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Effect of Chitosan Addition in Whey-based Biodegradable Films

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HIGHLIGHTS

- Dairy production generates large amounts of whey.
- Chitosan was added to verify the increase in film resistance to microbial decomposition.
- The films stored at room conditions did not decompose even after 5 months.
- Soil-exposed films were decomposed in a period of approximately eight days.

Abstract: Whey, a by-product of dairy industry, is a feedstock widely employed in the production of biodegradable films. However, these films present some limitations when considering the performance of synthetic polymers, especially biological transformation by decomposition. This work aimed to evaluate the effects of chitosan addition to whey-based films to improve films physical-chemical properties and resistance to microbial degradation. The results showed that there was an interaction effect between the chitosan concentration and the storage time for the physical-chemical properties of elongation at break and opacity. There was statistical difference among the formulations; however, for the moisture content and film thickness, there was no interaction effect between the formulation and the storage time. The films with 1.5 and 3.0 wt.% chitosan presented a yellowish hue, characteristic of the polysaccharide; this could also be detected by SEM analysis. The films presented an excellent biodegradability, being decomposed in about 8 days. Considering all chitosan contents tested had similar performances, the chitosan content of 0.15 wt.% was the one with the better cost-benefit relation.

Keywords: biopolymers; biodegradable films; antimicrobial agent; shelf life.

INTRODUCTION

Cheese is one the most produced and consumed dairy products in the world. In its production, there is also the production of whey, which can also be obtained by casein extraction [1]. It is estimated that, on average, for each kilogram of produced cheese, are used 10 L of milk, being obtained 9 L of whey [2].

Whey represents about 90% of the milk amounts used in dairy products and casein fabrication, having a low value and a high biochemical oxygen demand (BOD); this renders whey a product whose treatment and disposal is somewhat complicated. Aiming to minimize these impacts and also due to the considerable

amounts of nutritional compounds, whey is being used in the research and development of new products, such as in food industry [3], ethanol production [4], production of polymeric edible films [5], among other uses.

Although the concentrated whey has market as a supplement and protein source, the process of water removal is quite expensive, which may render the concentrated product (dry whey) economically unsuitable for film and packaging applications [6,7]. A possible way to save production costs is to use the whey in the diluted form (or only partially dried to increase whey concentration), generally a byproduct from waste streams of dairy and food industries. This approach may render a waste that needs special treatment prior to disposal to a feedstock for the production of films, membranes, and packaging [6,8].

Whey-based biopolymers can form films by changing the solution physical-chemical conditions, such as salt addition, heating, and pH-changing. The main method for film production is by solubilizing the biopolymer, followed by solvent evaporation. Solvent evaporation allows the polymeric chains to interact by electrostatic interaction, hydrogen bonding, and Van der Waals forces, generating the polymeric structure [9].

Due to some properties, whey-based films were studied as active and biodegradable packaging for food products [9]. However, these films may suffer microbial degradation during the product shelf life. To avoid this, several natural antimicrobial agents were proposed and tested; among them can be cited garlic and oregano essential oils and extracts [10], chitosan [11-12], nisin [13], and organic acids [14]. Among these, chitosan highlights itself to be used in whey-based films.

Chitosan is a biopolymer, obtained by the alkaline deacetylation of chitin, which constitute the exoskeleton of crustaceans and arachnids. Its biodegradable, antimicrobial, and non-toxic character renders it a promising material to be employed in food applications [15]. Due to its physiological, biological, and pharmacological properties, the use of chitosan in novel areas has grown recently, such as in cosmetics and food industries and also in the production of semi-permeable membranes [16].

Due to the antimicrobial character, chitosan is being widely studied in the production of biodegradable films and active packaging [17-19], which allow the slow release of a preservative or an antimicrobial agent on the surface of the food, inhibiting the growth of microorganisms. The chitosan may also act as a crosslinker, rendering the films more resistant, flexible, and with less wear, extending its durability and, consequently, the product shelf life [20].

Brink and coauthors [21], working with whey-chitosan films, reported an increase of about six days in the shelf-life of turkey meat packed in the film; the authors also reported an inhibition of the growth of the microorganisms *L. sakei*, *L. plantarum*, and *C. jejuni*, and a reduction of the total bacteria count in relation to the no-packed turkey meat. Shojaei and coauthors [22], working with composite cellulose-whey films with addition of chitosan nanoparticles (CNP), reported an increase of films elastic modulus, and a decrease of flexibility and water permeability. The addition of CNP also improved the films antimicrobial properties, with more pronounced effect on Gram-positive bacteria.

Shokri and Kamkar [23] evaluated the antioxidant properties of chitosan and whey, in which both whey-based and chitosan-based films presented very similar antioxidant activity, both regarding DPPH radical scavenging, and inhibition of linoleic acid oxidation. The authors also cited briefly the antimicrobial activity of chitosan and whey, without any evaluation.

Ye and Chen [24], who studied the interactions of chitosan-whey at different pH and heating conditions, reported a substantial influence of pH in both chitosan and whey microstructures, especially regarding film formation. According to the authors, at pH 4.0, the addition of small amounts of chitosan/whey protein isolate (C/WPI) at the ratio of 1:5 prevented the denaturation of the whey proteins after heating; higher amounts (1:2) of C/WPI led to a depletion flocculation. At a pH range of 5.5-6.0, the combination of chitosan and whey proteins was attractive form an electrostatic standpoint. With small chitosan contents, the complexes formed at pH 5.5 presented higher viscosity, complexes formed at pH 6.0 showed a shear-thinning behavior. With higher chitosan contents, the complexes showed high viscosity at pH 6.0. Heating led to a decrease of the viscosity and increase of turbidity of the complexes.

The present work aimed to evaluate the effect of the addition of several chitosan doses on physical-chemical, optical, and biodegradability properties of whey-based biodegradable films.

