 10 nm was used for the scanning process [28,29].

Spectroscopic analysis

Fourier Transform Infrared (FT-IR) mid-infrared (MID) spectroscopy tests were performed on raw materials and optimal active biofilms for qualitative purposes to detect chemical functional groups and assess the molecular interactions in the mixtures during the formulation of the active biofilms [25,26,27] and were carried out using a TruDefender™ FT (Ahura Scientific, USA) infrared spectrometer following the methodology proposed by Arrieta *et al*. [7], with modifications. The samples of the active biofilms were cut into 0.6 cm diameter circles, with average thicknesses of 150 ± 5 μm. For samples in powder form (sodium caseinate, dextrin, sorbitol, and citral microparticles), 100 ± 5 mg were added; and for liquid samples (citral mixture), 0.4 ± 0.05 mL were added; all of them were positioned directly in the equipment sample holder and analyzed at room temperature and 50% RH. Attenuated total reflectance spectra (ATR) were obtained in the region of 4000–600 cm^{-1} frequency, using 10 scans for each sample and a resolution of 3 cm^{-1}. A blank spectrum was obtained before each test to compensate for the effect of humidity and the presence of carbon dioxide in the air by subtraction of spectra.

Statistical analysis

The RSM experimental design matrix, data analysis, model development, and optimization were studied using Design Expert software (Version 12, Stat-Ease Inc., Minneapolis, USA). The numerical average and standard deviation of the responses were computed using Microsoft Excel.

The analysis of variance (ANOVA) for the transparency value of the optimal biofilms, evaluated at different storage times (3, 7 and 14 days), was performed at a 5% significance level. When significant differences were found, Tukey's multiple comparisons test ($\alpha \leq 5\%$) was applied using the software Statgraphics 15.2 (StatPoint Inc., USA, 2015).

Results and discussion

Optimization of active biofilms using response surface methodology

Table 1 shows the results of the variables evaluated in the active biofilms. The opacity percentage (Op) varied between 14.8 and 33.7%, the transparency value (TV) varied between 1.4 and 8.3 (A_{600}/mm), and the elastic modulus (*E*) between 52.7 and 493.0 MPa. These results fall within the ranges reported by Pires *et al*. [24] for biofilms made with hake proteins, glycerol, and thyme oil, which also had an opacity between 13 and 20%, and a transparency value between 1.5 and 4. Similarly, the *E* was within the ranges reported by Arrieta *et al*. [7] for biofilms of SC, glycerol, and carvacrol (10–200 MPa).

Only the interaction for the transparency value and the quadratic term of the SC:CM ratio for the opacity and elastic modulus was not significant, according to the ANOVA result (Table 2). Table 2 contains data regarding the estimates of the regression coefficients of the second-order polynomial models for all the dependent variables.

The opacity of a material refers to how much light can travel through it. The higher the opacity, the lower the amount of light that penetrates the material [23]. The model explained 96% of the variability (R^2 adjusted by degrees of freedom). The quadratic regression for opacity (Op) was represented by the following equation:

$$Op = 15.38 - 16.95\ SC{:}Sb + 12.07\ SC{:}CM - 19.62\ SC{:}Sb\ x\ SC{:}CM + 22.63\ SC{:}Sb^2 + 2.21\ SC{:}CM^2$$

The RSM plot (Figure 1) highlights the importance of the interaction effect (Table 2) by showing that the opacity is always low when biofilms are prepared with an SC:Sb ratio of 1:0.5 (-1), while at the high SC:Sb ratio, the SC:CM ratio should be at a high level (1:1.5) to allow for low opacity. The films produced in this work were of higher opacity than the films with SC, glycerol, and corn germ oil [16], as well as those with tilapia protein and glycerol biofilms (< 18%) [22]. The differences could be attributed to the fact that citral microparticles were incorporated in the current study, therefore the ingredients used in its elaboration (dextrin, soy lecithin, and citral) influenced its opacity, in addition to increasing the solutes in the solution.

