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A crayfish chitosan-based bioactive film to treat vaginal infections: A sustainable approach

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ABSTRACT

Polymicrobial communities are seen to be a sign of health, but they can turn detrimental when an excess of pathogenic species leads to recurring vaginal infections. This microbiological imbalance may decrease women's fertility, increasing also the risk of infection by Human Papillomavirus (HPV) and/or other sexually transmitted infections (STIs). There is a worldwide need for smart/sustainable solutions to tackle these types of infections. Hereupon, we investigated, as a potential solution, the use of crayfish chitosan-based membrane as a mucoadhesive, antimicrobial, biocompatible and biodegradable material. Chitosan was chemically extracted with a process yield of ca. 63 % and a degree of deacetylation of ca. 65 %. Further chitosan was characterized by FTIR, DSC, XRD and zeta potential. Antimicrobial and antioxidant activities were tested by microbicide concentration and ABTS methods. The extracted chitosan was confirmed to be antioxidant and antimicrobial against *Escherichia coli*, *Candida albicans*, *Staphylococcus aureus* (methicillin resistant and susceptible strains). Vaginal films using chitosan extracted from crayfish shells were produced by solvent casting, and the biological profile was tested in simulated vaginal fluid as a proof of concept. The main data showed that the vaginal films prepared were active against several microorganisms responsible for vaginal infections, demonstrating their potential in the field.

1. Introduction

Science responds to the needs of the community, not only in terms of advanced knowledge and technology, but in the development of goods, materials and services that better adjust to social trends. Health is a major area of study, in which microorganisms play a key-role, at its best or worst impact. Due to the emergence of new pathogens and their increased antibiotic resistance, there is the need for new and improved treatment methods. [1] Bacterial, yeast and viral infections are prevalent in various environments, with hospitals being a notable hotspot due to the diversity of pathogens. [2] In this context, infections caused by the growth of Gram-positive bacteria such as *Staphylococcus aureus* (e.g., methicillin-susceptible (MSSA), methicillin-resistant (MRSA)) and Gram-negative bacteria such as *Escherichia coli*, and fungi like *Candida albicans* are common and can be worrying. [3] Vaginal infections are caused by some pathogenic microorganisms overgrowth, primarily affecting health and woman fertility [3]. Bacterial vaginosis is a common type of infection resulting from uncontrolled growth of anaerobic bacteria in the vaginal microbiota. The uncontrolled functioning of the vaginal microbiota can also activate *C. albicans*, resulting in vulvovaginal candidiasis or aerobic vaginitis, characterized by an abnormal vaginal microbiota containing aerobic pathogens. [2] These infections present a challenge for effective local treatment, being the development of effective and tailored-made drug delivery systems necessary. Among those, pills, creams, gels, gel capsule and plasters are used. Vaginal films are solid delivery systems that quickly disintegrate in vaginal secretions and are not likely to cause leakage or other messiness. [4] Usually, the polymers chosen to formulate the film are based in polyacrylates, polyethylene glycol, polyvinyl alcohol or even cellulose derivates. However, there is a need for more sustainable solutions that can effectively fight infections with a low environmental footprint. One approach involves replacing conventional polymers with biodegradable polymers obtained from natural sources used to prepare bio-based films characterized by being thin, soft, and flexible. [4] In the past, there were

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formulations of films intended for vaginal use that included pectin from plants, especially fruits, or tragacanth gum obtained from the dried sap of several species of Middle Eastern legumes, [5] while others used chitosan, a chitin derived.

Chitin is a polysaccharide widely recognized as the second most abundant natural polymer, acting as a support material in the exoskeleton of arthropods and in the cell wall of fungi. Composed of a linear chain of *N*-acetylated glucosamine (GlcNAc) and glucosamine (GlcN) units, chitin is stabilized by β (1–4) glycosidic bonds. [6] The process of chitin extraction can involve both chemical and biological approaches, and typically includes three main steps: deproteinization, depigmentation, and demineralization. [7,8] These steps are crucial as the quality and quantity of chitin extracted depend greatly on the source material. [6] Due to its unique properties such as biodegradability, biocompatibility, and non-toxicity, chitin has versatile applications in diverse fields ranging from the polymer industry to agriculture and biomedicine. [6,7,9]

Chitosan, as the most common chitin derivative, is obtained from an alkaline deacetylation. It is a natural linear bio-polyaminosaccharide which, when in solid state, presents a semicrystalline structure, insoluble in water and organic solvents. [10,11] Nevertheless, it can be dissolved in dilute aqueous acidic medium. On the other hand, it is recognized by its physical-chemical and biological properties, such as non-toxicity, biodegradability, mucoadhesiveness, hemocompatibility, antioxidant and antimicrobial and anti-inflammatory activities. [12] It has been used in various formulations and materials, including solutions, suspensions, particles, nanoparticles, sponges, gels/hydrogels, membranes, and films, due to its physical-chemical and biological properties. [13]–[15] It is particularly advantageous for biomedical and pharmaceutical applications due to its biological properties, combined with in-situ gelling ability and controlled drug release in advanced systems. [12,13] This makes it a versatile and potential polymer option for the treatment of vaginal infections. [4]

Given the versatility of chitosan's applications, alternative sustainable sources for extracting the biopolymer with different physicochemical characteristics and biological properties have been searched for. One of these sources is crustaceans, such as shrimp, lobster, and crab. The red swamp crayfish is beginning to gain interest, as it is proving to be a very promising source that provides chitosan with exceptional properties, that is abundant as well. On the one hand, red swamp crayfish carapaces constitute an abundant residue from the food and food-processing industry enlarging landfilling routes. On the other hand, the need to mechanically remove this invasive species from affected ecosystems in non-native distribution ranges provides an additional generation of large biomass amounts that often have no other fate than landfilling, thus contributing to negative environmental impact.