MATERIAL AND METHODS

Preparation of the films

The whey-based films were prepared from an aqueous solution of 3.0% m/v of whey (whose composition in dry basis was 13.0 wt.% of proteins, 79.1 wt.% of carbohydrates, 0.3 wt.% of lipids, and 4.5 wt.% of ash, obtained from Relat Laticínios Renner S.A., located in the city of Estação, Rio Grande do Sul state, Brazil),

3.0% m/v of commercial gelatin (from animal source, purchased from Dinâmica, Brazil), and 0.6% v/v of glycerol (acting as crosslinker – 85% purity, from Merck), prepared using distilled water.

The solution was homogenized using a magnetic stirrer (ARE, Velp Scientifica) at 50 rpm, for 10 min, at 25±2 °C. The solution was heated in a water bath at 90 °C for 30 min, being stirred at 50 rpm. After, the solution was removed from the bath and allowed to cool to 25±2 °C. A chitosan solution at 1.0 wt.% was prepared by the dissolution of chitosan (Sigma-Aldrich, low-viscosity chitosan, obtained from crab shells, with deacetylation degree of 76%) in a solution of 1.0% v/v acetic acid, according to Ahmad and coauthors [25]. After cooling, different volumes of the chitosan solution were added to the whey/gelatin/glycerol solution, to obtain 0.00; 0.15; 1.50; and 3.00 wt.% of chitosan in relation to film weight. The resulting filmogenic solutions were homogenized by stirring at 50 rpm for 30 min; they presented a pH of 4.3 for all formulations. The degassing was carried out using an ultrasound bath (Q335D, Quimis, Brazil) at 40 kHz for 15 min [26].

The films were prepared by casting, using Petri dishes with diameter of 10 cm, coated with Teflon®. 10 mL of the chitosan-added solution was put in the Petri dishes, and the solution was spread. The spread solutions were air-dried in an air-conditioned room at 20±2 °C and RH of 33±5% for a period of 48 h [26].

Physical-chemical properties and morphology analysis

The film thickness was measured weekly, up to 22 weeks (five months), using a digital disk micrometer (MDC-SX, Mitutoyo, Japan), by measuring five different points of each sample. The moisture content was determined monthly, during 5 months, according to ASTM E104-02 standard [27]. The mechanical properties of the films were measured monthly, during 5 months, using an Emic texturometer, model DL2000, according to ASTM D882-10 standard [28], with the following adaptions: the film samples measured 70 x 20 mm; the initial separation between claws was 50 mm; and the traction speed was 1 mm/s, with a load cell of 30 N.

The microstructure (film surface and transversal area) characterization of the films was carried out by analyzing the samples surface using an EDS-scanning electron microscope (SSX 550, Shimadzu, Japan), with an acceleration voltage of 5 kV. The samples were prepared by metallization with a small gold layer. The films samples were analyzed with magnifications of 1000x. To obtain clean breaking points to evaluate the film microstructure throughout thickness, the films were frozen with liquid nitrogen and broken manually.

Optic properties

The visual aspect of the films was evaluated by simple visual inspection, being evaluated the surface homogeneity, film uniformity, color changes, and visible growth of fungi. The opacity was measured using a UV-Visible spectrophotometer (DU-530, Beckman Coulter, USA), being measured the absorbance (Abs) at the wavelength of 600 nm. The opacity was calculated by the formula: $\text{Opacity} = \text{Abs}/X$ (where 'X' is the film thickness in millimeters), proposed by Han and Floros [29] and Ramos and coauthors [30]. The films were evaluated weekly, up to 22 weeks (about 5 months).

Shelf life test

The prepared films were exposed to the environment, inside the Laboratory of Materials and Membranes (LAMEM) of the university, which has had an average temperature of 18 °C (max. 21 °C and min. 15 °C) and an average RH of 80% (max. 96% and min. 64%); the films were exposed to sunlight and also to microbial contamination by airborne microorganisms. The films were tested for 150 days (5 months). The opacity, elongation at break, moisture content, thickness, and microscopic morphology of the films were evaluated during this period.

Biologic degradation

For the biologic degradation test, film samples with dimensions of 60 x 40 mm were prepared. A 'simulated soil' was prepared, following the ASTM G160-12 standard [31]. Each film sample was put inside a polypropylene screen package with 80 x 100 mm. The simulated soil was put in twelve transparent polypropylene cups with 300 mL capacity; each film was evaluated in three replicates. The packages were put in the simulated soil, allowing contact between the soil and the films. The film samples were incubated for eight days, inside a common greenhouse. The films were weighed every day until the end of the incubation time.

The pH, volatile solids, total C, N, and P contents of the test soil were evaluated before and after the incorporation of the films. The pH was analyzed in accordance with ASTM D4972-19 standard [32], the volatile solids content was determined following the SMEWW 2540-G standard [33], total P content was

analyzed following SMEWW-4500 P, B and E standards [34], and both total C and N contents were determined in accordance with the methods described by Tedesco and coauthors [35].

Experimental design and statistical analysis

Each film formulation was prepared in four replicates. Each replicate sample was used in both physical-chemical, mechanical and optic characterization of the films. The obtained data underwent analysis of variance (ANOVA) and the means were compared by Tukey's multiple range test at 5% probability ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Characterization of the mechanical and optical properties of the films

The whey solution was used because it is a byproduct stream of dairy industry, and, therefore, do not require water removal when compared to whey protein concentrate as a feedstock. The commercial gelatin was added to improve the film plasticity and elasticity. The visual aspect of the whey-based films was related to their appearance during exposure period to the environment. Fresh-prepared films were translucid, without defects or cracks, with a yellowish hue to naked eye. Figure 1 presents the fresh-prepared films.