The transparency value represents the ability of biofilms to either retain or not retain the light rays that impinge on them; higher TV indicates less transparency [24]. According to the ANOVA result, it was revealed that the linear term of the SC:Sb ratio had the most important effect. Its quadratic and linear and quadratic terms were significant but of lower importance. The model explained 93% of the variability (R^2 adjusted; Table 2). The quadratic regression was represented by the following equation:

$$TV = 7.00 - 1.48\ SC{:}Sb - 11.02\ SC{:}CM - 1.05\ SC{:}Sb\ x\ SC{:}CM + 4.01\ SC{:}Sb^2 + 5.31\ SC{:}CM^2$$

The TV decreased with decreasing SC:Sb ratio and decreased slightly to intermediate SC:CM ratio, as seen in the RSM plot (Figure 1). The lower the Sb, the lower the TV of the biofilms, resulting in improved transparency, as partially observed in the opacity. As depicted by Pires *et al*. [24], who observed a reduction in transparency in hake protein biofilms with glycerol and thymol EO, transparency is altered more by the plasticizer (Sb), than by the addition of CM. However, Limpisophon *et al*. [30] reported higher transparency with increasing levels of stearic or oleic acid in shark protein biofilms.

The elastic modulus describes how an elastic material behaves in response to the direction in which a force is applied. Specifically, in a biofilm, this parameter quantifies the elasticity or stiffness of the material [2,7]. According to the ANOVA, the *E* was mainly influenced by the linear and quadratic terms of the SC:Sb ratio, followed by the interaction and the linear SC:CM ratio component. The model explained 98% of the variability (R^2 adjusted; Table 2). The quadratic regression equation that accurately describes the effect of the independent variables on *E* is as follows:

$$E = 505.95 – 968.50\ SC:Sb + 379.00\ SC:CM - 238.16\ SC:Sb\ x\ SC:CM + 442.70\ SC:Sb^2 - 10.57\ SC:CM^2$$

The surface response graph (Figure 1) demonstrates the reduction of *E* with a corresponding increase of the Sb content (higher SC:Sb ratios). Nevertheless, at low SC:Sb ratios, there is a linear decline in *E* as a function of the CM content (lower SC:CM ratio). With a higher SC:Sb ratio, the reliance of *E* on the CM content becomes less significant. Therefore, in agreement with Arrieta *et al*., the increase of the plasticizer (Sb) in the formulation showed a lower capacity of *E* to produce more flexible biofilms, [7]. In contrast, the CM increase at a low level of Sb altered the stiffness of the biofilms; Alarcón-Moyano *et al*. [9] observed greater rigidity in alginate biofilms with the increase of citral microcapsules.

The lack-of-fit tests for opacity and TV were significant ($p \leq 0.05$), indicating that the models should not be utilized to make predictions (R^2 prediction =0.72–0.78); the exclusion of the nonsignificant terms did not enhance the adjustment of the model. The very high repeatability of the Op and TV responses at the central conditions (0, 0, 0) contributed to this result. The models, on the other hand, were highly significant. The elastic modulus, however, had no significant lack of fit, indicating that the mathematical model matches the experimental data within the experimental domain.

