



Original article

Immobilization of bioactive compounds in *Cassia grandis* galactomannan-based films: Influence on physicochemical properties



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ABSTRACT

Galactomannan extracted from *Cassia grandis* seeds was used for the production of films containing different concentrations of the bioactive compounds lactoferrin (LF), bioactive peptides (BAPs), and phytosterols. SEM, FTIR, mechanical and thermal properties, colour, moisture content (MC), solubility, water vapour permeability (WVP), and contact angle (CA) were performed evaluating the effect of increasing concentrations of bioactive compounds on the films' physicochemical properties. The immobilization of bioactive compounds leads to films with roughness on their surface, as observed by SEM. The thermal events demonstrated that bioactive compounds avoided the establishment of more hydrogen bonds when compared to galactomannan control film; this behaviour was also confirmed by FTIR. All the studied films had a strong whiteness tendency as well as a yellowish appearance. The addition of LF reduced MC and solubility values and leads to an increase of WVP and CA values, while the addition of BAPs and phytosterols did not change the films' solubility. The mechanical properties were affected by the addition of bioactive compounds, which improved the stiffness of the films. Galactomannan-based films from *C. grandis* showed to be a promising structure for the immobilization of biomolecules, pointing at a significant number of possible applications in food and pharmaceutical industries.

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1. Introduction

Polymers, whether natural or synthetic, are large molecules whose chains are able to produce continuous matrices that will influence the successful formation of films, membranes and coatings. Among natural polymers, polysaccharides have been used as an alternative material for the development of biodegradable and non-toxic films and membranes that can be used in different applications. In the pharmaceutical field, films based on alginate [1], chitosan [2], and POLICAJU [3] have been used as wound dressing due to their capacity of adhesion and controlled release of

biomolecules; while galactomannan-based films have been applied as supports for the immobilization of Concanavalin A in immunological experiments, aiming the evaluation of interactions between polysaccharides, proteins and dengue virus [4]. In food industry, the use of polysaccharides for the production of films and coatings became an alternative for synthetic and wax materials, being cellulose derivatives, chitosan, agar and starch some of the most common used materials. They can act in the maintenance of safety and quality of foods, being used as carriers of biomolecules, not only for control of the microbial contaminations, but also to enhance the health benefits of food products [5].

Galactomannans are hydrophilic polysaccharides derived from leguminous seeds that possess a central core of (1 → 4)-linked D-mannopyranose units to which (1 → 6)-linked D-galactopyranose units are attached. They present as main advantages their emulsifying, thickening, and gelling capacity. In addition, they can be used as alternative sources for the production of edible films based on their edibility and biodegradability [6,7]. The galactomannan

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extracted from the seeds of *Cassia grandis*, a typical Brazilian tree, has been previously characterized by our lab [8]. Methylation analysis and NMR spectroscopy reported that the polysaccharide has a central core composed of 4-linked β -mannose units with branches of galactose, linked to the carbohydrate core through $\alpha(1-6)$ linkage. The rheological characterization indicated the galactomannan as an alternative source for conventional galactomannans due to its ability to present both liquid and gel behaviour suggesting that, due to its rheological properties, it can be useful in various biotechnological applications.

A great number of works reported the use of galactomannans from conventional [9] and non-conventional [10] sources to produce films. Other galactomannan-based films have also been explored as effective support for the immobilization of different functional compounds, such as peptides [11], antioxidants [12] and lectins [4]. The incorporation of bioactive compounds with functional properties in the galactomannan-based films can bring several advantages for pharmaceutical and food industries. By combining the excellent biological characteristics of the bioactive compounds and the physicochemical properties of the films, functional galactomannan-based films could bring several advantages when compared with the synthetic alternatives and thus should be explored.

Lactoferrin (Lf) is a monomeric, globular, and basic 80 kDa iron-binding glycoprotein of the transferrin family. It is abundant in milk and in most biological fluids and holds important biological activities including anticancer, anti-inflammatory and immune modulator, as well as antimicrobial properties against a large number of microorganisms. These properties are mostly due to its ability to bind iron and to interact with cellular and molecular compounds of hosts and pathogens [13]. Bioactive peptides (BAPs) are specific fragments of proteins with activities similar to drugs and/or hormones, capable of modulating physiological functions by binding to specific target cell receptors, leading to induction of physiologic responses. Milk proteins are a good source of BAPs, which can positively affect various health biomarkers *in vitro* [14]. Regarding the described effects in the cardiovascular system, BAPs induce anti-hypertensive activity by inhibiting the angiotensin converting enzyme, a key enzyme in the regulation of blood pressure that converts angiotensin I into angiotensin II, a potent vasoconstrictor. Plant sterols, also called phytosterols, have been reported to include over than 250 distinct sterols and related compounds in various plant and marine materials; in addition, they have similar chemical structures and biological functions as cholesterol. Phytosterols contain an extra methyl group, ethyl group, or double bond, and they are known to have hypocholesterolemic properties. Phytosterols analogues are suggested to lower cholesterol absorption and thus the serum cholesterol level in humans, leading to cardiologic health benefits [15].

Several studies have reported the immobilization of Lf [16,17], BAPs [18], and phytosterols [19] in pharmaceutical solid dosage forms such as capsules, beads, particles and tablets, however the immobilization of those bioactive compounds in galactomannan-based films emerges as an effective way for biotechnological applications in pharmaceutical and food industries, since they can be an alternative, for example, for individuals with dermal wounds or with difficulty in swallowing those unit doses.

The objective of this work was to evaluate the effect of three different bioactive compounds (LF, BAPs and phytosterols) on the properties of galactomannan-based films. The effect of increasing concentrations of bioactive compounds was evaluated through scanning electron microscopy (SEM), Fourier-transform infrared (FTIR) spectroscopy analyses and thermal properties. Colour, moisture content, solubility, water vapour permeability, water contact angle, and mechanical properties were also performed.