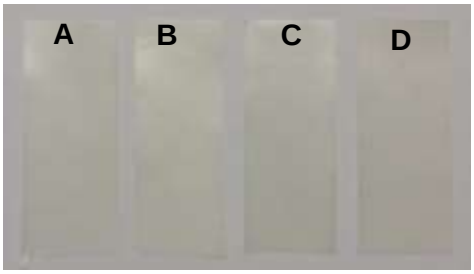

Figure 1. Visual aspect of the fresh-prepared films. A – film without chitosan; B – addition of 0.15 wt.% chitosan; C – addition 1.50 wt.% chitosan; D – addition 3.00 wt.% chitosan.

Considering film thickness, it is noteworthy the similarity of the behavior among the formulations. Once film thickness is related to the volume of filmogenic solution that is put in each Petry dish for casting, there was no important difference in film thickness after long periods of environmental exposure. Table 1 presents the statistical analysis for the film thickness in relation to the formulations and storage time.

Table 1. Statistical analysis of film thickness in relation to the film formulation and storage time (CV = 5.21%).

Formulation – chitosan content	Average thickness (μm)
0.00 wt.%	76.01 C
0.15 wt.%	77.87 B
1.50 wt.%	75.25 C
3.00 wt.%	79.81 A
Storage time (weeks)	Average thickness (μm)
0	71.08 bc
1	68.25 c
2	76.83 ab
3	79.76 a
4	75.25 ab
5	77.83 a
6	76.93 ab
7	78.75 a
8	77.99 a
9	79.08 a
10	7868 a
11	77.42 a
12	77.83 a
13	77.58 a
14	76.85 ab
15	78.08 a
16	78.96 a
17	79.24 a
18	77.45 a
19	78.01 a
20	78.02 a
21	77.80 a
22	78.70 a

Means followed by the same letter do not differ statistically by Tukey's multiple range test at 5% probability (α = 0.05). OBS: interaction effect not significant.

Considering film formulation, the one with 3.00 wt.% presented the highest thickness, probably due to moisture retention. However, the formulation of 0.00 (chitosan-free) and 1.50 wt.% have not differed statistically, indicating that the preparation procedure and/or filmogenic solution properties during film casting may influence the final film thickness.

For the storage time, there is no important difference among the measurements; only in the first week after film preparation there was a significant difference from the other weeks. This may be result of film rearrangement and/or maturation in contact with external moisture, after film saturation/equilibration with the environment the thickness remained nearly unchanged, as observed in Table 1.

All films presented a decrease in thickness in the first week of evaluation, it increased in the second week and stabilized after the first month (fourth week). This variation may be attributed to the drying process employed in film production or even a possible moisture absorption by the films. It was observed a more unstable behavior of film thickness in the initial weeks, when the films have the highest water content in their formulation after drying and film formation; this can be seen by analyzing the moisture content of the material and by differences in the film flexibility.

Comparing the thicknesses of the fresh-prepared films, Leceta and coauthors [36] reported for chitosan-based films an average thickness of 64.7±3.0 μm. Fráguas and coauthors [37] reported, for films containing 1.5 wt.% chitosan, thickness ranging from 60 to 68 μm. Considering the fresh-prepared films produced in the present work presented thickness ranging from 62 to 85 μm, it can be seen that the average thickness of the present films was similar to other works.

Concerning the mechanical properties of the films, there were differences among the formulations. Increasing values of chitosan decreased the elongation at break. The chitosan-free film presented a percentual elongation of 49.01%, whereas the film with 3.0 wt.% chitosan presented a percentual elongation of 28.60%. Table 2 presents the statistical analysis regarding the films' elongation at break.

Table 2. Statistical analysis of the elongation at break (%) considering film formulation and storage time (CV = 17.41%).

Storage time (month)	Formulation – chitosan content			
	0.00 wt.%	0.15 wt.%	1.50 wt.%	3.00 wt.%
0	49.01 Aa	35.81 Ca	40.55 Ba	28.60 Da
1	8.07 Ab	4.74 Ac	5.76 Ab	9.01 Ab
2	5.30 Cb	11.99 Ab	10.49 Ab	6.86 BCb
3	4.64 Ab	5.41 Ac	6.18 Ab	4.80 Ab
4	6.97 Ab	5.20 Ac	6.22 Ab	5.72 Ab
5	6.70 Ab	7.97 Abc	10.39 Ab	7.86 Ab

Means followed by the same letter, uppercase in row (formulation) and lowercase in column (storage time) do not differ statistically by Tukey's multiple range test at 5% probability ($\alpha = 0.05$).

As can be seen in Table 2, for film formulation, only in the months '0' and '2' there was statistical difference between treatments. In month '0' the chitosan-free film presented the highest elongation (49.01%), whereas the film with 3.00 wt.% chitosan had the lowest (28.60%). However, in month '2', the chitosan-free film presented the lowest elongation (5.30%), followed by the film with 3.00 wt.% chitosan (6.86%); the other two formulations (0.15 and 1.50 wt.% chitosan) have had similar elongation values (11.99 and 10.49%, respectively). For the storage time, only in month '0' there was an important difference from the other months; from month '1' on there was no statistical difference in the formulations, with exception of the 0.15 wt.% chitosan, which presented a statistical difference on month '2'.

Enhancement of film and membrane flexibility is directly related to the content of plasticizer agents within the polymeric structure, weakening the intermolecular forces in the three-dimensional structure and allowing wider movements among the polymeric chains. Some plasticizers have as a characteristic to decrease the film/membrane elastic modulus and increase the strain percentage due to an increase in water absorption by the biopolymer (moisture content); this may contribute for a wider mobility in a chitosan-added film [38-39]. According to Azevedo and coauthors [39], the use of glycerol as a plasticizer agent induces an increase of film flexibility and elasticity (smaller elastic modulus), this may explain the higher elongation at break of all formulations.

As reported by several previous works, chitosan-based films presented themselves fragile and brittle, therefore, plasticizer agents must be employed to promote better flexibility and malleability. The more brittle behavior (lower elongation at break percentages) for the films with higher chitosan contents may be explained by this phenomenon, where chitosan may be clustering and/or interacting with the polymeric base (whey) in a more crystalline way [40-42].