309 The optimal SC:Sb and SC:CM ratios for biofilms production with the lowest
310 percentage opacity, transparency value, and elastic modulus were determined using
311 numerical optimization. The optimal SC:Sb ratio of 1:0.91 and SC:CM ratio of 1:0.95, both
312 in the middle of the range studied, had a desirability value of 0.88. It is important to note
313 that the optimal conditions obtained for biofilms according to the statistical design are
314 specific to this system and cannot be applied when other plasticizers and ingredients are used
315 in the formulation [22]. The predicted values of the responses obtained at the optimized
316 ratios were 15.2±1.1% of opacity (confidence limits, CI: from 14.0 to 16.4 and prediction
317 limits, PL: from 8.8 to 21.6); 2.4±0.6 A_{600}/mm of transparency value (CI: from 1.7 to 3.1
318 and PL: from -1.2 to 6.0); 135.8±20.2 MPa of *E* modulus (CI: from 113.5 to 158.2 and PL:
319 from 18.4 to 253.2).
320
321 **Optimal active biofilms characterization**
322 New active biofilms were prepared with the optimal SC:Sb (1:0.91) and SC:CM (1:0.95)
323 ratio. Low opacity values (16.6%), transparency value at 14 days of 4.0 (A_{600}/mm), and
324 elastic modulus (74.6 MPa) were similar (Table 3) to the values predicted by the model for
325 opacity and TV, but lower for elastic modulus (135.38 MPa) despite being within the range
326 of prediction limits.
327 The thickness of active biofilms obtained under optimal conditions was 150±1.21 µm
328 (Table 3), which was higher than those reported by Arrieta *et al*. [7, 8] for SC biofilms with
329 free carvacrol EO (88±16 µm). This is likely due to the presence of dextrin and soy lecithin
330 in the citral microparticles of the current study, solutes that may increase thickness, as well
331 as, by the moisture content based on the same major polymer matrix.
332
333 Optical characterization

The biofilm showed (Table 3) high luminosity (L^*), with a slight deviation towards green (low negative values of the coordinate a^*) and a certain yellow component (high b^*), maybe because of citral EO and the incorporation of soy lecithin emulsifier in microencapsulation. Importantly, the yellowish component was visually perceived and with greater intensity in the central part of the biofilm, which is primarily due to the greater thickness in the central part (40 μm on average) compared to the thickness of the edges, due to the heterogeneity of the Petri dish surface where the drying process was carried out (Supplementary Figure 1). The results of C^*, h^*, W, and ΔE^* are also depicted in Table 3.

The SC biofilms with glycerol and carvacrol obtained by Arrieta *et al*. [8] showed similar L^* and a^* but lower b^* values. Biofilms made with hake protein and thyme EO [24] presented similar L^* and a^* values; lower b^*, C^*, and ΔE values; on the contrary, the values of h^* and W were higher. In general, the SC and CM biofilms were less white than those prepared with SC and free cinnamon and ginger EOs [31], hake proteins [24], wheat protein [32], and soy protein [33]. These differences could be due to the type of protein, origin, and form of EO incorporation, which influence its physical qualities, such as color and transparency [32,34].

The opacity results depicted in Table 3 were similar to the results obtained by Pires *et al*. [24] in biofilms with hake proteins, glycerol, and thyme EO (15–16%). These same results were also higher than those reported by García and Sobral [22] in tilapia protein and glycerol biofilms (4 and 10%). This could be mainly due to the presence of EO in the formulation. Matsakidou *et al*. [16] concluded that the opacity of biofilms was mainly influenced by the EO, thereby giving an appearance of higher opacity.

After three days of processing, the value for the TV (A_{600}/mm) was around 1.4 cm^{-1} (Table 3) and similar to the report obtained for other biofilms made with SC and encapsulated shrimp peptides [35]. It was however slightly higher (less transparent) than the

biofilms from fish proteins [22,28]. The TV behavior was similar to that of the opacity, which was influenced by the incorporation of solutes and EO in the formulation. The transparency of SC biofilms is reduced by the presence of EO in the formulations, and even more if they are microencapsulated [8,17,36].

During storage, the TV of the biofilms increased after 14 days, leading to a less transparent biofilm, and suggesting an alteration of the biopolymer possibly due to partial release and oxidation of citral. Similar behavior was reported for biofilms with SC and corn germ oil [16], or with soy protein [37].

Mechanical characterization

The elastic modulus (*E*), elongation at break (ε_B), and tensile strength (TS) of the biofilms obtained under optimal conditions are shown in Table 3; the results were within the range reported for biofilms with SC, glycerol, and carvacrol [7,8] and biofilms of SC, glycerol, and corn germ EO, except for the *E* (200–800 MPa) [16]. On the contrary, the results of the present study were lower than those reported for SC and tung EO biofilms [17] maybe because of the different types, forms, and concentrations of the additives used in the formulation.

