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Bacterial cellulose/cashew gum films as probiotic carriers

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ABSTRACT

This study was carried out to obtain probiotic films with good stability by combining spore-forming, resistant bacteria (*Bacillus coagulans*) with a biopolymer mix (bacterial cellulose – BC and cashew gum - CG) as a carrier matrix. Fructooligosaccharides (FOS) were used as prebiotic. Four different films were produced, namely, *Co* (control), *Pro* (added with probiotic), *Pre* (containing the prebiotic FOS), and Syn (synbiotic films containing probiotic and FOS). Although the tensile and barrier properties of films have been undermined by probiotic and FOS, those properties have remained within the values needed for food applications. Most films (except *Pre*) exhibited hydrophobic character (contact angles $> 90°$). FOS enhanced probiotic viability upon processing. The storage stability of probiotics was very good; even at 37 °C, the viability loss did not surpass 1 log cycle, due to the resistance of *B. coagulans* and the protective role of BC. Moreover, no cytotoxic effect of the films was observed on Caco-2 cells.

1. Introduction

The global probiotics market has been estimated at USD 49.4 billion in 2018, and projected to reach USD 69 billion by 2023 (Markets and Markets, 2020). Probiotics may be consumed either as supplements (e.g. capsules, tablets or sachets) or food components, the global sales for probiotic foods far outweighing those of supplements (Lonza, 2019). Although most probiotic foods in the market are dairy products, there has been an increasing number of people with restrictions to dairy foods, leading to an increasing demand for non-dairy probiotic products.

In this context, some studies have been carried out on edible films and coatings containing probiotics (Soukoulis, Behboudi-Jobbehdar, Macnaughtan, Parmenter, & Fisk, 2017; Soukoulis, Singh, Macnaughtan, Parmenter, & Fisk, 2016). Those films and coatings may be used as packaging aids, acting as well as protectant matrices to the probiotics, and presenting bioactive properties, contributing to consumers' health. Moreover, they may extend food microbial stability due to the competitive effects of probiotics against spoilage microorganisms (Espitia, Batista, Azeredo, & Otoni, 2016), as already demonstrated in inhibition zone tests (Karimi, Alizadeh, Almasi, & Hanifian, 2020) or in stability tests with fish (López de Lacey, López-Caballero, Gómez-Estaca, Gómez-Guillén, & Montero, 2012; Mozaffarzogh, Misaghi, Shahbazi, & Kamkar, 2020). The presence of prebiotics, which are non-digestible ingredients that selectively stimulate growth and/or activity of probiotics, such as fructooligosaccharides (FOS) and inulin, has been reported to enhance the viability of the probiotic bacteria in food products (Okuro, Thomazini, Balieiro, Liberal, & Fávaro-Trindade, 2013), including edible films (Pereira et al., 2019).

The ability of an edible film to keep a good probiotic stability upon processing and storage depends on the ability of the film components to protect the bacteria, and also on the ability of the bacteria themselves to survive processing and storage conditions.

In this study, bacterial cellulose (BC) was chosen as the film matrix, since it has been presented as a good matrix for immobilization of probiotics, protecting them against gastric juices and bile salts (Fijałkowski, Peitler, Rakoczy, & Żywicka, 2016). BC is synthesized by some bacteria (especially from the *Komagataeibacter* genus) as a network of cellulose nanofibrils forming a membrane. Although a BC-pectin composite has been reported as an encapsulating matrix for

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probiotics, providing better probiotic stability when compared to pectin alone (Khorasani & Shojaosadati, 2016), BC has never been reported as a matrix for probiotic films. On the other hand, BC has already been presented as a matrix capable of forming films with better tensile, barrier, and water resistance properties than the corresponding pectin films (Viana, Sá, Barros, Borges, & Azeredo, 2018). However, a limitation of nanofibrillated BC (NFBC) as a film-forming material is that its water suspensions are too viscous, making homogenization and spreading difficult. Cashew gum (CG), which is a low-viscosity heteropolysaccharide extracted from cashew tree bark, was then combined to NFBC for viscosity adjustment. CG has already been used to form films, mostly in combination with other polysaccharides (Azeredo et al., 2012; Britto, Rizzo, & Assis, 2012), since its low viscosity is not suitable for film formation by itself (Rodrigues et al., 2014).

The bacteria of choice in most studies on probiotic films are *Lactobacillus* or *Bifidobacterium* (Ebrahimi et al., 2018; Pereira et al., 2019; Soukoulis et al., 2016, 2017). However, most of them do not have the ability to form spores, which makes them sensitive to harsh conditions. The spore-forming ability of *Bacillus* species has been explored, since spores are highly resistant to technological stresses as well as to low pH (Shinde et al., 2019). In this context, *Bacillus coagulans* probiotic strains have been presented as promising alternatives, highly tolerant to processing, storage, and digestion (Marcial-Coba, Pjaca, Andersen, Knøchel, & Nielsen, 2019; Shinde et al., 2019).

This is the first study to report the development of probiotic films based on bacterial cellulose and using a spore-forming probiotic species. The objective was to obtain probiotic edible films with good stability to processing and storage conditions, by using a biopolymer matrix with excellent tensile and barrier properties (nanofibrillated bacterial cellulose, which was combined to cashew gum as a viscosity adjuster) and a spore-forming probiotic species (*Bacillus coagulans*), combined or not to prebiotic fructooligosaccharides (FOS).

2. Materials and methods

2.1. Production of nanofibrillated bacterial cellulose (NFBC)

A pre-inoculum from an isolated colony of *Komagataeibacter xylinus* ATCC 53582 was prepared in a flask containing 50 mL of sterile HS medium (Hestrin & Schramm, 1954) and incubated at 30 °C for 72 h. The pre-inoculum was added to HS culture medium (at 3 vol%), and each 500 mL of the medium were placed on a glass tray and statically incubated at 30 °C for 10 days. Each resulting BC pellicle was washed in 2 L of boiling water for 1 h, then immersed into 2 L of a NaOH 2% (w/v) solution at 80 °C for 1 h. Those two steps were repeated twice, and then the pellicles were washed in distilled water until pH 7. The pellicles were then dried in an air circulation oven at 105 °C for 24 h, then ground in a Vita-Prep 3 blender (Vitamix Corp., Cleveland, OH, USA) at 24,000 rpm for 5 min.