2. Materials and methods

2.1. Materials

The pods of *C. grandis* were collected at the rural zone of Pernambuco state, in the city of Angelim (Brazil), in July 2011. Ethanol (99.8%), acetone (PA) and sodium chloride were obtained from Vetec Fine Chemicals Ltda. (Brazil). Lf was obtained from DMV International (USA). The reported composition expressed as a dry weigh percentage was: 96% protein, 0.5% ash, 3.5% moisture and an iron content around 120 ppm. Glycerol 87% (Panreac, Spain), Lowpept[®] (hydrolysed milk protein, Innaves SA, Spain) and Lowcol[®] (phytosterols, Innaves SA, Spain) were obtained from commercial sources. Lowpept[®] (hydrolysed milk proteins, potassium chloride, cellulose microcrystalline as bulking agent, xantham gum as carrier and magnesium stearate and silicon dioxide as anti-caking agents) and Lowcol[®] (phytosterols, cellulose microcrystalline as bulking agent, xantham gum as carrier and magnesium stearate and silicon dioxide as anti-caking agents) were used as sources of BAPs and phytosterols, respectively. All other chemicals were of analytical grade.

2.2. Film preparation

The galactomannan contained in *C. grandis* seeds was obtained according to Albuquerque et al. (2014) [8], with an extraction yield of $36 \pm 8\%$ and a composition of 71.0% mannose and 29.0% galactose. Briefly, the purification process was performed by immersion of the pods of *C. grandis* in distilled water at 25 °C for 18 h; the pods were then separated in a half part, revealing the seeds which were removed and dried until reaching a constant weight. The dry seeds were boiled in distilled water 1:5 (w/v) at 100 °C for 1 h for enzyme inactivation and maintained in water by 18 h at 25 °C to facilitate removal of the hull. After that, the hull was removed and the residual was triturated in a blender with 0.1 M NaCl 5% (w/v) at 25 °C, filtered through a veil tissue and after using a screen printing cloth, and precipitated with 46% ethanol 1:3 (v/v) for 18 h. The white precipitate obtained was washed with 100% ethanol 1:3 (w/v) for 30 min and two times with acetone 1:3 (w/v) for 30 min, been filtered on screen printing cloth between each washing. The precipitate was dried until constant weight, milled and finally called galactomannan.

The film forming solutions were prepared in distilled water with 0.8% (w/v) of galactomannan and 0.2% (v/v) of glycerol, maintained under magnetic stirring (500 rpm) for 18 h, at room temperature (20 ± 2 °C), and finally called film A. Galactomannan and glycerol concentrations were chosen based on preliminary analyses (results not shown).

Lf (0.1% for B1 and 0.2% for B2), Lowpept[®] (0.1% for C1 and 0.2% for C2) and Lowcol[®] (0.1% D1 and 0.2% for D2) were added to the film forming solutions (w/v), and left under magnetic stirring (500 rpm) for 5 h, at room temperature (20 ± 2 °C). A constant amount (15 mL) of each of the obtained film forming solutions was cast onto a 90 mm diameter polystyrene Petri dish. The films were then dried in an oven at 33 °C for 9 h and maintained at 20 °C and 54% relative humidity (RH) until further characterization.

2.3. Film thickness

Film thickness was measured with a digital micrometer (No. 293–561, Mitutoyo, Japan). Five different randomly chosen points were performed on each film and the mean values were used in the calculations of water vapour permeability (WVP) and mechanical properties.

2.4. Scanning electron microscopy

The Scanning Electron Microscopy (SEM) surface scans of the films were performed on a scanning electron microscope (Nova NanoSEM 200, Netherlands) with an accelerating voltage of 10 kV under vacuum conditions. Each film was attached to a coverslip via a coated thin film of chromium and carbon. The coating also worked to prevent the accumulation of static electric charge on the surface during electron irradiation and to avoid scanning faults and other image artefacts. The samples were sputter-coated with colloidal gold particles and then left drying before scanning. The images were presented with 5000× of magnification and 20 μm of amplitude.

2.5. Fourier-transform infrared (FTIR) spectroscopy

The films were characterized by Fourier Transform Infrared (FTIR) Spectroscopy on a Bruker FT-IR VERTEX 80/80v (Boston, USA) in Attenuated Total Reflectance mode (ATR) with a platinum crystal accessory between 400 and 4000 cm⁻¹, using 16 scans at a resolution of 4 cm⁻¹. Before analysis, an open beam background spectrum was recorded as a blank. The galactomannan control film (A) was used as control and each spectrum recorded for the films with immobilized bioactive compounds (B1, B2, C1, C2, D1 and D2) was subtracted from the control spectrum. Data analysis was performed with GraphPad Prism 5.00.288 (GraphPad Software, Inc., San Diego, CA, USA).

2.6. Thermogravimetric analysis

Thermogravimetric analysis (TGA) was completed with a PerkinElmer TGA 4000 (PerkinElmer, Massachusetts, EUA). Samples were placed in the balance system and heated from 20 °C to 450 °C at a heating rate of 10 °C min⁻¹ under a nitrogen atmosphere.

2.7. Colour and opacity

The colour and opacity were determined using a digital colorimeter (Konica Minolta, model Chroma Meter CR-400, Osaka, Japan) calibrated at illuminant C with a white standard. The parameters determined were L^* ($L^*=0$ [black] and $L^*=100$ [white]), a^* ($-a^*$ =greenness and $+a^*$ =redness) and b^* ($-b^*$ =blueness and $+b^*$ =yellowness). These parameters are the ones recommended by the International Commission on Illumination. Films opacity was calculated as the ratio between the opacity of each sample on the black standard (Y_b) and the opacity of each sample on the white standard (Y_w). Five measurements were determined at random Y_b and Y_w and an average of them was used for calculations. The experiment was done in triplicate and the results were expressed as percentage and determined through:

$$Y(\%) = (Y_b/Y_w) \cdot 100 \quad (1)$$

2.8. Moisture content

The moisture content (MC) was expressed as the percentage of water removed from the initial mass sample. MC was determined gravimetrically by drying the films at 105 °C in an oven with forced air circulation for 24 h. The experiments were performed in triplicate.

2.9. Solubility

The measurement of solubility was determined according to Gontard, Duchez, Cuq, Guilberts (1994) [20]. Triplicates of each film were cut with a circular mould of 2 cm diameter, weighted

and dried at 105 °C in an oven for 24 h. After reweighting, the films were placed in cups with 50 mL of distilled water, sealed over with parafilm and homogenized at 60 rpm for 24 h. The non-soluble part of each film was dried at 105 °C for 24 h and weighted again.