On the other hand, some studies report that EO, such as citral, carvacrol, and thymol, somehow affect the interactions between macromolecular chains in the polymer matrix; this effect may be related to electrostatic interactions between the SC and the EO due to the different charge distributions in the protein chains. It can be affirmed that caseinates act as macro anions at the experimental pH (6.3–6.6), while EO such as citral, an acyclic aldehyde with a formyl functional group (-*CHO*), could behave as proton carriers, exchanging their proton for another cation, such as positively charged sodium [7]. Furthermore, the EO embedded in the SC matrix can act as deformable filler particles, improving the tensile

properties of biofilms [16]. The positive effects of EO on the elasticity of biofilms are greater when incorporated in a free form, but they are significantly reduced when microencapsulates are added [9].

Several studies on SC, plasticizers, and EO agree that the mechanical properties of biofilms are positively influenced by the incorporation of a plasticizer; non-plasticized films have high *E* and TS but low ε_B; an opposite behavior was recorded in samples with glycerol [7,16,17,35] and sorbitol [38], confirming the beneficial role of the plasticizer in biofilms.

It is generally known that food packaging films require great flexibility at room temperature to avoid unnecessary breakage during use [39]; in this sense, it was demonstrated that the biofilms of the present study have an adequate mechanical response for food packaging.

Biofilms microstructure

The arrangement of the different components in the emulsion, as well as the interactions that occur between them during drying, influence the resulting structure of biofilms. Microstructural examination of films provides relevant information on the disposition of these components and aids in understanding the mechanisms of water vapor movement through biofilms, as well as their interaction with light, which determines the optical properties [36]. SEM was used to quantitatively examine the microstructure of the biofilms obtained under ideal conditions. The micrograph of the surface (Figure 2A) displayed whitish external microparticles, adhered during the conditioning of the sample for SEM evaluation, as well as surface discontinuities in the form of small holes. In the micrographs taken from the cross-section (Figure 2B), the micro-holes were observed also. In both cases, it may be due to air bubbles present in the solution, which explode on the surface and inside the biofilm during the drying process, when water vaporization occurs [40]. The biofilms in this study had a greater number of micromoles compared to other works with SC and free or

410 microencapsulated EO [7,8,17,36], probably because in all these works, ultrasound baths
411 were used to eliminate bubbles from the solution. In the current research, ultrasound with
412 sonicating probes was used; it seems that, compared to ultrasonic baths, this system is less
413 efficient for the de-aeration of the solutions. Some studies report a higher presence of micro
414 holes in SC biofilms with essential oil than in biofilms without EO [17,36]. Contrarily, since
415 SEM is a qualitative analysis, it is not easy to evidence the integrity degree of the citral
416 microparticles in the biofilm, but the water-soluble encapsulating material probably
417 dissolved in the solution, keeping the soy-citral lecithin emulsion intact.

418 AFM was also used to analyze the structure of the biofilm surface. Figure 2 illustrates
419 the AFM micrographs of the biofilms obtained under optimal conditions, with observation
420 fields ranging from 2x2 µm (Figure 2C), 4x4 µm (Figure 2D), and 10x10 µm (Figure 2E);
421 and a Z scale ranging from 0.12 to 0.43 µm. The samples had an average thickness of 150
422 µm, with an average quadratic roughness of 90±5 nm. In all cases, irregular topographies
423 were observed with the presence of peaks and roughness similar to those observed in other
424 studies with SC, plasticizer, and EO; the presence of EO, depending on the form of addition
425 (free or encapsulated) and the type, favored the formation of irregular topographies,
426 compared to those of only SC and plasticizer which generally have a smoother surface [36].
427

428 **The spectroscopic study by FT-IR analysis**

429 Raw materials

430 Molecular interactions in mixtures were studied through their FT-IR spectra. The analysis
431 of the raw materials used to obtain the citral microparticles (citral, dextrin, and soy lecithin)
432 and raw materials to produce active biofilms (citral microparticles, sorbitol, and sodium
433 casein) are shown in Figure 3.