The BC was then submitted to oxidation mediated by 2,2,6,6-tetramethyl-1-piperidinoxyl (TEMPO radical), according to the method proposed by Saito, Kimura, Nishiyama, and Isogai (2007). Each 10 g of ground BC (10 g) was immersed in 1 L of an aqueous solution containing 0.16 g TEMPO and 1 g sodium bromide. The oxidation was started by the addition of a NaClO 11% solution (in such an amount as to have 5 mmol NaClO per g of BC). After 20 min of stirring (500 rpm), the pH was adjusted to 10 using NaOH 1 M solution, and the suspension was kept under stirring (500 rpm) for 2 h at 25 °C. The BC was removed from the oxidizing solution, washed in distilled water until pH 7, and processed on Vita-Prep® 3 blender at 24,000 rpm for three 10-min steps interspersed with 10-min intervals. The final NFBC dispersion (with about 1 wt% solids) was kept in a cold chamber at 4 °C.

2.2. Purification of CG

CG was obtained from exudates from cashew trees (Embrapa Tropical Agroindustry Experimental Field, Pacajus, Ceara State, Brazil) and purified by a method described by Torquato et al. (2004), with modifications. The exudate was ground, dried in an air-circulation oven (60 °C, 24 h), dissolved in distilled water (exudate:water w/v ratio, 1:3) at 24 °C for 24 h, vacuum filtered through a 325-mesh sieve, and precipitated with commercial 96°GL ethanol (ethanol:exudate weight ratio, 3:1). The precipitate was drained and dried in a fume hood. The dried CG was ground with an analytical mill (A11 Basic, Ika, Staufen, Germany) and passed through a 212-μm sieve.

2.3. Preparation of the probiotic strain

Freeze-dried *Bacillus coagulans* BC4 10 MLD spores (lot C192580A) standardized with maltodextrin and containing about 10^{11} CFU g^{-1} were provided by Sacco (Cadorago, Italy). A stock culture was prepared by inoculating 1 g of the freeze-dried culture in 20 mL of tryptone glucose yeast extract (TGY) broth, incubating it in a shaker at 37 °C and 200 rpm for 48 h, centrifuging it, then inoculating the *B. coagulans* biomass into 40 mL of TGY broth, incubating it again (37 °C, 200 rpm, 48 h), centrifuging it, and finally inoculating the biomass into 40 mL of TGY broth added with 10 mL glycerol. The stock culture was stirred in vortex tubes and transferred onto cryogenic tubes for storage at −80 °C.

A 5 mL sample of the frozen stock culture was transferred to 45 mL of TGY medium and incubated for 24 h at 39 °C in an incubator shaker at 200 rpm. After centrifugation (3000 g, 15 min), the supernatant was discarded, and the bacterial biomass was inoculated in 50 mL of a spore-forming medium (5 mL Corn Steep Liquor, 1 g dextrose, 0.056 g manganese sulfate, 0.05 g calcium carbonate, and 0.5 g ammonium sulfate per liter) at 39 °C, 200 rpm for 48 h. After viable cell counting, the sporulated culture was stored at −18 °C until use.

The viable cells were counted by the spread plate method. 0.1 mL samples (in triplicate) were immersed into 0.9 mL of a sterile NaCl solution (0.85%), homogenized in a vortex for 1 min, 6-fold serially diluted in saline solution, plated (in triplicate) on TGY agar, and incubated at 37 °C. Colonies were counted after 24 h.

2.4. Preparation of films

A BC-only film was tested as a vehicle for the probiotic bacteria, but its high viscosity limited the BC film-forming dispersion to 1% w/v. From such a dispersion, the initial (wet) thickness to cast had to be about 10 mm to obtain a film with a final thickness of about 100 μm, which required a very long time (and/or very high temperature) to dry, reducing the viable cell counts in about 3 log cycles. That is why CG was added.

A film-forming dispersion was made from the BC suspension (in such an amount as to contain 20 g BC), CG (20 g), and glycerol (12 g). The dispersion was homogenized in an Ultra-Turrax T25 (Ika, Germany) at 10,000 rpm for 10 min, then divided into four parts, each one containing 5 g of BC and 5 g of CG, which were transferred into filtering flasks already with stir bars (to facilitate homogenization and degassing). The dispersions were autoclaved at 121 °C for 15 min and allowed to cool at 25 °C. The four treatments are hereinafter referred to as *Co* (to produce a control film), *Pre* (with added FOS, Sigma-Aldrich, Saint Louis, USA), *Pro* (with added *B. coagulans*), and *Syn* (synbiotic, i.e., with FOS and *B. coagulans*).

The *Co* dispersion was vacuum degassed for 25 min for bubble removal, cast onto petri dishes (80 mL per 14-cm dish), and dried at 80 °C for 150 min in an MA030/12 oven (Marconi, Piracicaba, Brazil) with a vacuum pump (Marconi MA057/1) connected to it, with a pressure of −200 mmHg.

For the *Pre* film, 1 g FOS was added to the dispersion, which was stirred (500 rpm, 15 min, 25 °C). The dispersion was vacuum degassed, cast, and dried under the same conditions as for the *Co* film.