2.10. Water vapour permeability

The measurement of water vapour permeability (WVP) was determined gravimetrically based on ASTM E96-92 method [21,22]. The film was sealed on the top of a permeation cell containing distilled water (100% RH; 2337 Pa vapour pressure at 20 °C), placed in a desiccator at 20 °C and 0% RH (0 Pa water vapour pressure) containing silica. The cells were weighted at intervals of 2 h for 10 h. Steady-state and uniform water pressure conditions were assumed by maintaining the air circulation constant outside the test cell by using a miniature fan inside the desiccator [29]. The slope of weight loss versus time was obtained by linear regression. Three replicates were obtained for each sample.

2.11. Contact angle

Contact angle was measured in a face contact angle meter (OCA 20, Dataphysics, Germany). The samples of the films were taken with a 500 μL syringe (Hamilton, Switzerland), with a needle of 0.75 mm of diameter. The contact angle at the film surfaces was measured by the sessile drop method [23]. Measurements were made in less than 5 s. Thirty replicates of contact angle measurements were obtained at 19.8 (±0.3) °C.

2.12 Mechanical properties: Young's modulus, tensile strength and elongation-at-break

Young's modulus (YM), tensile strength (TS) and elongation-at-break (EB) were measured using a TA-HD plus Texture Analyzer (Serial RS232, Stable Micro Systems, Surrey, UK) following the guidelines of ASTM D 882-02 (2010). According to the ASTM standard, film strips with a length of 100 mm and a width of 20 mm were used and the average film thickness was measured as previously described in Section 2.3. The initial grip separation was set at 100 mm and the crosshead speed was set at 5 mm min⁻¹. YM was related as the slope of the elastic region in a plot of stress x strain. TS was expressed in MPa and was calculated by dividing the maximum load (N) by the initial cross-sectional area (m²) of the specimen. EB was calculated as the ratio of the final length at the point of sample rupture to the initial length of a specimen (100 mm) and expressed as a percentage. At least six replicates were performed for each sample.

2.13. Statistical analyses

Statistical analyses were performed using Analysis of Variance (ANOVA) and linear regression analysis. The Tukey test ($\alpha=0.05$) was used to determine any significance of differences between specific means (GraphPad Prism 5.00.288, GraphPad Software, Inc., San Diego, CA, USA).

3. Results and discussion

3.1. Scanning electron microscopy (SEM)

SEM is a technique that allows visualizing the surface morphology of the films, mainly the distribution of the immobilized bioactive compounds in the microstructure of the film matrix. Fig. 1 shows the surface morphology of the galactomannan-based films. Fig. 1A shows the image of the film without any bioactive compound (A), presenting a more uniform structure than the films