The citral spectrum (Figure 3A) showed an FT-IR profile similar to that reported by NIST [41]; typical bands for aldehydes were observed, with absorbance peaks associated with the *CH-flexion-extension* interaction in the range 2800–3000 cm^{-1} and with the *CHO-extension* interaction of the formyl functional group present in the citral, in the range 1100–1300 cm^{-1} [42].

The dextrin spectrum (Figure 3B) also showed a typical FT-IR profile of the polymer, similar to that reported by Garcia *et al*. [43] and Yousefi *et al*. [44,45,46] with absorbance bands associated with *OH-extension* and *CH-flexion-extension* in the ranges 3150–3600 cm^{-1} and 2800–3000 cm^{-1}, respectively; additionally, other bands between 995 and 1640 cm^{-1} were observed [42,43,45]. The soy lecithin spectrum showed a typical FT-IR profile of phospholipids (Figure 3C), with absorbance bands associated with the *OH-extension* region of 3150–3600 cm^{-1} and *CH-flexion-extension* region from 2800–3000 cm^{-1} [44,45]. The peaks observed in the ranges of 1400–1750 and 900–1100 cm^{-1} correspond to the *COO-extension* region and the carboxylate group present in phospholipids. Vibration peaks were observed at 1237 and 1167 cm^{-1} in the *CHO-extension* region (*CO-extension*), typical in soy phospholipids [42,45,47].

The spectrum of citral microparticles, made under optimal conditions with citral (Ct), dextrin (Dx), and soy lecithin (SL) by spray drying, is shown in Figure 3D. Three different spectral zones were observed: 3600–3150 cm^{-1}, 2800–3000 cm^{-1}, and 900–1750 cm^{-1}, the first two correspond to the *OH-extension* region and *CH-flexion-extension* region, respectively; present in the structure of dextrin (Figure 3B) and soy lecithin (Figure 3C); the last region corresponds to the extension of the *CHO* and *COO* group links [42]. Coincidences between some absorbance peaks of the ingredients used in the formulation were observed with the peaks of citral microparticles. In the Dx and microparticle profiles, coincidences were observed at peaks 2930, 1240, 1338, 1152, and 1080 cm^{-1}; with SL the coincidences

were in 1740, 1237, and 992 cm^{-1}; with Ct at 2856, 1444, and 1302 cm^{-1}. In the spectrum of pure SL (Figure 3C), a higher peak was observed at 1054 cm^{-1}, and in the microparticles spectrum, the highest intensity peak was obtained at 1025 cm^{-1}, probably due to the molecular rearrangement because of the interaction of the components used in the formulation [4]. The spectral profile of the present study was like that reported for microencapsulated EO of lemon verbena with capsule [9].

A spectrum of sorbitol (Figure 3E) similar to that reported by NIST [48] was obtained, showing typical bands for alcohols, with absorbance peaks associated with hydroxyl groups (*OH-extension*) in the range 3150–3600 cm^{-1}, similar to the results by Yousefi *et al*. [44,45,46], while the characteristic *CO-extension* peaks of primary and secondary alcohols were observed at 1046 and 1084 cm^{-1}, respectively. Bands in the 1150–1420 cm^{-1} range could be assigned to the *OH-flexion* interaction. In addition, asymmetric and symmetrical vibrations associated with *CH-flexion-extension* bonds in the range were identified as 2800 y 3000 cm^{-1} [42,45].