For the *Pro* film, a sample (about 6 mL) of the *B. coagulans* culture

was unfrozen, homogenized in vortex for 30 s, centrifuged (3000 g, 15 min), and the precipitate (bacterial biomass) was washed with sterile distilled water and centrifuged again, until the supernatant was transparent. The separated bacterial biomass was incorporated into the film-forming dispersion (in an amount defined so as to provide the film with a viable cell count of about 9 log CFU.g^{-1}, according to the previous viable cell count as described in item 2.3) and stirred (500 rpm, 15 min, 25 °C) using a previously UV-sterilized magnetic stir bar. The dispersion was then vacuum degassed and cast onto previously sterilized petri dishes. The dispersion was dried under the same conditions as for the *Co* film, except that the oven was previously sanitized with a benzalkonium chloride 0.01% (w/v) solution and then with ethanol 70%. For the *Syn* film, the same procedure as for the *Pro* film was carried out, except that 1 g FOS was added to the dispersion along with the bacterial biomass.

2.5. Physical determinations on films

Film samples were cut and detached from the surface. Before characterization, the free-standing samples were conditioned for at least 40 h at 23 °C and 50% RH following ASTM standard (as described in method D882-12, ASTM, 2012).

The film thickness was measured using a digital micrometer (Mitutoyo—QuantuMike IP65, Japan). Five measures were performed for each test film for determination of tensile properties and water vapor permeability.

2.5.1. Tensile properties

Tensile properties of 80 × 20 mm film strips (with ten replicates) were measured according to D882-12 (ASTM, 2012), using a 4500 Universal Testing Machine (Instron Corporation, Canton, USA), with a load cell of 100 N, initial grip separation of 50 mm, and crosshead speed of 100 mm min^{-1}.

2.5.2. Water vapor permeability (WVP)

The WVP determination, with six replicates, was based on the method E96/E96M-16 (ASTM, 2016) at 25 °C, using silica gel as desiccant (0% RH) in an Arsec DCV-040 vertical desiccator (outside the permeation cells) and water (100% RH) inside the permeation cells. Eight measurements were taken within 24 h.

2.5.3. Opacity

Opacity was determined in a Minolta colorimeter (CR 400, Minolta, Japan) as the ratio between the opacity on a black standard and on a white standard. Five measurements were taken for each of five film samples, and the results were expressed as a percentage.

2.5.4. Water contact angle (WCA)

The contact angles (WCA) were measured in a face contact angle meter (OCA 20, Dataphysics, Germany) at 20 °C, by the sessile drop method, using a syringe equipped with a needle with internal diameter of 0.71 mm (Hamilton, Switzerland). Contact angle measurements were performed immediately after placing a drop (3 μL) of ultrapure water on the film surface. Images were captured by CCD video camera (resolution of 752 × 582 pixels) and processed by C20 software. Twelve measurements were performed for each sample to obtain an average value.

2.5.5. Fourier-transform infrared spectroscopy (FTIR)

The FTIR spectra of the films was recorded with a Bruker FTIR VERTEX 80/80v (Boston, USA) in Attenuated Total Reflectance mode (ATR) with a platinum crystal accessory in the 4000-400 cm^{-1} wavelength range, with a resolution of 4 cm^{-1} over 16 scans. Before the analysis, an open bean background spectrum was recorded as a blank. All measurements were performed in triplicate.

2.5.6. Scanning electron microscopy (SEM)

The SEM micrographs of film surfaces were taken using a QUANTA FEG 650 (FEI Inc., Hillsboro, USA) microscope. The samples were mounted on an aluminum stub using carbon-coated double-sided adhesive tape, sputter-coated with gold, and examined using an accelerating voltage of 5 kV and a magnification of 10,000 times.

2.5.7. Statistical analyses

For tensile properties, WVP, WCA, and opacity, the data were submitted to one-way ANOVA and Tukey's test for multiple comparisons ($p < 0.05$).

2.6. Cytotoxicity of films

The cell compatibility of *Co* and *Pre* films was assessed using human colorectal adenocarcinoma Caco-2 cells. Several cell lines may be used as *in vitro* models for the intestinal epithelium, but Caco-2 cell line is the most widely used model for toxicity evaluation of edible nanomaterials (Tibolla et al., 2019), since it mimics many characteristics of intestinal enterocytes (Antunes, Sandrade, Araújo, Ferreira, & Sarmento, 2013), such as expression of many enzymes and transport proteins (Lea, 2018), being thus recognized as the gold standard for simulating intestinal absorption of test substances *in vitro*. The use of Caco-2 further circumvents the limitations of using primary cells, such as the difficulty to obtain and culture them, and substantial batch to batch variations (Hardy et al., 2018).

Caco-2 cells, clone HTB-37™, from human colon carcinoma, were obtained from the American Type Culture Collection (ATCC®). Caco-2 cells (passage 25–40) were cultured in minimum essential medium (MEM) supplemented with 20% fetal bovine serum (FBS), 1% sodium pyruvate and 1% penicillin/streptomycin. The cells were kept at 37 °C and 5% CO_2 in 75 cm^2 flasks. For the cell compatibility assessment, confluent cells were detached using 0.25% trypsin-EDTA solution, then precipitated by centrifugation at 6500*g* for 5 min and resuspended in MEM at 10^5 cells.mL^{-1}. Cells were seeded onto 96-wells plates at a density of 1×10^4 cells (100 μL of cellular suspension) per well and left adhering overnight in a humidified atmosphere of 5% CO_2 in air at 37 °C.

Film samples were magnetically stirred in Milli-Q water, placed in an ultrasonic bath (37 kHz, 104 W) for 15 min, and sterilized with ultraviolet light for 30 min. The dispersions were diluted in the culture medium (20 vol%). Cells growing in the culture medium were the negative control (100% cell viability), whereas 30 vol% dimethyl sulfoxide (DMSO) was the positive control. Films were tested at different final concentrations: 0.5, 1.0, or 2.0 mg/mL, incubated for 24 h or 48 h with resazurin (0.01 mg/mL), which is a redox indicator that, once entering a viable cell, is irreversibly reduced to the fluorescent resofurin. The fluorescence intensity (proportional to the number of viable cells) was measured using a Microplate Fluorescence Reader (Synergy, BioTek H1, USA) at excitation and emission wavelengths of 560 nm and 590 nm respectively, after 3 h of incubation. The cell viability was expressed as the percentage of fluorescence in treated cells in relation to fluorescence of cells growing in the culture medium.