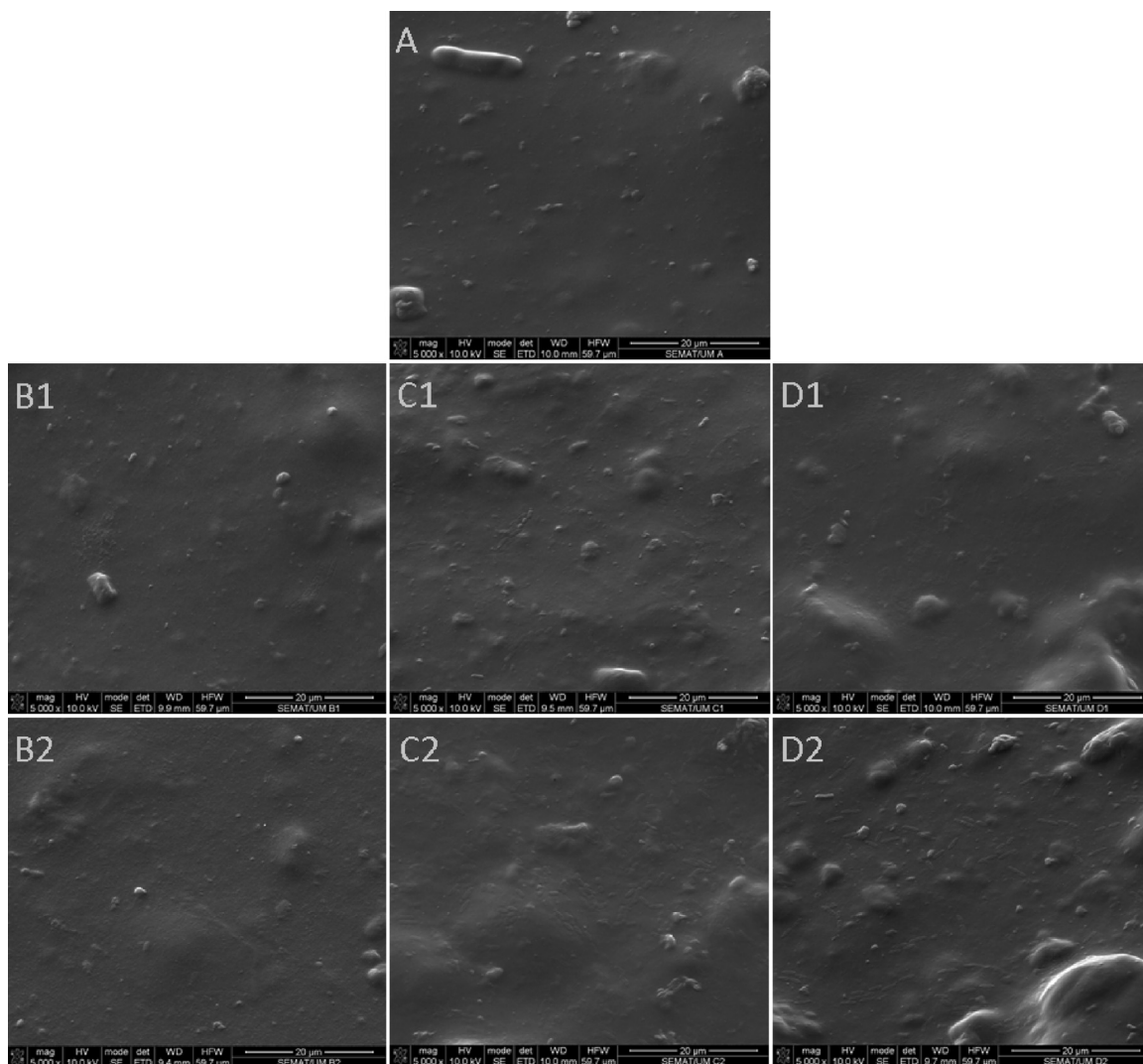


Fig. 1. SEM images showing the surface structure of (A) the control galactomannan film and after immobilization of (B1) Lf 0.1% (w/v), (B2) Lf 0.2% (w/v), (C1) Lowpept[®] 0.1% (w/v), (C2) Lowpept[®] 0.2% (w/v), (D1) Lowcol[®] 0.1% (w/v), and (D2) Lowcol[®] 0.2% (w/v) in the film-forming mixture.

with immobilized bioactive compounds (B–D). It is possible to observe by SEM some granules that maybe related to contaminating residues remaining from the galactomannan extraction. However, it is important to highlight that during galactomannan extraction NaCl was used to increase the solubility of the contaminating free proteins; also washing with ethanol allows to remove contaminants and a further wash with acetone that allows the removal of proteins [24]. In addition, galactomannan films with Lowpept[®] (C1 and C2) and Lowcol[®] (D1 and D2) showed more granules than B1 and B2 (immobilized with Lf) probably due to the presence of insoluble parts of these biomolecules. Is clear the presence of the immobilized bioactive compounds in the microstructure of the film matrix and their change according to the type of biomolecule.

For instance, working with the immobilization of bioactive compounds in galactomannan-based films, Cerqueira et al. (2010) [12] used different extracts and showed by SEM that the composite films present vesicles with spherical shapes, smooth surfaces and apparently free of visible cracks and pores. It is possible to observe in these results that Lf was homogenously distributed in the film matrix, while Lowpept[®] (Fig. 1C1 and C2) and Lowcol[®] (Fig. 1D1 and D2) presented a non-uniform pattern of distribution. In addition, according to the results presented in Table 1, film thickness was only affected ($p < 0.05$) by Lowcol[®] immobilization, maybe

Table 1

Bioactive compounds concentration used in films formulation and the corresponding values of film thickness.

Films	Bioactive compound (w/v)	Thickness (mm)
A	–	0.031 ± 0.001^a
B1	Lf 0.1%	0.041 ± 0.008^a
B2	Lf 0.2%	0.044 ± 0.005^a
C1	Lowpept [®] 0.1%	0.038 ± 0.001^a
C2	Lowpept [®] 0.2%	0.044 ± 0.000^a
D1	Lowcol [®] 0.1%	0.099 ± 0.006^b
D2	Lowcol [®] 0.2%	0.099 ± 0.008^b

^{a–b}Different superscript letters in the same column indicate a statistically significant difference ($p < 0.05$).

associated with voids formed during film preparation and which leads to films with certain roughness on their surface and thus higher values of thickness.

3.2. FTIR

FTIR was used for the evaluation of possible chemical interactions between Lf, Lowpept[®], Lowcol[®] and the film matrix and possible modifications in their structure (Fig. 2).

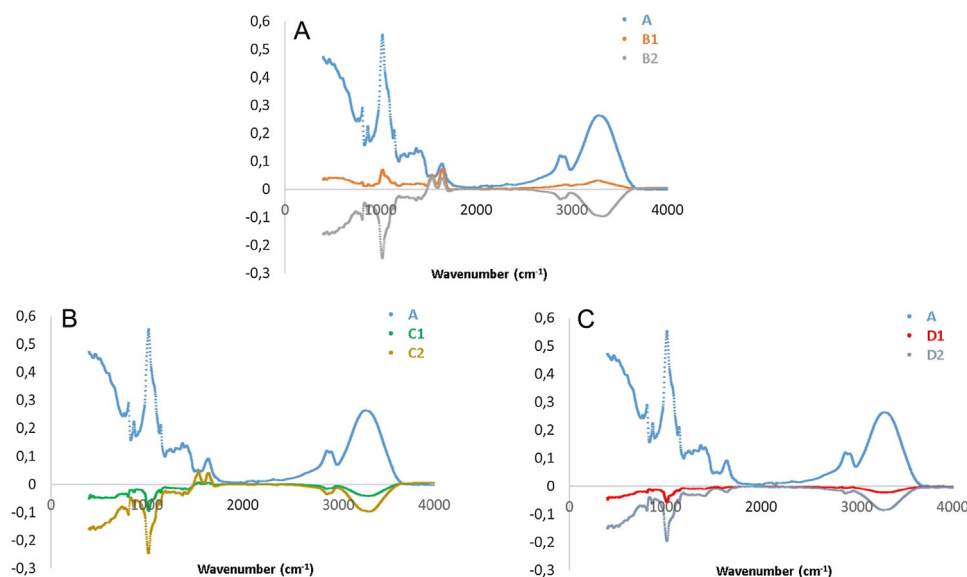


Fig. 2. FTIR spectra of the galactomannan control film (A) as blank and the subtracted spectra of the films after immobilization of (B1) Lf 0.1% (w/v), (B2) Lf 0.2% (w/v), (C1) Lowpept[®] 0.1% (w/v), (C2) Lowpept[®] 0.2% (w/v), (D1) Lowcol[®] 0.1% (w/v), and (D2) Lowcol[®] 0.2% (w/v) in the film-forming mixture.