Taking into account that a more brittle property is associated to lower elongation at break, it can be seen that the formulation with 1.5 and 3.0 wt.% of chitosan presented themselves more brittle; in this case the chitosan may act as a barrier to defect flow within film structure, reducing its plasticity and rendering it more brittle [22]. Smaller stresses at break may also indicate a more fragile film structure, in which the storage time may have deleterious effect on film stabilization, being by moisture reduction or by oxidative processes due to exposure to atmospheric oxygen [43].

During the environmental exposure test, all films presented a similar behavior, with a steadily reduction rate of elongation at break in the first month. From the first to the third month there was a relatively quadratic trend, with a local maximum at approximately 70 days; this parameter stabilized after approximately three months. Afterward, there was a small trend of increase. After five months (150 days) the elongation percentages ranged between 6.70% (chitosan-free film) and 10.39% (film with 1.5 wt.% chitosan).

The decreasing trend of the elongation at break may be attributed both to a surface crystallization and to moisture removal (water evaporation) of the films during the storage test. Suyatma and coauthors [44], who studied the effects of hydrophilic plasticizer agents on thermal, mechanical, and surface properties of chitosan-based films, reported a similar behavior.

Film opacity, which was evaluated weekly, presented absorbance values at the wavelength of 600 nm ranging from 1.380±0.180 Abs_{600}/mm for the films with 0.15 wt.% chitosan to 11.136±0.749 Abs_{600}/mm for the films with 3.00 wt.% chitosan. Table 3 presents the statistical analysis of the opacity for the formulations and the storage time.

Table 3. Statistical analysis for opacity values regarding film formulation and storage time (CV = 6.89%).

Storage time (weeks)	Formulation – chitosan content			
	0.00 wt.%	0.15 wt.%	1.50 wt.%	3.00 wt.%
0	1.41 Ae	1.38 Ae	1.81 Ae	1.57 Ac
1	3.89 Ad	3.59 Ad	3.60 Ad	2.89 Ac
2	5.06 Acd	5.75 Ac	3.89 Bcd	4.98 Ab
3	5.38 Acd	6.07 Abc	5.28 Abc	5.15 Ab
4	5.95 Abc	6.85 Aabc	5.84 Ab	5.48 Bb
5	7.28 Bab	7.41 Bab	9.58 Aa	9.54 Aa
6	7.75 Ba	7.95 Ba	10.46 Aa	11.14 Aa
7	7.66 Ba	7.73 Ba	10.43 Aa	10.43 Aa
8	7.62 Ba	7.71 Ba	10.26 Aa	10.69 Aa
9	7.54 Bab	7.59 Bab	9.91 Aa	10.53 Aa
10	7.42 Bab	7.63 Bab	10.05 Aa	10.44 Aa
11	7.46 Bab	7.56 Bab	10.00 Aa	10.35 Aa
12	7.42 Bab	7.75 Ba	9.71 Aa	10.37 Aa
13	7.40 Bab	7.59 Bab	9.59 Aa	10.29 Aa
14	7.48 Bab	7.53 Bab	9.78 Aa	10.36 Aa
15	7.38 Bab	7.42 Bab	9.33 Aa	10.14 Aa
16	7.36 Bab	7.36 Bab	9.31 Aa	10.22 Aa
17	7.30 Bab	7.37 Bab	9.24 Aa	10.09 Aa
18	7.63 Ba	7.73 Ba	9.36 Aa	10.31 Aa
19	7.34 Bab	7.42 Bab	9.46 Aa	10.35 Aa
20	7.24 Bab	7.51 Bab	9.48 Aa	10.26 Aa
21	7.31 Bab	7.52 Bab	9.33 Aa	10.08 Aa
22	7.26 Bab	7.29 Babc	9.29 Aa	10.11 Aa

Means followed by the same letter, uppercase in row (formulation) and lowercase in column (storage time) do not differ statistically by Tukey's multiple range test at 5% probability ($\alpha = 0.05$).

By analyzing Table 3, it is possible to verify that, in the first weeks after film preparation, there was no statistical change of the opacity in function of the formulation. However, from about the fourth/fifth week on there was a clear difference among the films. The ones with 1.50 and 3.00 wt.% had similar behaviors, having higher opacity values, with a yellowish hue. For the films with zero and 0.15 wt.% chitosan, the opacity values were lower, but for these two formulations there was no statistical difference between them.

Considering the storage time, a similar trend was observed, in which the opacity values stabilized approximately in the fifth/sixth week, not differing statistically among measurements from week 6 up to the end of the experiment (week 22).

By analyzing Table 3, it is also possible to verify an increase in film opacity of all formulations during the environmental exposure. The opacity values stabilized after approximately six weeks; from this period of time on it is possible to observe higher opacity values for the formulations with 1.50 and 3.00 wt.% chitosan, the formulation with 0.15 wt.% chitosan presented practically the same opacity of the film without chitosan.

According to Chen [45], biopolymer opacity is a result of the material morphology. Fakhoury and coauthors [46] cited that amorphous materials enhance light transmittance, presenting less opacity and, consequently, being more transparent when compared to materials with a more crystalline structure in the morphology. The influence of chitosan addition on films was also cited in other works, such as the ones from Pelissari and coauthors [47], Bourtoom and Chinnan [48], Chillo and coauthors [49], and García and coauthors [50], who employed chitosan as an additive to other polymers, such as cassava, tapioca, and rice starch and methylcellulose.

Relative to the moisture content of the films, there was a trend of decrease with storage. The fresh-prepared films presented moisture contents that ranged between 17.9 wt.% (chitosan-free film) and 19.7 wt.% (film with 3.00 wt.% chitosan). The statistical analysis of the moisture content is presented in Table 4.

Table 4. Statistical analysis of moisture content considering film formulation and storage time (CV = 9.38%).