2.7. Viability of probiotics on films

The viability of probiotics on *Pro* and *Syn* films was followed through processing (drying) and storage.

The probiotic viability loss on film drying was assessed by counting the viable cells on film-forming dispersions (three 1-mL samples taken before casting) and dried films (three 0.1 g samples). The counts of *Pro* and *Syn* films were compared by paired t-tests.

The viability loss of probiotics on storage was assessed by storing film samples at three different temperatures (4 °C, 20 °C, and 37 °C), and taking three 0.1 g samples of them at 0, 7, 15, 30, and 45 days for viable cell counting. For each temperature, the two films were

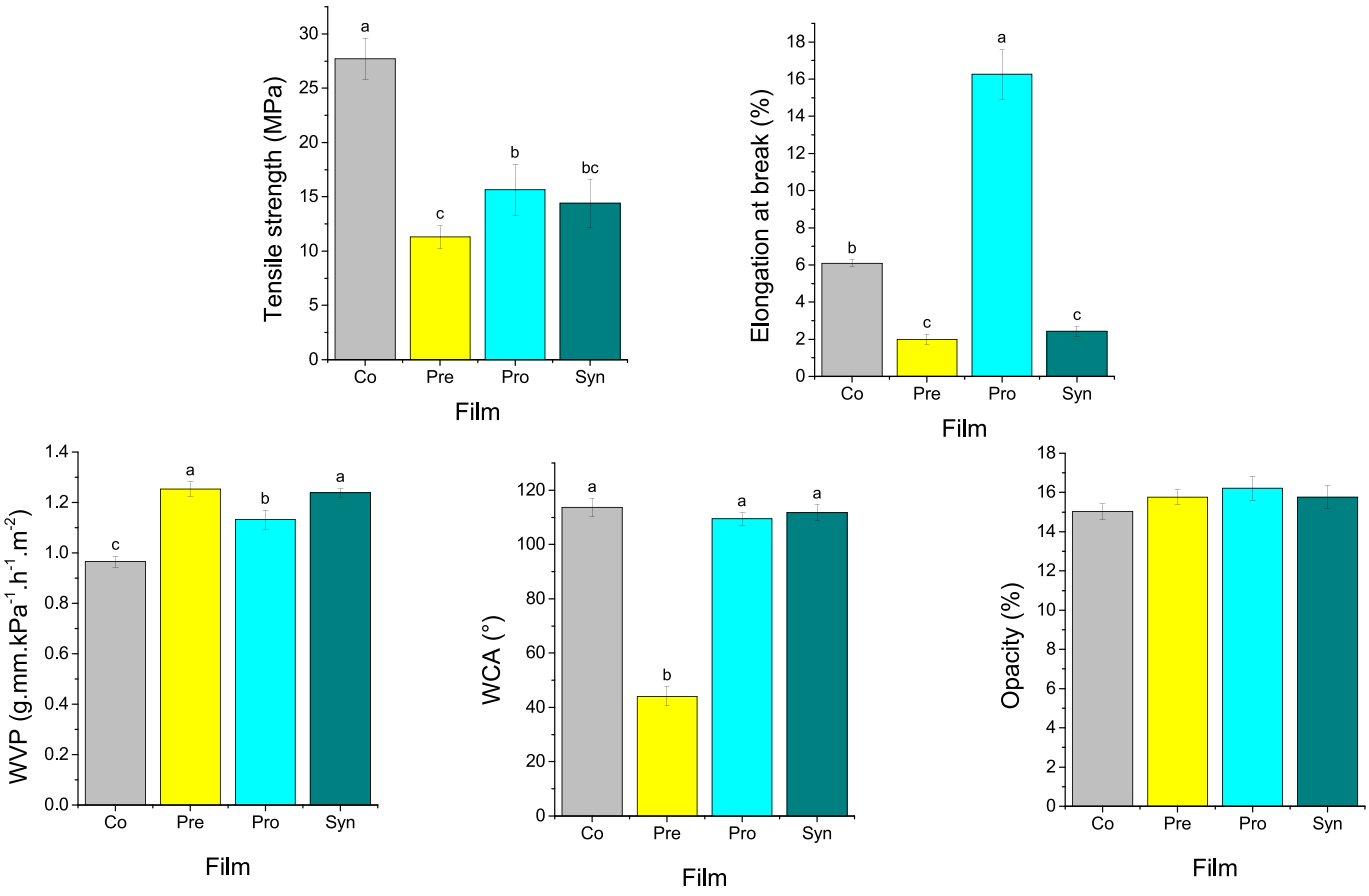

Fig. 1. Physical properties the films. WVP: water vapor permeability. WCA: water contact angle. Values in the same graph followed by at least one common letter (or not followed by any letters) are not significantly different ($p < 0.05$).

compared to each other in terms of overall variations (defined as the average cell counts at different storage times) by paired t-tests, at the confidence level of 0.05.