The control film (A), composed only of galactomannan, displayed a FTIR spectrum similar to the result obtained by Albuquerque et al. (2014) [8], confirming that the control film is originated exclusively by the polysaccharide purified from *C. grandis* seeds; however, particular differences were observed for the films with immobilized bioactive compounds. The spectra recorded for these films were subtracted from the spectrum obtained for the galactomannan control film (please see Section 2.5). In general, all films presented a broad band between the regions 3100–3500 cm⁻¹, that represents O–H stretching vibration formed by hydroxyl groups of the galactomannan and water involved in hydrogen bonding. In addition, a broad band around 2800–3000 cm⁻¹, assigned to C–H stretching vibration, is also presented in all samples [25]. The incorporation of bioactive compounds leads to a great variation in the region related to hydroxyl groups, which can be related with the hydrophilicity character of the films. The addition of 0.2% (w/v) of Lf (B2), 0.1 and 0.2% (w/v) of Lowpept[®] (C1 and C2) and 0.1 and 0.2% (w/v) Lowcol[®] (D1 and D2) decreased the intensity of the bands, being representative of the lower availability of hydroxyl groups on these films and thus a less interaction with water. A different behaviour was observed for 0.1% (w/v) of Lf (B1), where the intensity of the O–H stretching vibration was similar to the galactomannan film (A) used as control.

The peak near to 1000 cm⁻¹ corresponds to the stretching vibration of C–O in C–O–C bonds and appears in all studied films [26], while the anomeric region (950–700 cm⁻¹) and peaks near to 870 and 810 cm⁻¹ are representative of the α -D-galactopyranose and β -D-mannopyranose units, respectively [27]. Despite the maintenance of these peaks for all films confirming the main structure of the galactomannan, it is possible to observe that the spectra of films with high concentrations of bioactive compounds (B2, C2 and D2) show an inflection of the peak (intensity decrease), which can be related to their influence in the galactomannan's film structure.

According to the literature [28], signals at 1650 and 1550 cm⁻¹ are assigned to the axial deformation of the C=O bond (amide I) and the angular deformation of the N–H bond (amide II), proposed for identification of proteins. These peaks could be clearly observed in films with 0.1 and 0.2% (w/v) of Lf and 0.2% (w/v) of Lowpept[®], respectively B1, B2 and C2, once the spectra of these films lead to an increase of the peak intensity in this range. The addition of Lowpept[®] at 0.1% (w/v), namely C1) and Lowcol[®] (0.1

and 0.2%, w/v, respectively for D1 and D2) displays no signals at 1650 and 1550 cm⁻¹, corresponding to a similar spectrum to the galactomannan control film (A).

3.3. Thermogravimetric analysis

The thermogravimetric analysis (TGA) for galactomannan films show at least three thermal events, being the first attributed to water evaporation, the second attributed to the presence of glycerol, and the third related to polysaccharide decomposition [29,30]. TGA experiments performed in this study are in accordance with those reported by the literature and show three mass loss events for the galactomannan films (Table 2). The first thermal event occurred near 65 °C and corresponds to dehydration, which may be attributed to the evaporation process, a characteristic phenomenon of a polysaccharide with a hydrophilic nature. The second, around 188 °C, is related to the weight loss associated with glycerol decomposition. For these results no statistically significant differences were observed. The third peak is associated with the dehydration, depolymerization and pyrolytic decomposition of the polysaccharide backbone [30,31]. The galactomannan control film (A) presented a peak of 294.19 ± 0.95 °C and a weight loss of $45.17 \pm 2.33\%$, similar to the results obtained for other galactomannans [10,31,32]. In addition, B2, C1 and C2 were statistically different to the control film (A), i.e., the presence of Lf (0.2%, w/v) and Lowcol[®] (0.1 and 0.2%, w/v) avoided the establishment of more hydrogen bonds in the galactomannan film matrix, as confirmed by FTIR spectra analyses.

3.4. Colour and opacity

Colour and opacity are important features for the acceptance of products from pharmaceutical or food industry. Table 3 presents the colour parameters and opacity of the films. All studied films were bright with a strong whiteness tendency as presented by L^* coordinate values, and a yellowness appearance represented by b^* coordinate; for those parameters films did not present statistically significant differences ($p > 0.05$) among them. Results showed that the presence of Lf in higher concentrations (B2) lead to the increase ($p < 0.05$) of a^* values, i.e., the increasing concentrations of Lf lead

Table 2
Thermogravimetric behaviour of the galactomannan control film (A) and the films after immobilization of (B1) Lf 0.1% (w/v), (B2) Lf 0.2% (w/v), (C1) Lowpept[®] 0.1% (w/v), (C2) Lowpept[®] 0.2% (w/v), (D1) Lowcol[®] 0.1% (w/v), and (D2) Lowcol[®] 0.2% (w/v) in the film-forming mixture. The values of the peaks correspond to the values of derivative thermograms obtained by the TGA curve between 20 and 450 °C.

Sample	Peak 1 (°C)	ΔY 1 (%)	Peak 2 (°C)	ΔY 2 (%)	Peak 3 (°C)	ΔY 3 (%)
A	67.75 ± 6.94 ^a	14.28 ± 4.08 ^a	193.10 ± 11.50 ^a	15.94 ± 0.78 ^b	294.19 ± 0.95 ^{b,e}	45.17 ± 2.33 ^{d,b}
B1	67.18 ± 3.84 ^a	12.81 ± 4.86 ^a	189.87 ± 10.10 ^a	14.54 ± 0.15 ^{b,c}	289.90 ± 3.73 ^{b,c}	46.90 ± 3.35 ^{d,b}
B2	66.93 ± 2.15 ^a	17.40 ± 0.64 ^a	187.38 ± 3.00 ^a	12.67 ± 1.15 ^c	286.61 ± 1.53 ^{c,d}	43.39 ± 1.20 ^b
C1	63.45 ± 1.63 ^a	16.09 ± 2.02 ^a	188.74 ± 9.06 ^a	12.21 ± 0.6 ^c	282.33 ± 0.57 ^d	46.28 ± 1.76 ^{d,b}
C2	64.46 ± 3.10 ^a	13.42 ± 4.08 ^a	192.96 ± 2.88 ^a	9.43 ± 0.76 ^a	272.50 ± 0.55 ^a	45.79 ± 3.10 ^{d,b}
D1	63.80 ± 2.42 ^a	16.99 ± 1.19 ^a	183.63 ± 4.51 ^a	13.11 ± 0.95 ^c	295.34 ± 1.15 ^e	48.89 ± 1.13 ^{d,b}
D2	65.36 ± 4.38 ^a	16.89 ± 2.71 ^a	187.93 ± 1.72 ^a	12.61 ± 1.36 ^c	296.33 ± 0.33 ^e	50.55 ± 2.87 ^a

^{a–e}Values with the same superscript letters within a column are not significantly different ($p < 0.05$).