The spectrum for sodium caseinate (Figure 3F) showed a profile similar to that reported by Arrieta *et al*. [7]. Higher absorbance peaks associated with amide I and amide II were observed between 1500–1650 cm^{-1}, as well as the *NH-extension* group between 2850 and 3400 cm^{-1} characteristics of amino acids, as reported by Arrieta *et al*. [7], Oliver *et al*. [49] and Pereda *et al*. [5]. The peaks at 1638 cm^{-1} in the amide I region and 1515 cm^{-1} in the amide II region could be associated with the carbonyl group (*C=O-extension*) [50] and the symmetrical vibrations of the *NC=O-extension* bonds, respectively. The bands around 1395 and 1445 cm^{-1} could be assigned to the carboxylate group (*O-C-O*) [51] while the bands 1174 and 1066 cm^{-1} correspond to *CO-extension* in *C-OH* bonds [52]. The band at 976 cm^{-1} could be due to mono-cationic interactions with Na^{+} [7].

Optimal active biofilms

485 The FT-IR spectra corresponding to the optimal active biofilms are shown in Figure 3G. Due
486 to the nature of bovine caseinates and their ability to form extensive intermolecular hydrogen
487 bonds, their mixes with sorbitol and modified starches increased the cohesion of the
488 macromolecules [5]. In this sense, the wide absorbance band observed between the 3000 and
489 3600 cm^{-1} range could be attributed to the hydrogen bonds formed between the SC and the
490 hydroxyl groups of dextrin, soy lecithin, and sorbitol [4,52], as well as to the presence of
491 non-grouped *NH* groups [4]. Similar characteristics were observed in the region vibrations
492 of the *CH-extension* bonds between 2800 and 3000 cm^{-1}, to which citral interaction was
493 added. In contrast to the pure SC spectrum, a higher absorbance peak of amide I (1638 cm^{-1})
494 to amide II was observed in the biofilm. The latter changed from 1515 to 1540 cm^{-1}; these
495 changes could be associated with the existence of conformational rearrangements in the
496 protein caused by the addition of sorbitol and components of the citral microparticle. This
497 results in a decrease of inter and intramolecular hydrogen bonds. Similar spectral changes
498 for amide bands I and II were observed in caseinate/sorbitol films [4].

499 Some absorbance peaks corresponding to sorbitol (Figure 3E) remained in the biofilm
500 spectrum, such as the characteristic band of secondary alcohols (1084 cm^{-1}) and an
501 absorbance peak in the carboxylate group region, which moved from 1412 to 1410 cm^{-1};
502 however, the peak of primary alcohols (1046 cm^{-1}) was not observed. The absorbance bands
503 of citral (Figure 3A) were observed to possess less intensity in the biofilms spectrum, in the
504 regions of the carboxylate group (1444 cm^{-1}) and of the formyl group (1302 cm^{-1}), the latter,
505 typical for aldehydes. Most of the peaks present in the SL spectrum (Figure 3C), although
506 having less intensity, were observed in the biofilm spectrum (1740, 1237, and 922 cm^{-1}).
507 When evaluating the biofilm spectrum, the absorbance peak observed in the citral
508 microparticle in the region of the carboxylate group maintained its intensity between 1024
509 and 1025 cm^{-1}. The observed variations in intensity and displacement in the wavenumber

could be primarily due to changes in the concentrations and overlapping of molecular interactions present in the ingredients of the formulation, as well as to a molecular rearrangement [4,7].

Potential applications of active biofilms

The application of active packaging systems based on biopolymers (sodium caseinate), plasticizer (sorbitol), and a microencapsulated antimicrobial active agent, such as the biofilm obtained in the current study, has an enormous potential to improve the quality, safety, and shelf life of foods. Due to its antimicrobial properties, it could be used for the packaging of perishable foods such as fresh fruits, fresh vegetables, meat products, fish, shellfish, and dairy products. In fresh fruits and vegetables, it may also decrease moisture loss, and reduce weight loss, firmness, deterioration, and respiratory rate, as suggested in former studies [8,53,54]. Due to their high protein content, meat, fish, and shellfish products are highly susceptible to contamination and microbial growth. Also, for these products (red meat, chicken, turkey, pork, shrimp, salmon, and trout fillet, among others), the use of antimicrobial biofilms was reported to guarantee good quality and safety [10,54,55]. Exploiting these active biofilms in the packaging of dairy products is capable of reducing the microbial load, protecting against moisture loss, oxygen, and light, and preventing undesirable changes such as oxidation, microbial contamination, discoloration, and formation of stains and off-flavors which lead to rapid spoilage of dairy products [53,54,56-58]. However, the main limitation of active biofilms may be their solubility, thus, their use is recommended in solid foods that do not release free water and come into direct contact with the container. To overcome this limitation, further investigations are needed to evaluate hydrophobic inputs, such as vegetable oils or waxes, which can reduce solubility as a result of direct contact with water.