This work describes the extraction and characterization of chitin from crayfish shells, its reaction to chitosan and the development of a bioactive film using the extracted biopolymer, chitosan, to combat vaginal infections. Here, a sustainable method for producing vaginal bio-based films using chitosan obtained from crayfish chitin was studied in vitro with simulated vaginal fluid, as a proof of concept. The results showed the potential of this chitosan-based film as a new sustainable vaginal sustained drug delivery system.

2. Materials and methods

2.1. Biomass and reagents

The raw material used was the crayfish (*Procambarus clarkii*), caught in different zones of Portugal. The biomass came from both male and female individuals, mainly young adults. The shells were cleaned, dried, and grounded before use. [16] All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless mentioned otherwise.

2.2. Experimental procedure

2.2.1. Chitin extraction

The chitin extraction procedure used in this work was an adaptation of a methodology described in literature. [17] Briefly, the frozen biomass of crayfish was weighed to calculate the required volume of each solution needed. After its optimization (unpublished data), in this work a ratio of 20 g of biomass *per* 646 mL of solvent was adopted, which represents a solid-liquid ratio of 0.31. [17] Simultaneously, the solutions of HCl, NaOH and NaClO were placed in a water bath at 100 °C. The first step of the process was performed between the biomass and the acetic acid glacial, occurring at room temperature for 10 min with agitation. After occurring the extraction, the solution was decanted, and the extract obtained was retained for further use. This extract obtained contained chitin contaminated with astaxanthin, a red-coloured pigment present in the crayfish shells. The sample was washed twice under agitation for 5 min with distilled water to remove the acetic acid residues. Then, a 3 % (*v*/v), NaClO solution was added at 100 °C to the sample after washing and put to react for 10 min under controlled temperature and stirring. This step allowed bleaching the sample, consequently permitting to remove most of its reddish-brown content, which was proved by the removal of the sample coloration. Finally, the sample was washed again with distilled water, twice with continuous agitation. To remove calcium carbonate (demineralization process), the sample was reacted with HCl (1 M) at 75 °C for 15 min with agitation, a procedure that ended with several washes with distilled water under stirring. Finally, a 1 M NaOH solution was added at 100 °C for 20 min with agitation to remove the proteins (deproteinization process). The extraction process was concluded with at least two washes to ensure the removal of any remaining reagents. Finally, the solution was decanted and dried in an oven at 70 °C for at least 24 h. [17] Using the parameters, mass of biomass ($m_{Biomass}$) and the extracted chitin (m_{CT}), the yield of extraction was determined using Eq. 1.

$$\%Yield\ (CT) = \frac{m_{CT}}{m_{Biomass}} \times 100 \quad (1)$$

2.2.2. Deacetylation of chitin

The production of chitosan consisted on the chitin deacetylation using a concentrated NaOH solution. [18] Several reaction conditions were tested according to the Table 1.

A NaOH solution was prepared with a ratio of 50 % (w/v) between the mass sample and the volume of solution. The sample was placed in a flask and submerged in an oil bath, under either an oxygen or a nitrogen atmosphere. At the end of the reaction period, the solution was vacuum filtered and rinsed with water until a neutral pH of around 7 was achieved. The sample was then left to dry for 24 h at 50 °C. [18] In the end, the reaction's yield was calculated using the initial mass of chitin (m_{CT}) and the resultant chitosan mass (m_{CS}), as described in Eq. 2.

$$\%Yield\ (CS) = \frac{m_{CS}}{m_{CT}} \times 100 \quad (2)$$

2.3. Characterization of the extracted chitin and chitosan

2.3.1. Percentage of chitin extracted

Gas chromatography (GC) was performed to determine the percentage of chitin extracted preceded by an acidic hydrolysis, followed by the reduction and acetylation of the samples. The first step comprised the dissolution of samples in HCl, 6 M. In a heating block at 100 °C, the samples were left to hydrolyse for 6 and 24 h. [19] Reduction and acetylation steps were initiated by adding 200 μL of an internal standard, the 2-deoxyglucose at 1 $mg.mL^{-1}$. After stirring, 1 mL was transferred to new culture tubes, neutralized with 200 μL of NH_3 25 % (*v*/v), following the same protocols described in literature. [20] The reduction reaction occurred by adding 100 μL of $NaBH_4$ (15 % (*w*/*v*) in NH_3, 3 M), left on a heating block at 30 °C for 1 h, and cooled in an ice bath. With

Table 1
Deacetylation reaction conditions, NaOH concentration, temperature, time, ratio, and atmosphere.

Process	NaOH Concentration [%(w/v)]	Temperature (°C)	Time (h)	Ratio ($w_{chitosan}/V_{NaOH}$)	Atmosphere
1	50	100	2	1:10	O_2
2	50	100	24	1:10	O_2
3	50	110	5	1:15	N_2
4	50	130	5	1:15	N_2
5	50	100 130	5 5	1:15	N_2

the samples cold, 2 × 50 μL of glacial acetic acid was added. Then, 300 μL were transferred to *Souriel* tubes and placed in an ice bath again. After, 450 μL of 1-methylimidazole and 3 mL of acetic anhydride were added, stirred to homogenize, and placed on the heating block at 30 °C. After 30 min, the sample was placed on ice, then 3 mL of distilled water and 2.5 mL of dichloromethane were added, with the sample being shacked and centrifuged for 30 s at 3000 rpm. When phases were separated, the aqueous phase was removed. The addition of distilled water and dichloromethane, centrifugation, and aspiration of the aqueous phase was repeated and followed by a washing step, performed twice, with distilled water only. After washing, the organic phase was transferred to tubes appropriate for *Speedvac* and the dichloromethane was evaporated. [21] At the end, anhydrous acetone was added and evaporated, twice. The last step was a GC analysis using a DB-225 capillary column, and Perkin Elmer-Clarus 400 chromatograph. The operational conditions were an injection rate of 2 μL, injection temperature of 220 °C, temperature of 230 °C, range 1, attenuation 6, and split 10 min. The temperature programs had a total time of 9 min, initial temperature of 200 °C, ramp 1 worked at 40 °C.min^{-1} until 220 °C, time of action 7 min at 220 °C, then starts ramp 2, working at 20 °C.min^{-1} until 230 °C, for 1 min.