Table 3
Colour parameters L^* (luminosity), a^* ($-a^*$ = greenness and $+a^*$ = redness), b^* ($-b^*$ = blueness and $+b^*$ = yellowness) and Y (opacity) for the galactomannan control film (A) and the films after immobilization of (B1) Lf 0.1% (w/v), (B2) Lf 0.2% (w/v), (C1) Lowpept[®] 0.1% (w/v), (C2) Lowpept[®] 0.2% (w/v), (D1) Lowcol[®] 0.1% (w/v), and (D2) Lowcol[®] 0.2% (w/v) in the film-forming mixture (values expressed as average ± standard deviation).

Film	L^*	a^*	b^*	Y (%)
A	95.59 ± 0.40 ^a	0.917 ± 0.01 ^{b,c}	7.037 ± 0.40 ^a	11.83 ± 0.25 ^a
B1	95.05 ± 0.42 ^a	1.197 ± 0.13 ^{a,b}	6.823 ± 0.78 ^a	11.71 ± 0.26 ^a
B2	95.18 ± 0.09 ^a	1.270 ± 0.01 ^a	6.593 ± 0.04 ^a	11.53 ± 0.15 ^a
C1	95.47 ± 0.32 ^a	0.903 ± 0.11 ^{b,c}	7.607 ± 0.40 ^a	11.84 ± 0.19 ^a
C2	95.29 ± 0.24 ^a	0.843 ± 0.19 ^c	7.980 ± 1.08 ^a	12.37 ± 0.16 ^{a,b}
D1	95.30 ± 0.15 ^a	1.040 ± 0.06 ^{a,b,c}	7.257 ± 0.28 ^a	13.43 ± 0.16 ^b
D2	94.51 ± 0.90 ^a	1.133 ± 0.11 ^{a,b,c}	8.063 ± 0.91 ^a	15.41 ± 1.40 ^c

^{a–c}Different superscript letters in the same column indicate a statistically significant difference ($p < 0.05$).

Table 4
Moisture content (MC), solubility (Sol), water vapour permeability (WVP) and contact angle (CA) values of the galactomannan control film (A) and the films after immobilization of (B1) Lf 0.1% (w/v), (B2) Lf 0.2% (w/v), (C1) Lowpept[®] 0.1% (w/v), (C2) Lowpept[®] 0.2% (w/v), (D1) Lowcol[®] 0.1% (w/v), and (D2) Lowcol[®] 0.2% (w/v) in the film-forming mixture.

Films	MC (%)	Solubility (%)	WVP × 10 ⁻⁷ (g h ⁻¹ m ⁻¹ Pa ⁻¹)	CA (°)
A	29.33 ± 2.31 ^{a,b}	74.00 ± 7.21 ^a	5.60 ± 0.39 ^a	68.72 ± 4.26 ^a
B1	17.67 ± 5.51 ^{b,c}	55.67 ± 7.02 ^{b,c}	8.85 ± 1.37 ^{a,b}	123.0 ± 4.27 ^b
B2	15.67 ± 5.51 ^c	51.67 ± 4.16 ^c	9.39 ± 1.31 ^b	122.8 ± 9.18 ^b
C1	31.67 ± 4.04 ^a	69.67 ± 7.77 ^{a,b}	7.75 ± 0.75 ^{a,b}	83.60 ± 3.46 ^c
C2	26.33 ± 5.69 ^{a,b,c}	63.67 ± 7.50 ^{a,b,c}	8.72 ± 0.52 ^{a,b}	93.05 ± 3.20 ^c
D1	20.33 ± 3.79 ^{a,b,c}	69.33 ± 4.93 ^{a,b,c}	22.43 ± 0.21 ^c	67.34 ± 7.07 ^a
D2	18.00 ± 2.65 ^{b,c}	56.00 ± 5.00 ^{b,c}	21.29 ± 2.55 ^c	62.06 ± 2.17 ^a

^{a–c}Values with the same superscript letters within a column are not significantly different ($p < 0.05$).

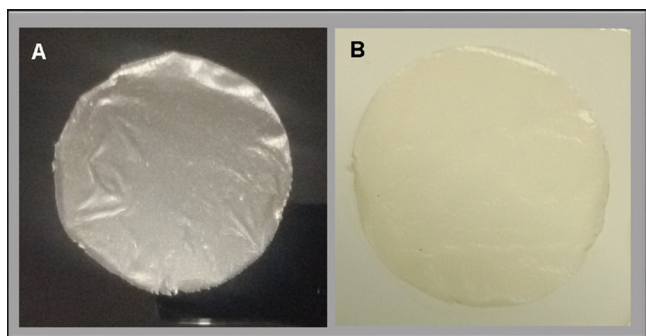


Fig. 3. Representative image of the galactomannan film with immobilized Lowpept[®] at 0.2% (w/v) in black (A) and white (B) background (D2).

to a redness appearance of the film, even considering their evident yellowness tendency.

All the films were slightly opaque (Table 3), however only galactomannan films with immobilized Lowcol[®] (D1 and D2) exhibited significantly higher ($p < 0.05$) values of opacity when compared to the control film (Fig. 3). This is in agreement with other published works [11,12,33], where it has been showed that the addition of bioactive compounds to the films could lead to an increase of opacity values.

3.5. Moisture content (MC), solubility (Sol), water vapour permeability (WVP) and contact angle (CA)

Moisture-binding abilities of films can affect significantly their physical and barrier properties, thus the knowledge of the content and affinity of film matrix to water is a key parameter when choosing a film for specific applications [34].