3. Results and discussion

3.1. Physical properties of films

The *Co* film presented very good tensile strength, even higher than the one reported by Khanna and Srivastava (2005) for low density polyethylene (LDPE), although the elongation was very low (Fig. 1). The incorporation of probiotics made the films less strong, corroborating previous studies (Ebrahimi et al., 2018; Kanmani & Lim, 2013), but the strength was still kept quite above 4 MPa, which is considered as the minimum value for food packaging (Tajeddin, Rahman, & Abdulah, 2010). A positive effect of the bacteria on the tensile properties was an increase in elongation, contrasting with the elongation-decreasing effect reported by Ebrahimi et al. (2018) and Kanmani and Lim (2013), although Shahrampour, Khomeiri, Razavi, and Kashiri (2020) reported that some films presented improved elongation when added with *Lactobacillus plantarum*. The bacteria effects on tensile properties are compatible with plasticizing effects, ascribed to decreasing intermolecular attractions between adjacent polymeric chains, enhancing film flexibility but decreasing strength. On the other hand, when FOS were added, not only the strength was impaired, but also the elongation. The decreased strength was expected due to the low glass transition temperature (T_g) of FOS (Rajam & Anandharamakrishnan, 2015), related to the plasticizing effects of FOS, as previously reported in starch (Bersaneti, Mantovan, Magri, Mali, & Celligoi, 2016), methylcellulose (Romano et al., 2014), and whey protein films (Fernandes et al., 2020), meaning that FOS interfered with the hydrogen bonds among hydroxyl groups of the matrix (Romano et al., 2014). The impaired elongation found in this study as resulting from FOS addition corroborates results by Karimi et al. (2020) with polydextrose in films, but contrasts with those reported by other authors from FOS or other oligosaccharides (Bersaneti et al., 2016; Fernandes et al., 2020; Orozco-Parra, Mejía, & Villa, 2020), and suggests that the interaction of FOS with the matrix was probably very poor, weakening the matrix-FOS interface.

The addition of probiotic and/or FOS (mainly the latter) made the films more permeable to water vapor, as previously reported from the presence of other bacteria (Ebrahimi et al., 2018; Sánchez-González, Saavedra, & Chiralt, 2013) or prebiotic oligosaccharides (Orozco-Parra et al., 2020), which is ascribed to disruptions on the polymeric structure of the films, as already mentioned for tensile properties. In contrast to this study, Bersaneti et al. (2016) reported that FOS decreased the WVP of starch films, which the authors ascribed to lowering effects on water diffusivity through the matrix; on the other hand, those authors (as well as Orozo-Parra et al., 2020) reported that FOS increased the water solubility of the films, due to their hydrophilic character. Since permeability is defined as the product of diffusivity and solubility, one mechanism seems to have dominated the WVP results in the study by Bersaneti et al. (2016), and the other to have dominated WVP in the present study as well in the one by Orozco-Parra et al. (2020).

The WCA of most films (except *Pre*) was higher than 90°. The sharply decreased WCA resulting from FOS addition results from the high hydrophilicity of the oligosaccharides, as also observed by Fernandes et al. (2020) from the addition of galactooligosaccharides and xylooligosaccharides to whey protein films, and which also corroborates the increased WVP from FOS addition in this study. On the

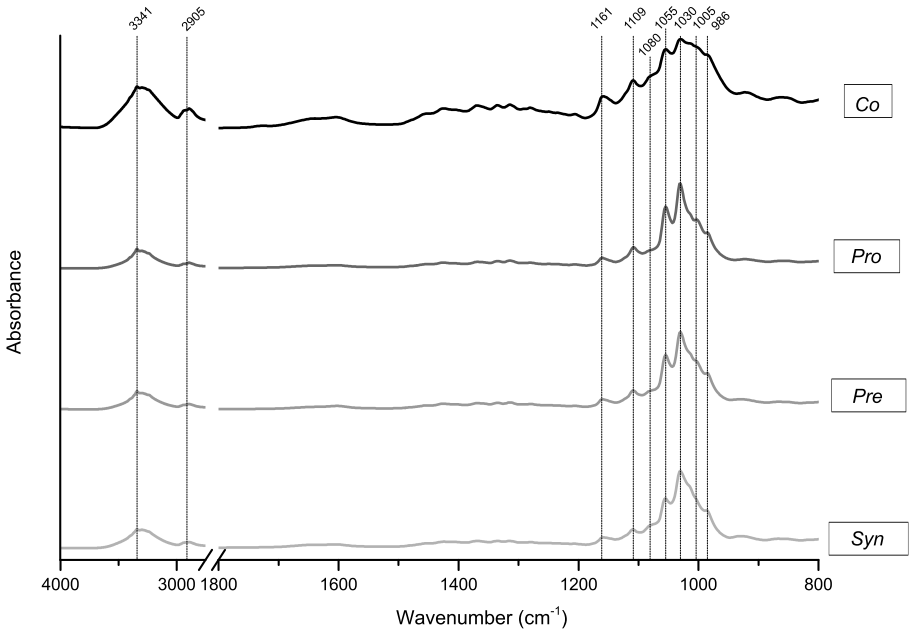

Fig. 2. FTIR spectra of films.

other hand, the film containing both probiotic and FOS presented similar WCA value to those of films without FOS, which may be ascribed to hydroxyl groups of FOS being possibly involved with bacterial surface polysaccharides, reducing film hydrophilicity.

The opacity was not affected by the addition of bacteria and/or FOS, that is to say, the active components did not impair film transparency. Other studies also reported that the incorporation of oligosaccharide prebiotics did not affect film transparency (Orozco-Parra et al., 2020; Sánchez-González et al., 2013), but, in contrast with this study, Orozco-Parra et al. (2020) found that the presence of bacteria (*L. casei*) increased film opacity.

3.2. FTIR spectra

FTIR is a rapid and non-destructive technique useful to identify functional groups of different chemical components of a mixture and spectral changes resulting from interactions between those components. The FTIR spectra of the films (Fig. 2) show that most bands are common to polysaccharide films, such as those from O–H stretching at 3341 cm^{-1}, C–H stretching at about 2900 cm^{-1}, asymmetric C–O–C stretching at 1161 cm^{-1} (Colom, Carrillo, Nogués, & Garriga, 2003), as well as C–O–C stretching at 1080 cm^{-1} (Liu, Thibodeaux, & Gamble, 2012), C–C stretching at 1109 cm^{-1}, and the C–O stretching and bending bands between 1055 and 1030 cm^{-1} (Agrebi, Ghorbel, Bresson, Abbas, & Kallel, 2019; Xiong, Li, Shi, & Ye, 2017).