2.3.2. Fourier transform infra-red spectroscopy (FTIR)

Fourier Transform Infra-Red Spectroscopy (FTIR) was used to analyse the extracted chitin and chitosan samples. The parameters used were frequency range between 400 and 4000 cm^{-1}, with 256 scans and resolution of 16 cm^{-1}. This allowed the calculation of the degree of acetylation (DA) in the case of chitin (Eq. 3), and the degree of deacetylation (DD) in the case of chitosan (Eq. 4). [16,22]

$$DA = \left(\frac{A_{1655}}{A_{3450}}\right) \times 100 \tag{3}$$

$$DD = 100 - \left(\frac{\frac{A_{1320}}{A_{1420}} - 0.03822}{0.03133}\right) \tag{4}$$

2.3.3. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) was done by using a DSC Hitachi model DSC7000X. The equipment was calibrated with several standards (decane, 4-nitrotoluene, naphthalene, benzoic acid, diphenyl acetic acid, indium, tin, caffeine, lead, zinc, potassium nitrate, water, and anthracene). Both commercial (CS-C) and extracted chitosan (CS-E) were analysed, means of 3 runs were performed. [23]

2.3.4. X-ray powder diffraction (XRD)

X-ray powder diffraction (XRD) analysis was performed on the extracted and commercial chitosan according to the general directions. It used a 2θ angle, between 2.99(9) ° and 80.022°, with generator voltage of 45 V, tube current 40 A, wavelength K-Alpha1 on 1.5405980 and K-Alpha2 on 1.5444260. It was a one reading test. The degree of crystallinity (CD) was calculated by Eq. 5, that relates the intensity of some characteristic angles, corresponding to the crystallinity peaks. Here, the I_{020} means the intensity on first peak, angle 10.7, and the I_{min} corresponds to the minimum value between the two significant peaks. [24]

$$CD = \left(\frac{I_{020} - I_{min}}{I_{020}}\right) \times 100 \tag{5}$$

2.3.5. Zeta-potential

For the zeta-potential analysis, about 10 mg of chitosan, both the commercial and extracted samples, were dissolved in acetic acid, 1 % (*v*/v). Since it is quite viscous, the samples had to be diluted, and then filtered through microfilters. Then, the *Malvern Zetasizer Nano ZSP* equipment was used, putting the sample inside a double cell, and then reading the zeta-potential value.

2.4. Bioactivities evaluation of chitosan

2.4.1. Antimicrobial activity

The microorganisms' consortium tested included Gram-positive such as *S. aureus* (MRSA and MSSA) and Gram-negative bacteria, represented by *E. coli*, and *C. albicans*. The antimicrobial potential was analysed for CS-C and CS-E in duplicate.

The microorganisms' growth was performed in Muller-Hinton agar, with an incubation period of 24 h, at 37 °C. Meanwhile, the chitosan solutions (20 mg.mL^{-1}) were prepared in acetic acid at 1 % (*v*/v) and sterilized in the autoclave before use. After that, antimicrobial evaluation tests were performed, by transferring microorganisms to a sterile saline solution, homogenized in the vortex and finally, performing the turbidity adjustment to 0.5 MacFarland scale (which corresponds to a cell density *at* circa 1.5 × 10^8 CFU.mL^{-1}). In Eppendorf tubes, chitosan solutions in a range of concentrations from 1 to 10 mg.mL^{-1} were prepared in Muller Hinton broth. Then, 2 % of each microbial inoculum was added and incubated at 37 °C for 24 h. The lowest concentration with no growth was considered the minimum inhibitory concentration (MIC). Then, a drop of 20 μL of each chitosan solution with no growth, previously prepared, was transferred to Muller Hilton agar plates. After incubation for 24 h at 37 °C, the results were observed, and the minimum microbicidal concentration (MMC) of chitosan was determined. MBC corresponds to the concentration of solution that kills 99.9 % of the microorganisms. [25]

2.4.2. Antioxidant activity

The antioxidant activity was evaluated using the ABTS [(2,20-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] assay in a 96-well microplate. The sample used consisted of 10 mg of (CS-C) and (CS-E) in 1 mL of 1 % (*v*/v) of acetic acid. Briefly, the production of the ABTS radical cation was made by the reaction of ABTS (7 mM) and 2.45 mM of potassium persulfate, during 16 h at room temperature in the dark. The absorbance of the ABTS solution was adjusted with distilled water until an absorbance of 0.70 ± 0.02 at 734 nm. Then, 20 μL of the sample or Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-caboxylic acid) was pipetted, and 180 μL of ABTS solution was added. After incubation for 5 min at 30 °C, the absorbance was determined at 734 nm using a multi-detection plate reader (*Synergy H1*, Vermont, USA). The percentage of inhibition was then calculated by comparing it with a standard curve of Trolox [25–175 μM]. All determinations were performed in triplicate. [26,27]

2.5. Chitosan-based antimicrobial films production

The antimicrobial films were produced using the solution casting method, the extracted and the commercial chitosan. For each membrane, several formulations were tested. The membranes were created either without or with the addition of glycerol (as plasticizer) at 0 %, 10 %, or 30 % of the mass of the chitosan. [28] The solution was placed under stirring for about 2 h and then deposited in Petri dishes. These were left to dry in an oven at 50 °C for 24 h.