Table 4 shows the values of moisture content (MC), solubility, water vapour permeability (WVP), and contact angle (CA) of the films without any immobilized bioactive compounds (A) and of

the films with immobilized Lf (B1 and B2), Lowpept[®] (C1 and C2), and Lowcol[®] (D1 and D2). MC results showed that galactomannan-based films with 0.2% (w/v) of Lf (B2) presented a significant ($p < 0.05$) reduction of MC values when compared to the control film (A). Bourbon et al. (2011) [5] reported that incorporating bioactive compounds in chitosan films led to an increase of MC, however this increase was not significant for films with Lf. Our results showed that the incorporation of Lf in its highest concentration decreased the MC, which can be explained by the fact that Lf presents both hydrophobic and hydrophilic parts in its conformation. At neutral pH, Lf has a net positive charge [35] with a hydrophobic domain [36], thus the higher concentration of Lf in the galactomannan-based film may have reduced the availability of hydroxyl groups to interact with water, resulting in a decrease of MC values, as confirmed by FTIR spectra analyses. Other explanation for the availability of hydroxyl groups on galactomannan composite films and a lower interaction with water was advanced by Antoniou et al. (2015) [6], who worked with tara gum-based films and showed the influence of the addition of compounds in the MC. They explained that the films with composites, nanoparticles in this case, presented a more compact structure, which allowed them to occupy more free volume in the polymer matrix, thus reducing the MC values.

Solubility results showed that the addition of Lf (B1 and B2) and Lowcol[®] in its highest concentration (D2) decreased significantly ($p < 0.05$) the solubility values when compared to the control film (A). Nevertheless, it is important to mention that when Lf, Lowpept[®], and Lowcol[®] were added to galactomannan films, they maintained their structure even after 24 h immersion in water. On the other hand, the control film (A) became a cracked mass due to a potential dissolution pattern. The reduction of the solubility for the galactomannan-based films with immobilized Lf is in agreement with the decrease of MC values observed for B2, i.e., the film could be considered more hydrophobic than the control film (A).

It is known that the presence of a plasticizer like glycerol leads to a reduction of the hydrophobicity of polysaccharide-based films

[37,38], but also the proportion and distribution of galactose units along the mannan chain can have an essential role in water content of galactomannans. The water solubility tends to increase with increasing content of galactose for galactomannans with M/G ratio up to 3; in turn, several studies reported that higher values of M/G ratio lead to films with lower solubility [39,40]. The MC and solubility values obtained for the control film (A) are higher than the results obtained for the galactomannan studied by Antoniou, Liu, Majeed, Qazi, & Zhong (2014) [41], who evaluated how the properties of tara gum-based films were influenced by polyols, including glycerol at a similar concentration to the one used in our work. They obtained solubility values ranging between 14.37 and 32.40% according to the glycerol concentration used. Cerqueira, Souza, Teixeira, & Vicente (2012) [31] also reported that the addition of glycerol increased the solubility and MC of films of *Gleditsia triacanthos* galactomannan when compared with other plasticizers. The galactomannan extracted from the seeds of *C. grandis* used in this work has a mannose/galactose ratio (2.44:1) different from the galactomannans used in the above mentioned works. This molar ratio, in addition to the presence of glycerol may have influenced the galactomannan film structure, justifying the differences obtained for MC and solubility when compared to other galactomannan-based films; but also the extraction process (different in this case) can lead to great difference in the solubility of obtained galactomannans.

WVP is the most extensively studied property of edible films mainly because of the importance of the water in deteriorative reactions [12]. WVP values ranged from $5.60 \pm 0.39 \times 10^{-7}$ (A) to $22.43 \pm 0.21 \times 10^{-7} \text{ g h}^{-1} \text{ m}^{-1} \text{ Pa}^{-1}$ (D1) (Table 2). The presence of phytosterols into galactomannan-based films (D1 and D2) increased significantly the WVP results, leading to values 3.9-fold higher than the control film (A). The same happened when Lf was added in a high concentration (B2), however in this case the increase was lower (1.7-fold). According to Antoniou, Liu, Majeed, Qazi, & Zhong (2014) [41], the high degree of hydrophilicity and the water attracted into the matrix contribute to create regions of higher water mobility, with greater inter-chain distances in the galactomannan film. Regarding the results for the film with immobilized Lowcol[®] in its highest concentration (D2), one can observe that it is more hydrophobic than the control film (A) in result of its reduced solubility, thus the increase in the WVP value can be associated to the irregular surface of the film, confirmed by SEM results (see Section 3.1). The presence of voids on the irregular surface of the film could function as site for water binding during moisture absorption, also allowing the water vapour to pass through the film. Finally, the increased WVP values reported in our results demonstrated that the contents of Lf in its highest concentration (B2), and phytosterols from Lowcol[®] (D1 and D2), in addition to the interaction with the galactomannan film, were the most significant factors affecting the WVP.

The determination of the contact-angle (CA) of films surface is a simple way to evaluate the degree of hydrophobicity of the films, since the CA values will increase for higher hydrophobic surfaces [42]. Generally, if surfaces have CA values less than 90°, they are considered hydrophilic. The CA of the control film (A) indicated that this film is essentially hydrophilic, which was quite similar to that reported for other galactomannan-based films [41,42]. Lf (B1 and B2) and Lowpept[®] (C1 and C2) immobilized in galactomannan films led to an increase ($p < 0.05$) of CA values of the control film (A), while no difference ($p > 0.05$) was observed for Lowcol[®] (D1 and D2). It is also important to note that the concentration effect did not affect each batch-film, i.e., there were no differences observed between B1 and B2, C1 and C2, and D1 and D2.

The results confirmed that the presence of Lf increased the hydrophobicity of the films at all concentrations (B1 and B2) with CA values almost 40° higher than the control films. The

Table 5

Effect of concentration on Young's modulus (YM), tensile strength (TS) and elongation at break (EB) of the galactomannan control film (A) and the films after immobilization of (B1) Lf 0.1% (w/v), (B2) Lf 0.2% (w/v), (C1) Lowpept[®] 0.1% (w/v), (C2) Lowpept[®] 0.2% (w/v), (D1) Lowcol[®] 0.1% (w/v), and (D2) Lowcol[®] 0.2% (w/v) in the film-forming mixture.