Formulation – chitosan content	Moisture (wt. %)
0.00 wt.%	13.57 AB
0.15 wt.%	13.48 B
1.50 wt.%	14.18 AB
3.00 wt.%	14.64 A
Storage time (month)	Moisture (wt. %)
0	18.84 a
1	17.88 a
2	11.36 bc
3	11.70 bc
4	12.83 b
5	11.18 c

Means in column followed by the same letter do not present statistical difference by Tukey's multiple range test at 5% probability (α = 0.05). OBS: interaction effect not significant.

According to Table 4, the average moisture content has not differed among all formulations; only the formulations of 0.15 and 3.00 wt.% presented statistical difference. Relative to the storage time, it was clear a decreasing trend, in which the first two months presented the highest moisture contents, with reduction of the film moisture, probably due to water loss to the environment. Despite some variation between the months that may be result of variation of the environmental humidity, the moisture content of the films stabilized from the second month on.

According to Rindlay-Westling and coauthors [51] and Leceta coauthors [36], the presence of glycerol (plasticizer agent) favors water absorption due to its intrinsic hygroscopicity. The relatively low differences among formulation may also be result of the storage conditions, which were an average temperature of 18 °C and relative humidity of 80%.

During storage, it was observed an important reduction in the moisture content of the films. This reduction may be a result of water diffusion/releasing to the environment as a slower and progressive drying process, with the removal of the remaining solvent trapped inside the polymeric structure. When reaching an equilibrium, the moisture content tends to stabilize; this was observed after approximately 70 days of storage. This change in the moisture content also affects the mechanical properties, as was observed with the elongation at break of the films.

Films surface microstructure characterization

The films surface microstructure was evaluated by SEM. Relative to the fresh-prepared films, it could be observed that the chitosan-free film and the film with 0.15 wt.% chitosan presented a relatively smooth surface, with the presence of small pores and other minor defects, very probably being result of crystallization of the polymeric compounds during film drying or even a poor homogenization of the filmogenic material. Figure 2 presents the SEM images of the films surface and transversal section (thickness) for all formulations.

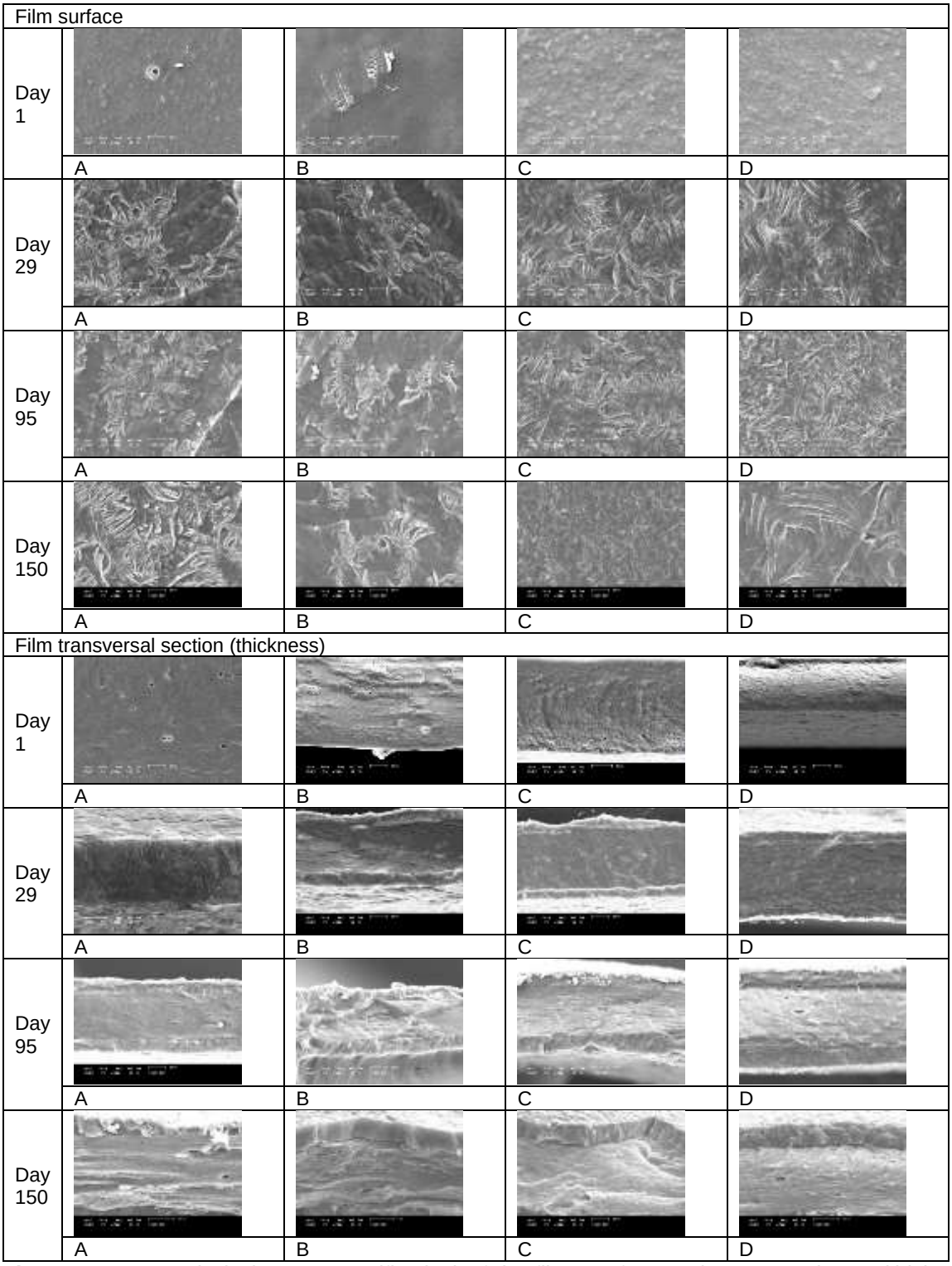

Figure 2. SEM analysis (1000x magnification) of the films surface and transversal area (thickness). A – film without chitosan; B – addition of 0.15 wt.% chitosan; C – addition 1.50 wt.% chitosan; D – addition 3.00 wt.% chitosan.