2.6. Degradation test and antimicrobial activity of the films

The films are intended to treat vaginal infections, and therefore it is essential to know if they maintain a viable antimicrobial activity, and how they behave when in contact with a simulated vaginal fluid.

2.6.1. Swelling and degradation test

Swelling and degradation assays were performed according to previous studies. [29,30] After the films being properly dried, they were cut into pieces of 2 × 2 cm, and then weighed individually. Next, films were immersed in 1 mL of simulated vaginal fluid. The simulated vaginal fluid was adapted from Owen & Katz, containing water, 0.5 % (*w/v*) of glucose, 0.351 % (w/v) of NaCl, 0.2 % (w/v) of lactic acid, 0.14 % (w/v) of KOH, 0.1 % (w/v) of acetic acid, 0.04 % (w/v) of urea, 0.022 % (w/v) of $Ca(OH)_2$, 0.016 % (w/v) of glycerine, and concentrated HCl to achieve pH 4.2. [29] They remained immersed for periods of 30 min, 1, 3, 6, and 24 h under agitation at 200 rpm and 37 °C in an orbital shaker. To evaluate the film swelling, the chitosan membranes were weighted after immersion in the simulated vaginal fluid in each timepoint. [29] To evaluate the film degradation, the chitosan membranes were also dried in an oven at 50 °C for 24 h and weighed again.

The swelling test was based on the dry membrane mass before immersion in the fluid (m_i) and the mass measured immediately after immersion (m_f), according to Eq. 6. The calculation of the percentage degradation and the weight loss (WL) required the initial and final drying membrane masses (m_d), Eq. 7.

$$HD = \frac{m_f - m_i}{m_f} \times 100 \quad (6)$$

$$WL = \frac{m_i - m_d}{m_i} \times 100 \quad (7)$$

At each timepoint, simulated vaginal fluid was also collected for further analysis of the antimicrobial activity of the degradation product.

2.6.2. Antimicrobial activity of the chitosan films

The degradation products in the simulated vaginal fluid collected during the different time points (30 min, 1, 3, 6, and 24 h) from the degradation test were used for the evaluation of the antimicrobial activity. For this purpose, pathogenic strains were grown for 24 h at 37 °C (*E. coli*, *C. albicans*, MSSA and MRSA) in Muller Hinton agar and for 48 h at 37 °C for *Lactobacillus acidophilus* in De Man, Rogosa and Sharpe *agar*. The last one was testes because it represents a microorganism from the normal vaginal microbiota. Then, plates of Muller-Hinton agar were seeded with an inoculum of 0.5 McFarland (1.5×10^8 CFU.mL^{-1}) of each microbial culture and then 20 μL of degradation product samples in the vaginal fluid were pipetted. Plates were incubated for 24 h or 48 h (depending on the microorganism) at 37 °C. The presence or absence of translucent halo zones were considered as positive or negative for antimicrobial activity, respectively. A control with the simulated vaginal fluid was made and the assays were performed in duplicate. [21]

2.7. Statistical analysis

All experiments were conducted at least twice, and data were expressed as mean ± standard deviation. Data were analysed using *t*-test at a significance level of 0.05.

3. Results and discussion

This work tackled two main goals: (i) the extraction and characterization of chitin/chitosan from crayfish shells and (ii) the production of bioactive chitosan films against different pathogenic microorganisms with potential to treat vaginal infections.

3.1. From crayfish shells to chitin extraction

For the chitin extraction, the biomass utilized corresponded to young adults. The method of extraction used in this work was an adaptation of the process proposed by Kaya and collaborators, in which the main steps were kept, but a depigmentation step with acetic acid was added. [17] The samples showed a composition of ca. 60 ± 2 % of chitin. Fig. 1 presents the FTIR spectra of the extracted chitin and of a commercial chitin, where for the commercial chitin the spectrum was obtained from literature. [16] As it can be seen, the three amide bands at 1656, 1555, and 1311 cm^{-1}, which correspond to the amide *I* stretching of CO, the amide *II* of NH, and the amide *III* of CN, respectively were observed in the FTIR spectrum of extracted chitin. Furthermore, it is also concluded that, in general terms, the chemical structure of the chitin extracted is similar to that of a commercial chitin reported on literature. [16]

Also, from the results obtained in FTIR, it is possible to calculate the DA of our sample. It is well known that, for various chitin sources, the DA may differ, which may has an impact on its characteristics. The DA of the chitin extracted in the present work was calculated and a value of 97.6 ± 0.2 % was reached, when a commercial chitin generally has a DA above 95 % according to literature. [16,31]

3.2. Deacetylation of chitin and chitosan characterization

After extracting the chitin from crayfish shells, the respective deacetylation reaction was carried out to obtain chitosan by a heterogeneous reaction at different temperatures (100–130 °C; Table 1). After the reaction and with the sample properly dried, the chitosan obtained was analysed by FTIR to verify the viability of its chemical structure. At temperatures of 100 °C for a short time (2 or 5 h), the deacetylation reaction wasn't occurring. Thus, it was necessary to optimize the deacetylation parameters by varying the temperature, time, alkaline solution, and the ratio between chitin mass and volume of NaOH solution. Meanwhile, the most suitable deacetylation was the heterogeneous reaction at 100 °C (Process 5), described by a first reaction of chitin with a NaOH solution, for 5 h. Then, a second stage was completed by another thermal reaction at 130 °C for about 5 h. After optimization, the ratio of mass of chitin *per* volume of NaOH solution was 1:15. The conditions of extraction utilized in this work were similar to others already described for other marine sources, namely shrimp and lobster. [18] In these works, the optimum temperatures were found to be around 100 and 140 °C, however, for longer times and with a high variability in terms of chitin ratio (*w/v*). Then, the CS-E was characterized, and the data compared with the results obtained for a CS-C sample. For a further characterization, FTIR, XRD, DSC and Zeta-Potential analysis were carried out.