Conclusion

The active biofilms made of sodium caseinate (SC), sorbitol (Sb), and citral microparticles (CM) had the best resistance and optical qualities when the SC:Sb ratio was 1:0.91, and the SC:CM ratio was 1:0.95. The optimized biofilm possessed a thickness of 150 μm, color coordinates of L^* = 89.0, a^* = -1.8 and b^* = 15.4, 16.6% opacity, transparency values ≤ 1.61 A_{600}/mm after 7 days of storage (and 3.97 after 14 days), elastic modulus of 74.6 MPa, 17.7% deformation at break, and 3.16 MPa tensile strength. The SEM and AFM micrographs revealed surface discontinuities, micro holes, and irregular topographies, which most likely are due to the incorporation of citral microparticles and the insufficient elimination of air bubbles during the formulation, as well as the vaporization of water during drying. However, the evaluated properties were within acceptable ranges. FT-IR data indicated intermolecular interactions between biofilm components. The findings strongly imply that biofilms with desirable properties could be used as active packaging material for the preservation of fresh foods/meals in the future.

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Declarations

Conflict of interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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743

744 **Table 1.** Experimental design conditions for the production of active biofilms with sodium
745 caseinate (SC), sorbitol (Sb), and citral microparticles (CM) and results of opacity,
746 transparency value, and elastic (E) modulus

Standard order	Independent variables		Response variables		
	X_1: SC:Sb ratio	X_2: SC:CM ratio	Y_1: Opacity (%)	Y_2: Transparency (A_{600}/mm)	Y_3: *E* modulus (MPa)
1	1:0.5 (-1)	1:0.5 (-1)	14.8 ± 0.7	2.40 ± 0.30	239.5 ± 17.6
2	1:1.5 (+1)	1:0.5 (-1)	33.7 ± 0.8	8.29 ± 0.45	52.7 ± 3.1
3	1:0.5 (-1)	1:1.5 (+1)	20.0 ± 0.3	1.99 ± 0.10	493.0 ± 44.1
4	1:1.5 (+1)	1:1.5 (+1)	19.2 ± 0.2	6.83 ± 0.78	68.0 ± 4.3
5	1:0.5 (-1)	1:1 (0)	17.4 ± 0.6	1.36 ± 0.39	407.5 ± 34.1
6	1:1.5 (+1)	1:1 (0)	25.4 ± 1.8	7.11 ± 0.57	55.3 ± 2.6
7	1:1 (0)	1:0.5 (-1)	16.3 ± 0.9	5.80 ± 1.07	72.9 ± 7.1
8	1:1 (0)	1:1.5 (+1)	16.2 ± 0.5	3.32 ± 0.57	163.2 ± 12.1
9	1:1 (0)	1:1 (0)	15.9 ± 0.4	2.70 ± 0.64	90.5 ± 7.0
10	1:1 (0)	1:1 (0)	15.9 ± 0.1	2.38 ± 0.66	104.4 ± 11.9
11	1:1 (0)	1:1 (0)	15.7 ± 0.2	2.31 ± 0.28	120.3 ± 7.6
12	1:1 (0)	1:1 (0)	15.3 ± 0.6	2.80 ± 0.85	105.8 ± 9.5