The spectra was mostly unchanged by the presence of the probiotic and/or FOS, except by some intensity changes, which may ascribed to minor interactions between components or even to dilution effects (by adding other components), but nothing that suggests noticeable interactions. Since FOS are also composed of saccharides, their structure has much in common with the matrix polysaccharides. Indeed, some of the above-mentioned bands have previously been detected as part of FOS spectra, as the ones at 1055 (Bomfim et al., 2020), 1030, and 986 cm^{-1} (Romano, Santos, Mobili, Vega, & Gómez-Zavaglia, 2016). So, in FOS-containing films, many bands originate from both matrix polysaccharides and FOS, as also observed in other studies with films containing oligosaccharides (Bersaneti et al., 2016; Orozco-Parra et al., 2020). The bacteria, on the other hand, have non-saccharide components in their cell walls, but their low concentration in the films (when compared to those of major components) may be the reason why they do not affect the spectra. Indeed, other studies with probiotic films also reported that their FTIR spectra were unaffected by the bacteria (Mozaffarzogh et al., 2020; Orozco-Parra et al., 2020; Pereira et al., 2016).

3.3. SEM micrographs

All films presented rough, mesh-like surfaces (Fig. 3), like other films containing NFBC (Lai, Sheng, Liao, Xi, & Zhang, 2013; Lin, Lien, Yeh, Yu, & Hsu, 2013). The presence of probiotic and/or FOS resulted in rougher surfaces, corroborating previous reports from effects of bacteria (Heinemann, Carvalho, & Favaro-Trindade, 2013; Mozaffarzogh et al., 2020) and FOS (Bersaneti et al., 2016), with discontinuities in the film structure that explain the impaired tensile strength and water vapor barrier of the films.

3.4. Stability of the probiotic during film drying

The drying of probiotic-containing film-forming dispersions into films involves osmotic stress and heat stress, which are potentially harmful to the bacteria. The protective effect of FOS on the bacteria during film drying has been demonstrated by the *Syn* film exhibiting higher bacterial viability than the *Pro* film (Fig. 4A), even though the initial cell counts of *Pro* and *Syn* film-forming dispersions (before drying) were not significantly different from each other (about 9 log $CFU.g^{-1}$). This protective effect of FOS during air drying corroborates previous studies with films (Romano et al., 2014; Soukoulis, Behboudi-Jobbehdar, Yonekura, Parmenter, & Fisk, 2014), as well as other reports of FOS and other prebiotics acting as thermal protective agents to probiotics (Karimi, Azizi, Ghasemlou, & Vaziri, 2015; Rodríguez-Huezo et al., 2014). The protective effect of FOS has been described by Romano, Schebor, Mobili, and Gómez-Zavaglia (2016) as resulting of a balance between sugar with different molecular weights in FOS mixtures - the smaller ones being efficient in protecting lipid membranes (by replacing water molecules during dehydration), and the larger ones favoring the vitrification (formation of glassy states in which high viscosity and low molecular mobility limit molecular interactions).

Anyway, even in the film without FOS, the viability loss upon film drying was only 1.2 log $CFU.g^{-1}$, lower than the 1.7 log $CFU.g^{-1}$ decrease reported by Soukoulis et al. (2016) for *L. rhamnosus* in a starch

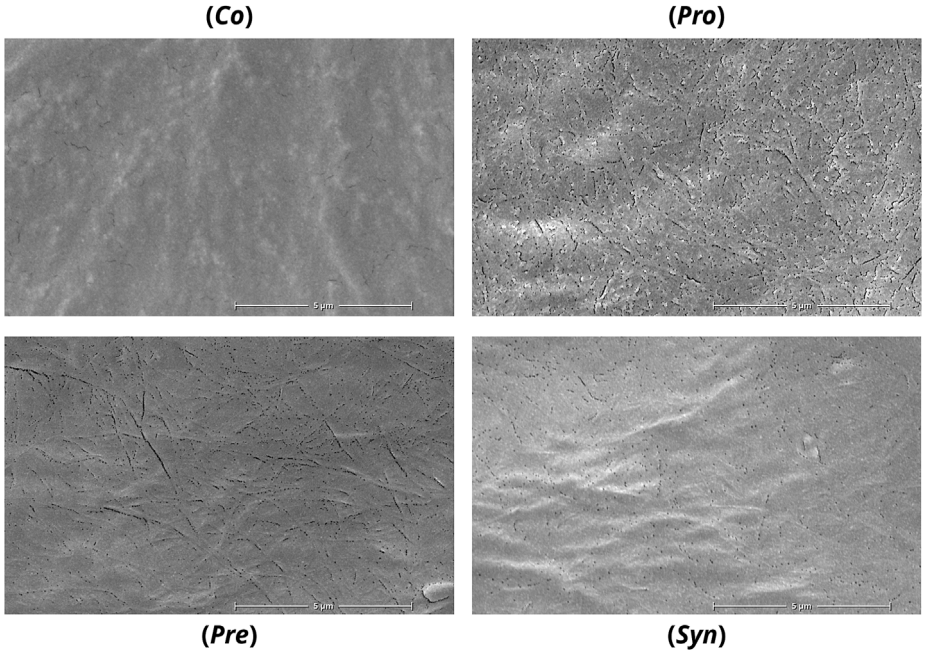

Fig. 3. Scanning electron micrographs of film surfaces.