3.3. Chitosan physico-chemical characterization

3.3.1. FTIR analysis

Fig. 2 shows the FTIR spectra of commercial chitin, commercial chitosan, and chitosan obtained from the chitin extracted from the crayfish shells. According to previous studies, chitosan exhibits its unique absorption bands at around 3450 cm^{-1} (O—H stretching), 1870–2880 cm^{-1} (CH stretching), 1656 cm^{-1} (Amide *I*), 1580 cm^{-1} (-NH_2 bending), 1550 cm^{-1} corresponds to N—H bending of amide II and 1320 cm^{-1} (Amide *III*). [32] The CH_2 bending and CH_3 symmetrical

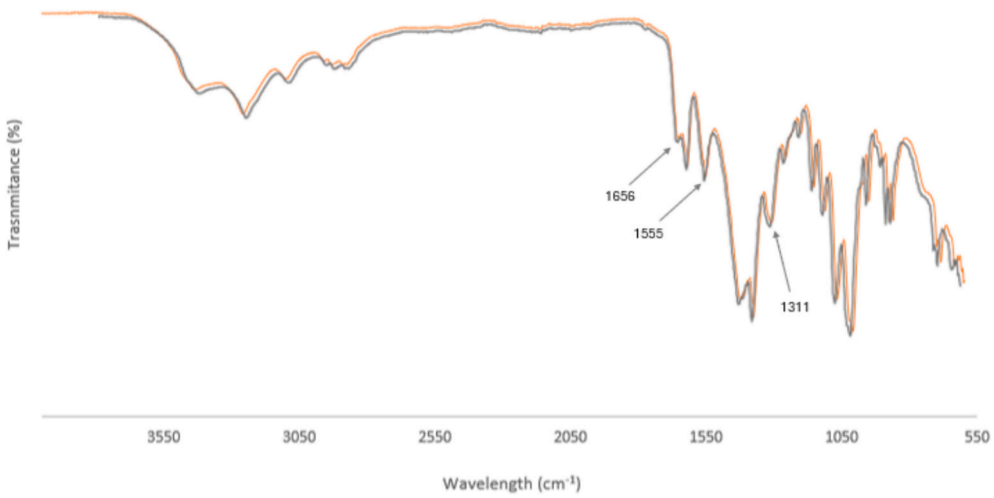

Fig. 1. FTIR spectra of extracted (—) and commercial (—) chitin. The spectrum representing the commercial chitin was obtained in literature. [16].

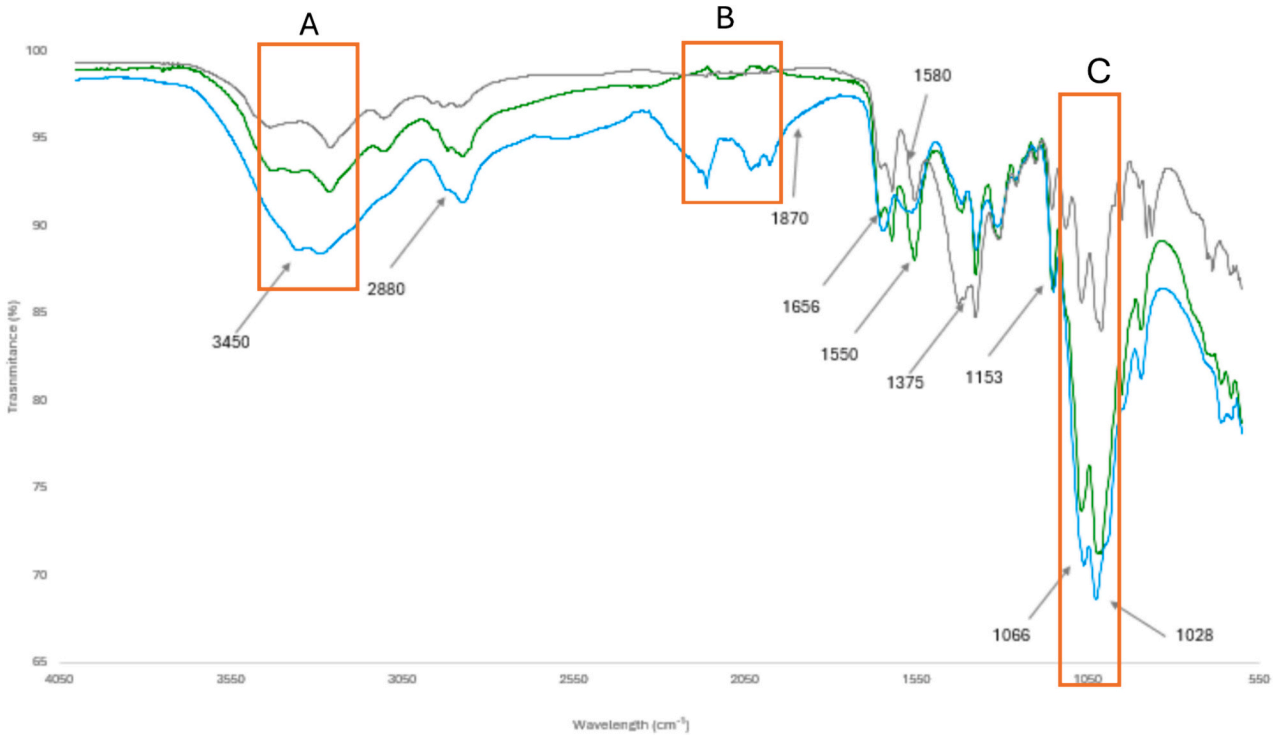

Fig. 2. FTIR spectrum of commercial chitin (—), CS-C (—), and CS-E (—).