747

748 **Table 2**. Estimates of the regression coefficients (Coeff.) of the second-order polynomial
749 models and analysis of variance (mean square-MS and significance) for opacity,
750 transparency value, and elastic modulus

		Opacity		Transparency value		Elastic modulus	
Source	d.f.	Coeff.	MS	Coeff.	MS	Coeff.	MS
Intercept		15.71		2.78		110.42	
Model	5		65.5 ***		11.9 ***		45336.6 ***
A-SC:Sb	1	4.34	113.1 ***	2.75	45.3 ***	-160.64	154800.0 ***
B-SC:CM	1	-1.57	14.8 *	-0.73	3.2 *	59.85	21495.7 ***
AB	1	-4.90	96.2 ***	-0.26	0.28	-59.54	14180.1 ***
A^2	1	5.66	85.3 ***	1.00	2.7 *	110.67	32663.1 ***
B^2	1	0.55	0.81	1.33	4.7 *	-2.64	18.6
Residual	6		1.22		0.38		407.7
Lack of Fit	3		2.37 *		0.70 *		666.1
Pure Error	3		0.07		0.06		149.2
R^2			0.98		0.96		0.99
Adj-R^2			0.96		0.93		0.98
Pred-R^2			0.78		0.72		0.92
C.V. %			5.86		15.6		12.28

751 SC:Sb, sodium caseinate:sorbitol ratio; SC:CM, sodium caseinate:citral microparticles ratio;
752 d.f., degrees of freedom; Adj-R^2, R^2 adjusted by d.f.; Pred-R^2, R^2 in prediction; *, $p \leq 0.05$;
753 **, $p \leq 0.01$; ***, $p \leq 0.001$.
754

755 **Table 3**. Characteristics of the active biofilms obtained under optimal conditions (sodium
756 caseinate:sorbitol, 1:0.91 and sodium caseinate: citral microparticles, 1:0.95 ratios).
757

Parameters	Mean ± standard deviation
Thickness (µm)	150.00 ± 1.21
Color parameters:	
*L**	88.95 ± 0.26
*a**	-1.81 ± 0.14
*b**	15.40 ± 0.27
*C**	15.51 ± 0.26
*h**	96.77 ± 0.59
W	80.96 ± 0.35
*ΔE**	12.36 ± 0.32
Opacity (%)	16.57 ± 0.13
Transparency value (A_{600}/mm):	
3 days	1.44^{b} ± 0.16
7 days	1.61^{b} ± 0.11
14 days	3.97^{a} ± 1.45
Mechanical properties:	
Elastic modulus-*E* (MPa)	74.55 ± 6.39
Deformation at break-ε_B (%)	17.66 ± 2.91
Tensile strength-TS (MPa)	3.16 ± 0.18

758
759 Different letters indicate significant differences among the transparency values at different
760 storage times, based on the Tukey test ($p \leq 0.05$).
761

762 **Captions to Figures**

763 **Figure 1**. Response surface plots for active biofilms: (A) opacity (%), (B) transparency value
764 (A_{600}/mm), (C) *E*-elastic modulus (MPa).

765

766 **Figure 2**. Micrographs of the active biofilms obtained under optimal conditions (sodium
767 caseinate:sorbitol, 1:0.91 and sodium caseinate: citral microparticles, 1:0.95 ratios) through
768 scanning electron microscopy (1250 x; A, view of the surface; B, view of the cross-section)
769 and atomic force microscopy at different sizes of the observation field (C, 2 x 2 μm; B, 4 x
770 4 μm; C, 10 x 10 μm).

771

772 **Figure 3**. FT-IR spectra of raw materials: citral (A), dextrin (B), soy lecithin (C), citral
773 microparticles (D), sorbitol (E), and sodium caseinate (F), as well as of the active biofilms
774 obtained under optimal conditions (G; sodium caseinate:sorbitol, 1:0.91 and sodium
775 caseinate: citral microparticles, 1:0.95 ratios).