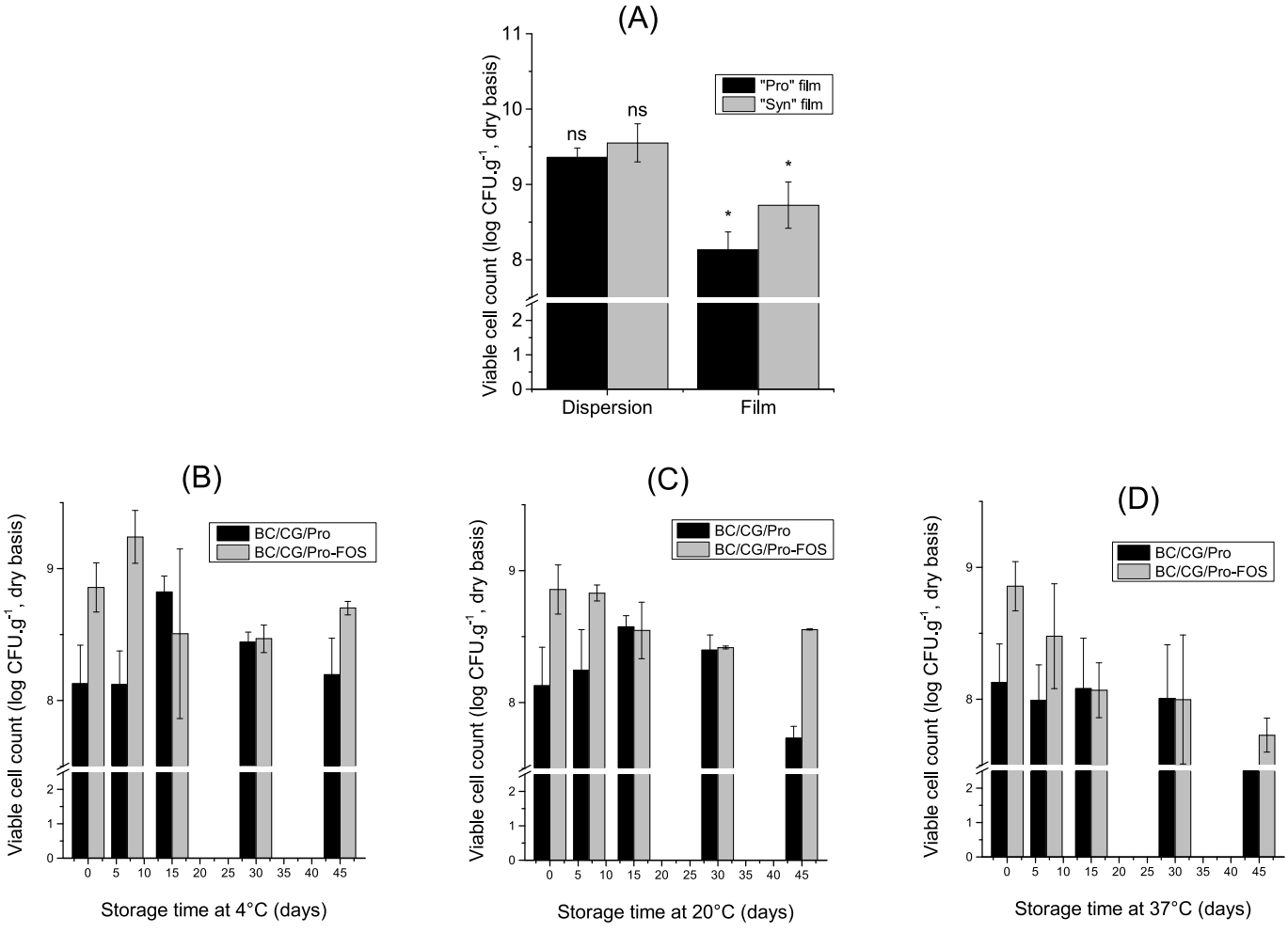

Fig. 4. Viable cell counts changes in *Pro* and *Syn* films: (A) on processing (^{ns} non-significant differences; * significant differences, $p < 0.05$); (B), (C), and (D), on storage at 4 °C, 20 °C, and 37 °C respectively, with p-values for overall *Pro* x *Syn* differences of 0.18, 0.08, and 0.10 respectively.

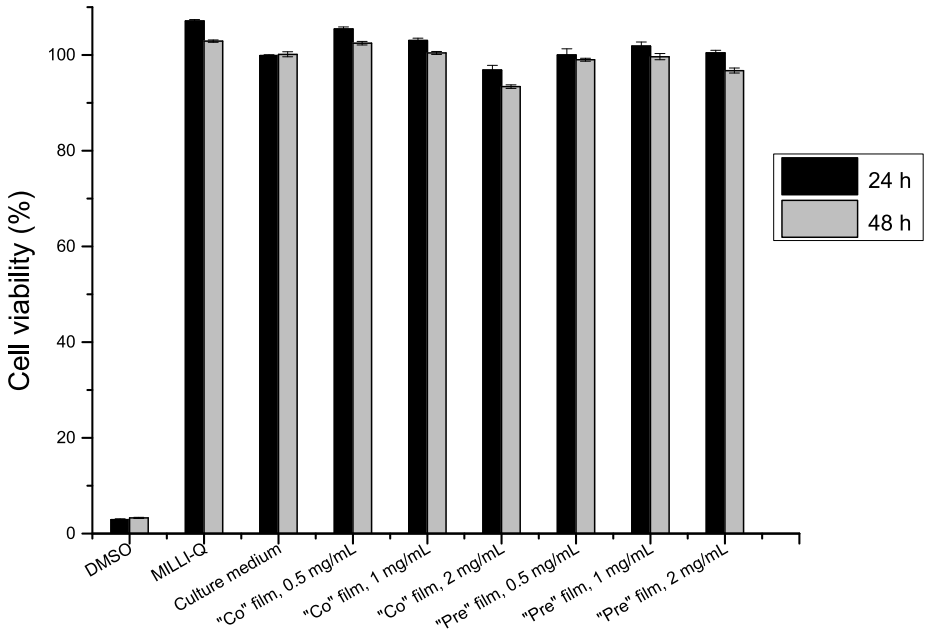

Fig. 5. Viability of Caco-2 cells after incubation (24 and 48 h) with *Co* and *Pre* films at different concentrations. DMSO: cells incubated with 40% of DMSO (positive control). Milli-Q: cells incubated with 20% Milli-Q (negative control).

film dried at a much lower temperature (37 °C, 15 h). Those authors (Soukoulis et al., 2016) reported that the presence of proteins in combination with starch resulted in lower viability losses on drying (0.9–1.1 log CFU.g^{-1}), which was similar to the viability loss of the *Syn* film of this study (0.8 log CFU.g^{-1}), although the drying temperature used in this study was much higher (80 °C, 150 min).