deformations were confirmed by the presence of bands at around 1423 and 1375 cm^{-1}, respectively. The absorption band at 1153 cm^{-1} can be attributed to asymmetric stretching of the C-O-C bridge. The bands at 1066 and 1028 cm^{-1} correspond to C—O stretching. [33] These are absorption bands also appearing in our data, thus in agreement with the data from literature, with the groups Amide *I* and *III* expressed at wavelengths around 1650–1300 cm^{-1}, and -NH_2 bending at 1570 cm^{-1}.

Nevertheless, there are some differences between both spectra which may be the result of the incomplete deacetylation reaction, but also potentially attributed to variations in sample preparation. Although we have ensured consistent preparation methods for each sample, the samples were not weighted, which may have some impact on the spectra obtained. Nevertheless, meticulous care was taken to ensure that each sample covered the detection point of the equipment uniformly and maintained a consistent thickness. This procedure minimized any potential discrepancies between the samples, but do not guarantee that these differences did not significantly affect the readings. Also, the lack of a perfect match between the different samples analysed may be due to the use of chitosan from different sources; the commercial sample was extracted from shrimp, while the samples obtained in this work were extracted from crayfish.

It can be observed that in the region between 3428 and 3258 cm^{-1} (rectangle A), there was no significant changes when comparing the spectrum of chitin and chitosan, indicating that deacetylation was not complete. In the same line of thought it is possible to identify some regions in which the difference between chitin and chitosan is very significant, indicating some degree of deacetylation (e.g the region enclosed in rectangle B). Indeed, in this study, CS-E and CS-C showed DD values of 64.5 ± 0.2 % and 69.70 ± 0.01 %, ($p > 0.05$), respectively. These values agree with some of the data previously reported in

literature, which reported values for DD between 29 % to 90 %. [34]–[36] However, despite small shifts and changes in peak intensity, there are regions (rectangle C) of the chitin and chitosan spectrum obtained from the deacetylation of extracted chitin that show alterations, proving the presence of chitosan from the spectra.

3.3.2. DSC analysis

The effect of temperature on chitosan was evaluated by DSC analysis. This evaluation helps to determine, not only the melting point, but also the characteristic endothermic and exothermic peaks of the sample. The exothermic peak indicates the beginning of the degradation of the compound, and may coincide with the melting point, which in this case was around 250 °C - 300 °C. The endothermic peak represents the evaporation of solvents that were not extracted in the drying process. CS-C showed an endothermic melting transition on the heating ramp, around 125.3 ± 8.9 °C, superior to what was formerly reported to crustaceans, which is around 100 °C. [37,38] This indication may also result from the loss of some residual water present in the sample through evaporation, which is also plausible considering the way chitin was obtained (extraction with aqueous solutions and sample washed with water several times) and chitosan was formed. Also, it seems that a small deviation of the endothermic peak to higher temperatures is observed when compared with the commercial sample, which may also be related with the presence of some residual NaOH used in the deacetylation procedure, a deviation already reported in literature to other samples (e.g. starch). [39] In contrast, no exothermic crystallization transition was observed during the cooling step. It does, however, exhibit some small peaks, which may indicate that due to the complexity and size of the compound, more time will be required to reach the solid state. Crystallization, exhibited in the form of an exothermic temperature peak, should not be obtained in a "one-step" analysis. The CS-E resulted in a similar behaviour showing the endothermic peak at 116.4 ± 4.7 °C. It described a less abrupt behaviour; however, no recrystallization was observed. Fig. 3 shows the endothermic peaks of CS-C and CS-E in a comparative way, which were statistically similar ($p = 0.39$). The statistical analysis was conducted using the *T*-Test feature available in Microsoft Excel, where the sample controller and the extract were compared. Significance was determined when the *p*-value exceeding 0.05, indicating a substantial similarity.

3.3.3. XRD analysis

The crystal structure of chitosan was evaluated through XRD analysis. Chitosan is characterized as a semi-crystalline solid, with amorphous and crystalline zones. The peaks represent crystalline zones, and the intermediate values correspond to the amorphous zones. Chitosan exhibited two peaks with angles around $2\theta = 10$ and $2\theta = 20$. [40] Fig. 4 represents the XRD spectrum of CS-C and CS-E, in which the two characteristic peaks are depicted for both. To CS-E $2\theta = 10.21$ and $2\theta = 20.06$, to CS-C $2\theta = 10.07$ and $2\theta = 20.10$. It indicates that CS-E has crystalline structures of types I and II. [41]

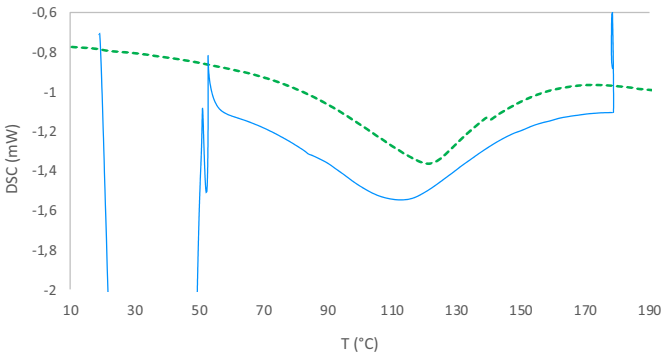

Fig. 3. DSC spectra of commercial (−) and extracted (−) chitosan samples.