In the films with higher chitosan contents, it was possible to observe surfaces with less homogeneity, more and larger pores, and more defects. This may be linked to the intrinsic crystallinity of chitosan, which may induce more pore and defect formation. Considering the chitosan used in the present work had a low purity, the presence of other substances may act as nucleation centers that induce pore and defect formation in both film surface and through the internal structure. Alberti and coauthors [52], working with addition of chitosan in starch and polylactic acid-based films, also reported the presence of clusters similar than the ones that occurred in the present work.

Considering the film with the lower chitosan content (0.15 wt.%), it presented itself more homogeneous (less pores and defects) when compared to the films with higher chitosan content. During storage was possible to see the presence of crystals that may be formed due to arrangements in the film structure as a result of moisture reduction (Figure 2).

Taking into account that chitosan has a semicrystalline profile, which is result of the strong intermolecular and intramolecular interactions, mainly originated from the hydrogen bonds among the hydroxyl, amino, and amide functional groups presented in the structure of chitosan. This favors a more compact and ordered organization of the polymeric chain, with some degree of crystallinity [53].

It can be assumed that a possible presence of clusters in the film without chitosan may be related to a poor homogenization of the filmogenic solution during film preparation, or an incomplete denaturation and dispersion of the whey proteins. According to Le Tien and coauthors [54], whey proteins have the capacity to crystallize during heating in film preparation. Abdelwahab and coauthors [55] and Souza and coauthors [56] also reported the influence of chitosan on the morphological characteristics of films based on biopolymers, with similar results.

Shelf life test

During the shelf life test, the films presented an increasing yellowish color; the ones with higher chitosan contents (1.50 and 3.00 wt.%) showed themselves more colored than the others. This also corresponded with the increasing opacity of the films, observed in the present work and also cited by Silva and coauthors [57], who reported more intense yellowish color with increase of the chitosan dose. The films presented themselves intact, without cracks, but developed a bent shape during environmental exposure. Figure 3 presents the films soon after production and after 30, 75, and 150 days of environmental exposure.

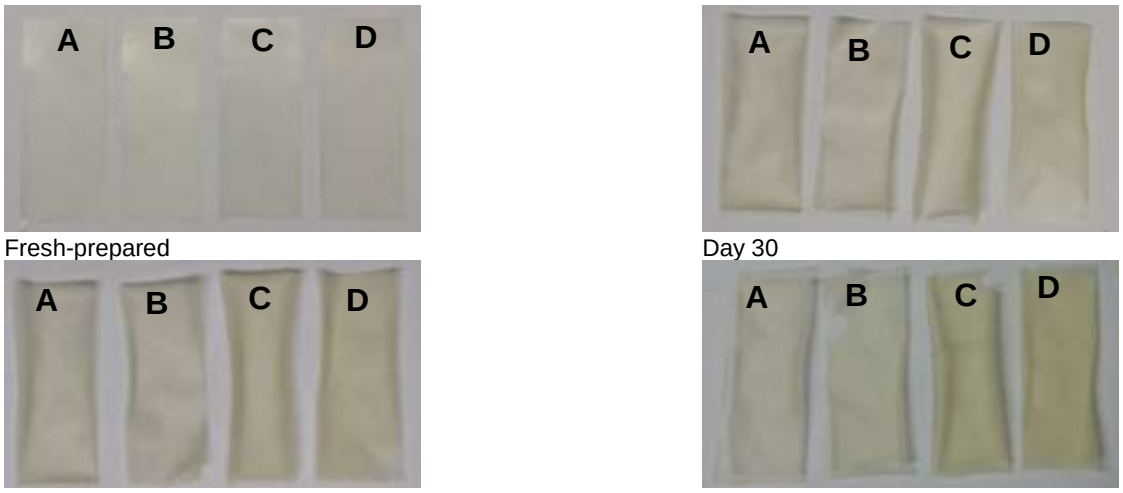

Figure 3. Visual aspect of the films during shelf life test. A – film without chitosan; B – addition of 0.15 wt.% chitosan; C – addition 1.50 wt.% chitosan; D – addition 3.00 wt.% chitosan.

However, with increase of the testing time, the films presented a more brittle behavior. This may be result of moisture reduction or even result of exposure to atmospheric oxygen, that may cause oxidative stress in the film structure. Considering the behavior of the films during storage, although there was a worsening of some properties (elongation at break, increased brittleness), there was an overall stabilization of the films' properties, especially moisture content and thickness.

Biological degradation of the films

A test soil was prepared in accordance to ASTM standard G160-12 [31] to be employed in the degradation tests of the films and to evaluate the physical-chemical changes in the reference soil due to film degradation and incorporation. Table 5 presents some physical-chemical parameters of the test soil prior and after film incorporation.

Table 5. Change of test soil physical-chemical parameters with film degradation and incorporation.

Parameter	Film-free soil	Soil with film incorporated
pH (25 °C)	6.88 a	6.99 a
Volatile solids (wt.%)	17.18 b	18.63 a
Total organic C (wt.%)	6.32 b	8.36 a
Total N (wt.%)	0.35 a	0.34 a
Total P (wt.%)	0.24 a	0.21 a

Means in row followed by the same letter do not present statistical difference when compared by Tukey's range test at 5% probability (α = 0.05).

According to ASTM G160-12 standard [31], the pH range of the soil must fall between 6.5 and 7.5, to provide microorganism survival; in the present work, the soil pH ranged from 6.88, prior to film incorporation, to 6.99 to the film-incorporated soil, keeping within the recommended pH range.