Films	YM (MPa)	TS (MPa)	EB (%)
A	0.29 ± 0.14 ^a	4.73 ± 1.96 ^a	18.06 ± 6.44 ^a
B1	4.58 ± 1.12 ^d	10.10 ± 1.13 ^{b,c}	3.76 ± 0.93 ^b
B2	5.08 ± 0.91 ^{c,d}	9.40 ± 2.25 ^{b,c}	3.37 ± 2.21 ^b
C1	3.83 ± 0.049 ^d	8.08 ± 1.05 ^c	3.64 ± 1.42 ^b
C2	6.24 ± 0.92 ^{b,c}	11.37 ± 1.70 ^b	3.16 ± 0.87 ^b
D1	6.77 ± 1.10 ^b	10.58 ± 1.97 ^{b,c}	2.36 ± 0.69 ^b
D2	4.51 ± 1.20 ^d	8.25 ± 1.40 ^c	3.29 ± 0.54 ^b

^{a-d}Values with the same superscript letters within a column are not significantly different ($p < 0.05$).

hydrophobic pattern observed for CA is also in agreement with the results obtained for MC and solubility of galactomannan-based films with immobilized Lf. Relatively to the addition of Lowpept[®], the hydrophilic nature of the films (C1 and C2) remains unclear since their CA values were approximately 90°. A different behaviour was observed for the addition of Lowcol[®]: the results showed a higher water affinity for D1 and D2. CA provides information regarding the sorption of water molecules to the film structure [39], being related with the capacity for water binding during moisture absorption for films with immobilized Lowcol[®], as confirmed by WVP results.

It is important to mention that, depending on the application of the films with immobilized bioactive compounds, the substantial differences in its physicochemical properties may be relevant for the biomolecules and the display of their activities, for instance, the relative hydrophobic pattern of the films with immobilized bioactive compounds allows its graduated release, once the compound guarantees its permanence into the film until the complete solubilisation of the matrix.

3.6. Mechanical properties

Table 5 shows the mechanical properties (Young's modulus – YM, tensile strength – TS and elongation at break – EB) of the galactomannan films without and with immobilized bioactive compounds. YM is a measure of the stiffness of a sample and a decrease in this value means a reduction of the film stiffness, i.e., the reduction of YM increases the deformability of the films [43]. Films with immobilized Lf (B1 and B2), Lowpept[®] (C1 and C2), and Lowcol[®] (D1 and D2) showed statistically significant ($p < 0.05$) difference in YM when compared to the control film (A), which means that the immobilized bioactive compounds improved the toughness of the films. Regarding the effect of the immobilized bioactive compounds concentration on the films, it is important to note that no significant ($p > 0.05$) impact occurred to the films with Lf, while for films with Lowpept[®] a concentration-dependent behaviour was observed, where films with higher concentrations of this biomolecule (C2) showed higher YM values than the films with 0.1% (C1). A distinct behaviour was observed for films with Lowcol[®], where YM values decreased for higher concentrations of the bioactive compound.

TS indicates the maximum tensile stress that the film can sustain. It is a parameter related to the chemical structure of the film and depends strongly on film composition, being directly influenced by water, plasticizers, surfactants and bioactive compounds immobilized in the film matrix [5]. Galactomannan-based films with Lf, Lowpept[®], and Lowcol[®] exhibited higher TS values compared to the control film (A), which means that the immobilization of bioactive compounds affected the structure of the films, improving the tensile stress to which the films may be subjected before deformation occurs. In addition, increasing the content of

Lowpept[®] leads to a significant increase of ($p < 0.05$) TS values of the films. The concentrations of the other bioactive compounds did not show effects on TS values.

EB is related with the film flexibility and the obtained results are presented in Table 5. Results show that films with immobilized bioactive compounds were less flexible ($p < 0.05$) than the control film (A). Nevertheless, the increase of the concentration of them did not affect EB. This is in agreement with other works, as the EB of galactomannan-based films decreased when biomolecules or other compounds were added to the matrix [7].

The M/G ratio of galactomannans significantly affects the mechanical properties of the films. For example, Mikkonen et al. (2007) [44] reported that films prepared from locust bean gum (M/G ratio of approximately 3.33) were stronger and more flexible than films prepared from guar gum (M/G ratio of approximately 1.67). Dos Santos et al. (2015) [39] produced films from five sources of galactomannans (*Adenantha pavonina*, *Cyamopsis tetragonolobus*, *Caesalpinia pulcherrima*, *Ceratonia siliqua* and *Sophora japonica*, presenting mannose/galactose ratios of 1.3, 1.7, 2.9, 3.4 and 5.6, respectively) and reported that EB increases with the increase of the M/G ratio for values up to 3.0. For higher molar ratios, the EB values showed a reduction. TS values generally follow the inverse of those obtained for EB; they are high at low M/G ratios, rising again for high M/G ratios. The result for TS of the galactomannan control film (A) is in agreement with published works for other galactomannan-based films [45].

The galactomannan extracted from *C. grandis* seeds used in this study (M/G ratio of 2.44) produced more flexible control films (A) than the above mentioned galactomannans; in addition, the immobilization of Lf, Lowpept[®], and Lowcol[®] affected the structure of the films, already reported for the physical characterization by MC, solubility, WVP and CA results, increasing the film's stiffness (higher YM) and tensile strength (TS), confirming the reinforcing effect of the bioactive compounds into the film matrix.

4. Conclusion

Lactoferrin, Lowpept[®], and Lowcol[®] can be added to galactomannan-based films maintaining their main structure and promoting significant variations on the physicochemical properties of the films. The incorporation of the bioactive compounds increased the films' stiffness and decreased their solubility, leading to a reinforcement of the films. It has also been shown how the structure, the surface and the hydrophilicity character of the films could be influenced by the presence of bioactive compounds through measurement of thermal properties (TGA), surface microstructure (SEM) and chemical structure (FTIR).

The immobilization of Lactoferrin (Lf), peptides (from Lowpept[®]) and phytosterols (from Lowcol[®]) in galactomannan-based films can be valuable for a great number of industrial applications. For example, in the pharmaceutical field, galactomannan-based films with immobilized Lf can be tested as a candidate for wound dressing by combining the physicochemical properties of the film and the excellent Lf biological activities. For biotechnological applications in food, the health benefits associated to BAPs and phytosterols could be achieved in galactomannan films with immobilized Lowpept[®] and Lowcol[®], respectively, which can be used as alternative for individuals with difficulties for swallowing pharmaceutical solid dosage forms.

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