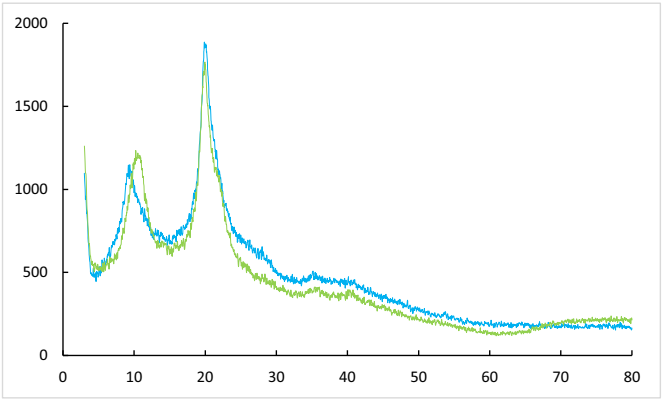

Fig. 4. XRD spectra of commercial (−) and extracted (−) chitosan samples.

In terms of the degree of crystallization, several works reported values around 23.6 % and 59.9 %. [23,42,43] In this study, the results obtained for the CS-C were around 41 % and for the CS-E around 53 %, which means that the CS-E has a higher degree of crystallization, which can result in improved mechanical properties, such as increased stiffness and strength, as well as enhanced barrier properties.

3.3.4. Zeta-potential analysis

The zeta-potential was determined to allow the evaluation of the surface charge of the material (Fig. 5), with influence on the chitosan stability. A particle is considered electrostatically stable if it has a zeta potential >30 mV or above −30 mV. [44]

In this study, the zeta potential values of CS-C and CS-E were, respectively, 66 ± 3 mV and 47.6 ± 0.3 mV ($p = 0.01$), these values being higher than the ones previously reported in literature of 39 ± 3 mV or 34 ± 5 mV. [45,46] An higher zeta-potential of CS-C suggests a better promotion of stronger electrostatic forces with molecules like proteins, DNA, or bacteria, or surfaces negatively charged.

3.4. Chitosan biological profile

The antimicrobial activity of crayfish shell chitosan was tested for a broad spectrum of microorganisms representative of Gram-negative - *E. coli,* Gram-positive - MSSA and MRSA, and yeast - *C. albicans*. Table 2 summarizes the minimum microbicidal concentration (MMC) values obtained for CS-E and for CS-C.

The results suggest that CS-E is more efficient as an antimicrobial agent because it has a lower MMC value against *E. coli*, *C. albicans*, and MSSA. In the case of MRSA, the MMC is the same for both samples of chitosan, 10 $mg.mL^{-1}$. In general, the MMC values are in agreement with literature, where higher efficiency against Gram-negative than Gram-positive was reported. [47] Different factors may affect the effectiveness of chitosan as an antimicrobial compound, which justifies the MMC values variation. These factors directly impact the chitosan' mechanisms of action against the microorganisms, being the most prevalent related with the binding of chitosan to the negatively charged cell walls causing disruption of the cell and consequently altering the membrane permeability. [48] Some authors reported activity against *E. coli* and *S. aureus* at 5 and 1.25 $mg.mL^{-1}$, [49,50] and for *C. albicans* at values between 0.2 and 0.8 $mg.mL^{-1}$. [51] However, the high MMC observed for MRSA compared to MSSA is justified by intrinsic multi-resistance of these *S. aureus*.

Antioxidant activity is studied worldwide because antioxidant compounds protect the body from free radical damage, which is a major factor in the development of many chronic illnesses. [52] It is well known that chitosan has some inherent antioxidant activity because the interaction of free radicals with its amino groups resulting in the production of stable molecules. The antioxidant activity (Table 3)

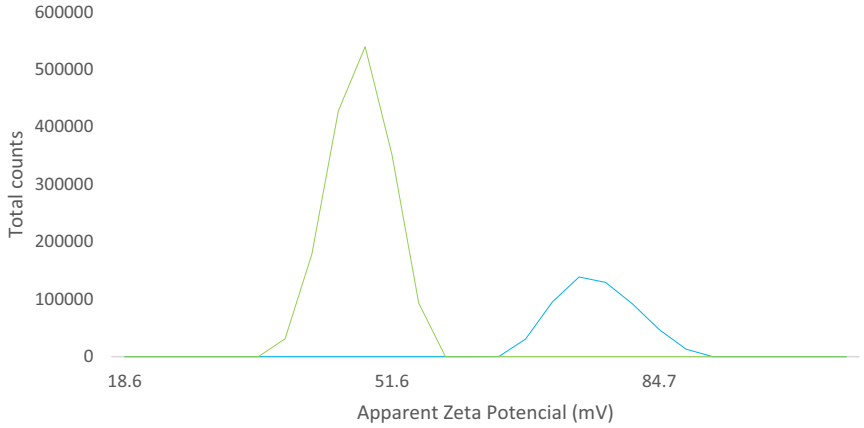

Fig. 5. Apparent zeta-potential of commercial (−) and extracted (−) chitosan.

Table 2
Results of the antimicrobial activity (MMC values) of both the extracted (CS-E) and commercial (CS-C) chitosan samples in the range of tested concentrations (0.25 to 10 mg.mL^{-1}).

	MMC (mg.mL^{-1})			
Type of chitosan	*E. coli*	MSSA	MRSA	*C. albicans*
CS-E	1	2	10	4
CS-C	8	8	10	4

Table 3
Results of antioxidant activity by ABTS assay (μmol Trolox equivalent/g chitosan) and % RSA (radical scavenging activity).