Beyond pH, carbon and nitrogen contents are also important for microbial growth; shortage of any of these elements may hinder the microbial activity, delaying the degradation of any disposed material. According to Pereira and Fialho [58], a C/N ratio of 30 is considered optimal for microbial growth. In the present work the starting C/N relation of the test soil was 18.06; the soil C/N relation at the end of the experiment was 24.59, indicating an increase in this ratio due to carbon incorporation from the film.

Due to film composition, the increase in C/N ratio is a normal result of the film incorporation by the soil. It is noteworthy that there was no change in the total N content (from 0.35 to 0.34 wt.%), this may be result of N biotransformation from the organic to ammoniacal and nitrate forms, which are readily absorbed by plants and the soil microbiota, or even a biotransformation to dinitrogen (N_2), leaving the soil to the atmosphere. It is also possible to verify a near steady-state behavior of N in the soil, characteristic of a relatively stabilized soil [59]. Relative to P soil content, it presented a slight decrease (from 0.24 to 0.21 wt.%) with film incorporation. This may be result of microbial activity (nutrient consumption) or due to the increase in soil pH, which reduces P availability [35,60].

The volatile solids content is a way of measuring the organic matter (OM) content, which includes the products of biological degradation of the whey-based films. It was observed an increase of the volatile solids content from 17.18 wt.% in the soil prior to film incorporation to 18.63 wt.% in the film-incorporated soil. Considering the increase in the volatile solids of the soil indicate that the microbial degradation of the films was quite quick.

Visual characterization

The visual aspect and integrity of the films were evaluated daily during the exposure time to the soil. The visual aspect of the four formulated films for the eight days of testing is presented in the Figure 4.

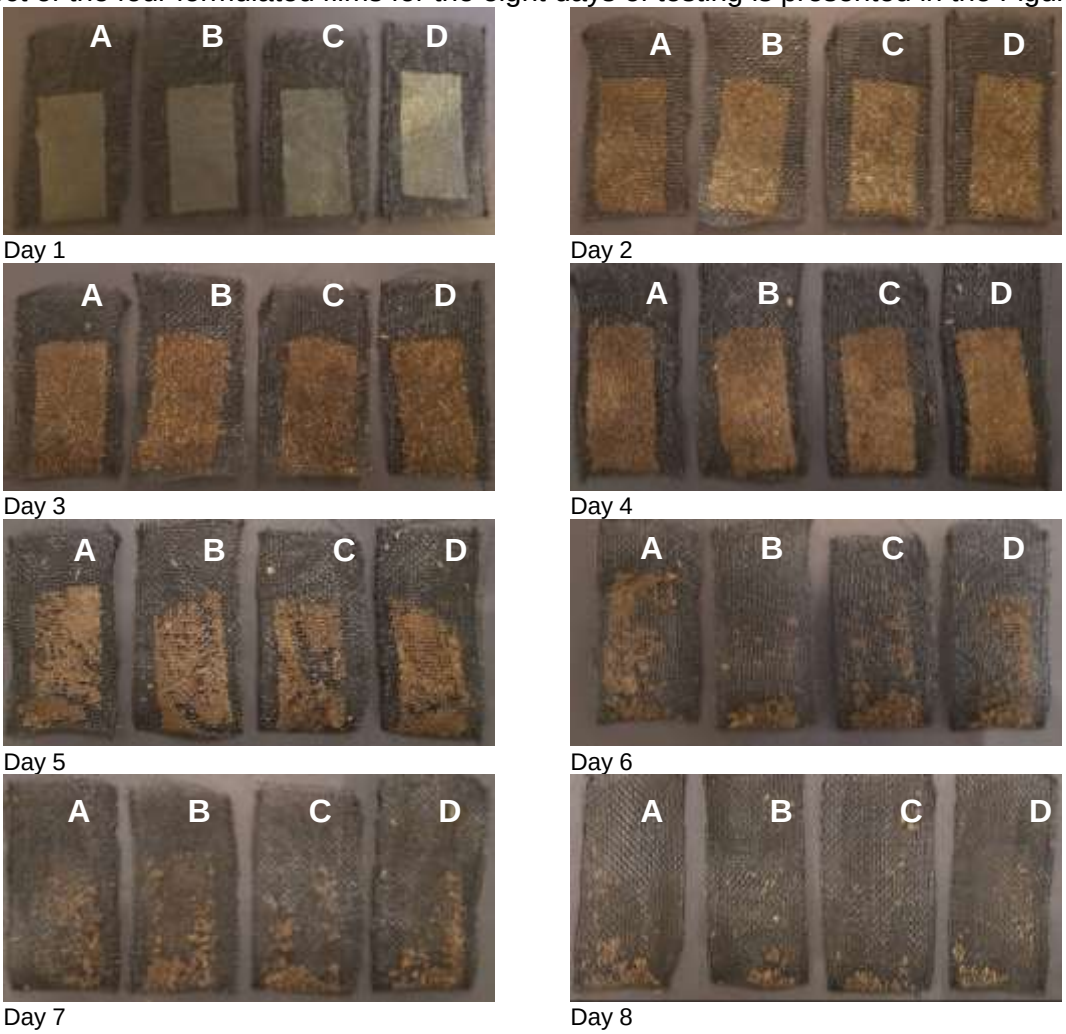

Figure 4. Visual aspect and degradation behavior of the films during exposure to the test soil. A – film without chitosan; B – addition of 0.15 wt.% chitosan; C – addition 1.50 wt.% chitosan; D – addition 3.00 wt.% chitosan.

It can be seen that all films, with and without chitosan, presented similar behavior in the soil exposure test. The color change in the second day is a strong indicative of the degradability of the films, for all formulations. From day five on, it is visible the disaggregation of the structure of the films, they starting to tear apart. The addition of chitosan presented no visible inhibition of the microbial activity on the films, being all formulation very degradable when exposed to soil (considering the test soil), being nearly completely decomposed after eight days.