Moreover, the viable cell counts of both films were still higher than 8 log CFU.g^{-1}. Singh et al. (2019) reported that, for carboxymethyl cellulose (CMC)-based films entrapped with *Lactobacillus rhamnosus*, a similar film-forming method (mixing bacteria with film components followed by casting and drying for 50 °C for 15 h) was too harsh for the bacteria, the final viable count being about 3 log CFU/mL^{-1}. Unfortunately, those authors (Singh et al., 2019) did not report the viable count of the film-forming dispersion, so the loss of viability obtained in this study cannot be compared to the one in that study.

3.5. Stability of the probiotic during film storage

The *B. coagulans* counts in both films were quite stable at 4 °C and 20 °C (Fig. 4B and C), demonstrating a probiotic stability much higher than those reported in other studies for films with *Lactobacillus* or *Bifidobacterium* species at similar temperatures, namely, 3–4 log CFU.g^{-1} loss in CMC films within 42 days at 4 °C or 25 °C (Ebrahimi et al., 2018), 1–5 log CFU.g^{-1} loss in starch or starch-protein films within 15–30 days at 4 °C or 25 °C (Soukoulis et al., 2016), and 3–4 log CFU.g^{-1} loss in whey protein isolate or alginate films within 60 days at 23 °C (Pereira et al., 2016, 2019). The viability of *L. plantarum* in an alginate film was stable for 30 days at 4 °C, but suffered a loss of about 3 log CFU.g^{-1} at 25 °C (Shahrampour et al., 2020). Altamirano-Fortoul, Moreno-Terrazas, Quezada-Gallo, and Rosell (2012) reported a poor storage stability of *L. acidophilus* in starch coatings applied to bread (1–2 log CFU/bread decrease in viability after just 24 h at room temperature).

Even at an abusive temperature of 37 °C (Fig. 4D), the cell counts were decreased by only about 1 log CFU.g^{-1} after 45 days of storage, demonstrating that the films proposed in this study may be submitted to harsh temperature conditions while still maintaining a reasonable probiotic stability, which is probably due to the spore-forming ability of *B. coagulans*, although the matrix may have also played a role as a good protecting matrix to the probiotics.

Although prebiotics have been reported to enhance the storage stability of probiotics in films (Pereira et al., 2019; Soukoulis et al., 2014), paired t-tests indicated that the presence of FOS in this study did not significantly enhance the overall storage stability of the probiotic in the films at any of the tested temperatures. Other studies reported that prebiotic oligosaccharides have failed in improving the probiotic survival during storage – for instance, oligofructose in probiotic orange juice (Costa et al., 2017), and inulin in cheese (Nejati, Gheisari, Hosseinzadeh, & Behbod, 2017). In this study, a plausible explanation for the lack of apparent effect of FOS on storage is that the stability of the probiotic in the *Pro* film (without FOS) during storage was already very high, so any FOS effect on that stability was small enough to be undetected by the paired t-tests. Anyway, all films presented viable counts higher than 7 log CFU.g^{-1} after 45 days of storage, even at 37 °C.

3.6. Cytotoxicity of films

One of the basic requirements for a film to be considered edible is the absence of cytotoxicity. The use of novel edible materials (including nanomaterials) raises concerns about eventual negative impacts to human health. So, in order to evaluate any potential cytotoxicity of unusual food components (in this case, NFBC and CG), Caco-2 cells were exposed to those components in the films at different concentrations for 24 or 48 h of incubation, and the cell viability was evaluated through the resazurin assay.

Both films (*Co* and *Pre*) demonstrated cell compatibility, since the Caco-2 cell viability was kept above 90% at the concentrations tested (up to 2 mg/mL) after 48 h of incubation (Fig. 5), whereas 70% of cell viability is considered the threshold for non-toxicity (ISO 10993-1). So, the films and their components did not produce any cytotoxic effect on those cells, corroborating previous results from materials containing NFBC (Lima et al., 2018; Padrão et al., 2016) and CG (Abreu et al., 2016).

4. Conclusions

A bacterial cellulose/cashew gum mixture was used as a polymer matrix for edible films added with probiotic *Bacillus coagulans* and/or

prebiotic fructooligosaccharides (FOS). The films presented good tensile and barrier properties, thanks to the remarkable performance of BC, although the probiotic and FOS have reduced tensile strength and increased permeability. The films exhibited hydrophobic character, except the one added with just FOS. The presence of FOS improved the probiotic viability upon film processing, although not upon storage. The viable cell counts upon storage at 4 °C and 20 °C were quite stable for at least 45 days, and even an abusive temperature of 37 °C resulted in reductions of no more than 1 log cycle. That storage stability was probably favored by the spore-forming ability of *B. coagulans* and the protective role of BC. The films presented viable cell counts higher than 7 log CFU.g^{-1} for at least 45 days of storage. Cytotoxicity assays on Caco-2 cells revealed that the film components did not produce any cytotoxic effects. The films may be applied as coatings or wrappings to a variety of foods, providing them with potential health benefits to the consumers, besides being potentially able to inhibit the growth of spoilage microorganisms on food surface, thus increasing food shelf life.

CRediT authorship contribution statement

Ana Vitória Oliveira-Alcântara: Investigation, Writing - original draft, Visualization. **Ana Angel S. Abreu:** Investigation. **Catarina Gonçalves:** Methodology, Validation, Supervision. **Pablo Fuciños:** Methodology. **Miguel A. Cerqueira:** Investigation, Validation. **Francisco M.P. Gama:** Supervision, Project administration. **Lorenzo M. Pastrana:** Resources, Funding acquisition. **Sueli Rodrigues:** Methodology, Formal analysis. **Henriette M.C. Azeredo:** Conceptualization, Writing - review & editing, Supervision, Project administration.

Declaration of competing interest

The authors have no conflict of interest to declare.

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