It was also observed a strong aggregation of soil particles to the films, must probably due to the high hydrophilicity and flexibility of the films. According to Shah and coauthors [61], visual changes in the surface of the films, such as pores, cracks, disaggregation, or color change does not prove definitely the occurrence of a microbial/biological degradation from a metabolic point of view; however, they can be assumed as the first signs of microbial activity on the films.

Considering that all films lost started to disaggregate in the fifth day, the films with higher chitosan contents aggregated more amounts of soil in their surfaces. According to Tonhi and Peplis [62] and Shah and coauthors [61], this is result of chitosan high hydrophilicity, result of the presence of hydroxyl and amino groups in the polysaccharide structure. Gigli and coauthors [63] and Paoli [64] cited a possible correlation between the biological degradation rate of a biodegradable film and its hydrophilicity degree.

Li and Chen [65], investigating the biological degradation of concentrated whey-based (CW) and isolated whey-based (IW) films, concluded that, when in contact with soil, the CW films were degraded more quickly than the IW ones. The IW films started to decompose after two days of incubation in the soil; after seven days, the mass loss of the films was more than 80%, indicating a nearly complete decomposition of the films.

Evaluation of loss mass of the films

The mass of the soil-exposed films was measured daily to verify the behavior of the mass loss of the films during biological decomposition. Table 6 presents the statistical analysis of the mass loss of the films regarding formulation and day of exposure.

Table 6. Statistical analysis of the mass loss of the films regarding film composition and days of exposure (CV = 8.79%).

Day	Formulation – chitosan content			
	0.00 wt.%	0.15 wt.%	1.50 wt.%	3.00 wt.%
1	0.624 Ade	0.661 Ade	0.643 Ae	0.659 Aef
2	1.300 Aa	1.412 Aa	1.314 Aa	1.363 Aab
3	1.077 Bb	1.127 Bb	1.161 Bab	1.488 Aa
4	0.951 Bbc	0.959 Bbc	1.022 Abc	1.168 Abc
5	0.849 Bc	0.827 Bcd	0.854 Abcd	1.018 Acd
6	0.774 Acd	0.715 Ade	0.743 Ade	0.831 Ade
7	0.646 Ade	0.634 Ade	0.680 Ade	0.662 Aef
8	0.516 Ae	0.611 Ae	0.590 Ae	0.616 Af

Means followed by the same letter, uppercase in row (formulation) and lowercase in column (day) do not differ statistically by Tukey's multiple range test at 5% probability ($\alpha = 0.05$).

Regarding film composition, it can be seen that for the films with 1.50 and 3.00 wt.% chitosan the total mass presented itself slightly higher than in other formulations; this may be result of water (moisture) capture due to chitosan high hydrophilicity. In relation to the days of exposure, there was an increase in film mass up to day two; this may be result of moisture absorption of even due to the strong aggregation of soil particles to the film. The decrease due to degradation occurred steadily for all formulations, but without important statistical difference in the last days. According to Brambilla [66], a mass increase is the first step towards biological degradation of a material, being a result of water absorption and, afterward, the starting of microbial colonization of the material surface and formation of the biofilm.

Tonhi and Peplis [62] cited the proportional relation between the chitosan amount added to a film and the capacity it to aggregate soil particles; this can be seen especially by analyzing Table 6, in which the formulation with 3.00 wt.% chitosan presented higher mass values when compared to other formulations. Assis and Silva [67] reported that increasing amounts of chitosan added to biodegradable films interfere significantly in the water amounts absorbed. The hydrophilicity of chitosan is considered the main reason for this phenomenon, considering that the hydroxyl and amino groups present strong affinity for polar molecules, such as water. This helps water absorption and diffusion throughout the film structure [36].

The predominance of amino groups in chitosan, which present covalent N-H bonds, generate sites of high polarity, which are places for alignment and rearrangement of water molecules. This characteristic is also associated to acetamido groups, which also present polar character, and confer chitosan a biopolymer with a high degree of hydrophilicity [67].

When the soil amount aggregated by the film reached its capacity, it was observed a reduction of film mass for all formulations, especially from day three on. The mass reduction, which indicated the biotransformation of the polymeric structure of the film, eventually led to the disaggregation of the entire structure, the films crumbling due to the weakened and decomposing polymeric chains; the presence of moisture in the soil may also help decomposition by enhancing the decomposition rate and also by helping solubilizing the smaller fragments of the films [68].

After eight days of analysis, despite the mass of the decomposed film was similar to the starting mass, the presence of fragments of aggregated soil may induce a false idea of no (or little) mass loss. By analyzing Figure 4, it can be seen that the films were nearly completely decomposed. This indicated that whey-based films with addition of chitosan present a very high biodegradability, being nearly completely decomposed after eight days of exposure to an appropriate decomposition medium (soil); however, as seen in the shelf-life test, the films did not decompose even after 150 days (5 months) without and appropriate medium.

CONCLUSION

There were changes in the physical-chemical and mechanical properties of the films, in which increasing chitosan content rendered a lower elongation at break, higher moisture content, opacity and film thickness. Most of the properties remained stable during the storage time (5 months). The addition of chitosan rendered a yellowish hue to the films along with an increase in film opacity, especially the formulations with higher chitosan content. The films with higher chitosan content (1.50 and 3.00 wt.%) presented more pores and surfaces with lower homogeneity; the one with 0.15 wt.% presented the best results considering homogeneity and crystallinity. Considering biological degradation, all films presented a high biodegradability, the ones with higher chitosan content presented a higher soil aggregation due to the increased hydrophilicity. The film degradation increased some soil parameters, such as volatile solids and total organic C; however, it has not changed soil pH, N, and P contents. All films presented a similar behavior concerning microbial degradation, where chitosan addition have not enhanced the film resistance to fungi attack. Taking into account that chitosan is a relatively expensive material, the chitosan content of 0.15 wt.% may be considered the best one to be employed in whey-based biodegradable films.

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