Chitosan samples	μmol Trolox equivalent/g chitosan	% RSA
CS-E	180.6 ± 3.3	27.8 ± 1.4
CS-C	159.0 ± 12.4	23.8 ± 1.7

determined with ABTS for CS-E was 180.6 ± 3.3 μmol Trolox equivalent *per* g chitosan, whereas the value achieved for the CS-C was 159.0 ± 12.4 μmol Trolox equivalent/g chitosan ($p = 0.04$), which means that the CS-E has an antioxidant activity slightly higher than the control, the commercial. Moreover, the radical scavenging activity of ABTS radicals was 27.8 ± 1.4 % for CS-E and 23.8 ± 1.7 % to CS-C. As it is well recognized, the antioxidant activity variation depends on many factors, where the concentration of chitosan present in the films is one of the most important conditions. The literature contains information on the radical scavenging activity (RSA) of ABTS radicals, with recorded values ranging from 20 % to 35 %. Therefore, the findings of this work are consistent with what has been previously reported, [53,54] even though the chitosan has a different source (crayfish shells). So, CS-E exhibits antimicrobial and antioxidant activities, making it an interesting compound with considerable potential for the development of biomaterials for vaginal infections control.

Research studies have already been done to produce hydrogels with chitosan, due to its natural tendency to form gels. [55] However, in this work, chitosan from crayfish shells was studied in the preparation of antimicrobial bio-active films aiming to prevent/treat vaginal infections.

3.5. Development and characterization of antimicrobial films to treat/prevent vaginal infections

>70 % of adult women have already had vaginal issues, making vaginal infections extremely common. Furthermore, complicated vaginal infections caused by a variety of pathogens, including fungi and bacteria, are linked to high recurrence, which is frequently caused by the absence of an effective treatment. [3] Chitosan's mucoadhesiveness allows for the extension of the formulation's residence duration at mucosal locations. [3,56] Therefore, chitosan films are formulations with potential to prevent/treat vaginal infections, that can be used as antimicrobial materials. There are already several film production methods, which in common have the dissolution of chitosan in acetic acid, and the addition of a plasticizer, usually glycerol. Glycerol can enhance the free volume and decrease the intermolecular pressure between the polymer chains, improving the flexibility of the film materials. [28,57,58] The formulations described in the previous methodology section were tested, and the best relation between chitosan and glycerol was evaluated. The films without glycerol were quite rigid and the low flexibility could hamper its application as a coating film. Meanwhile, the membranes with 10 % and 30 % (*w*/w, chitosan) of glycerol exhibited transparency with a clear view, flexibility, smoothness and colourlessness (Fig. 6). The surface analysis of the glycerol-plasticized chitosan film at the macroscopic level revealed compact and uniform structures free of any crack or fracture, demonstrating the miscibility of chitosan with glycerol.

The film containing 30 % (w/w, chitosan) of glycerol was selected because it had an adequate balance between stiffness and malleability after hydration, Fig. 6(a), which is an important feature for its subsequent application.

Consequently, all tests were performed with films formulated with 30 % (w/w, chitosan) of glycerol. Since the main objective of this work is to prove the advantages of these films in the prevention/treatment of vaginal infections, it was evaluated the behaviour of the films in contact with the simulated vaginal fluid. This study included the swelling/degradation of the film and the antimicrobial activity against the pathogenic microorganisms common in urogenital infections and also against commensal *Lactobacillus* spp., in normal vaginal microbiota.

The swelling and degradation tests were performed simultaneously. Fig. 7a shows the results of swelling degree for CS-C and CS-E films, while Fig. 7b describes the degradation behaviour of both films during 24 h, when immersed in the simulated vaginal fluid.

The percentage values of swelling after just 30 min in contact with the simulated vaginal fluid are high for both CS-E and CS-C membranes and are maintained generally constant after that through time. This is consistent with the literature, where a swelling constant after 30 min or 1 h has been reported. [59] In the degradation, the CS-C films degraded less than the CS-E films. CS-E membrane has a high weight loss in the first timepoint, 30 min, and a maximum degradation at 24 h representing 50.1 ± 0.4 %, with the degradation on CS-C occurring in a more moderated way. Previously, the degradation of chitosan based films with acidic solutions at pH 4.0 were reported, where a visible degradation occurred around 6 h. [60] On the other hand, at pH 4.5, by

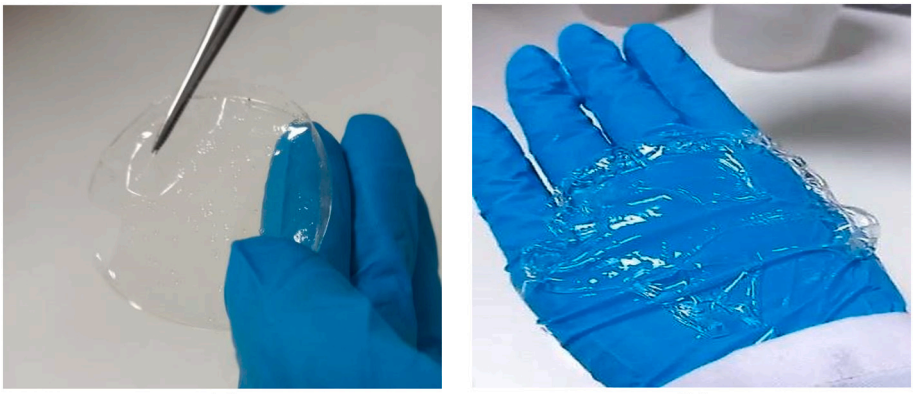

Fig. 6. Chitosan film prepared with 30 % (*w*/w, chitosan) of glycerol: (a) dried; (b) PBS hydrated to test vaginal tract malleability. For a clearer view of the malleability please consult the video in Supporting Information.

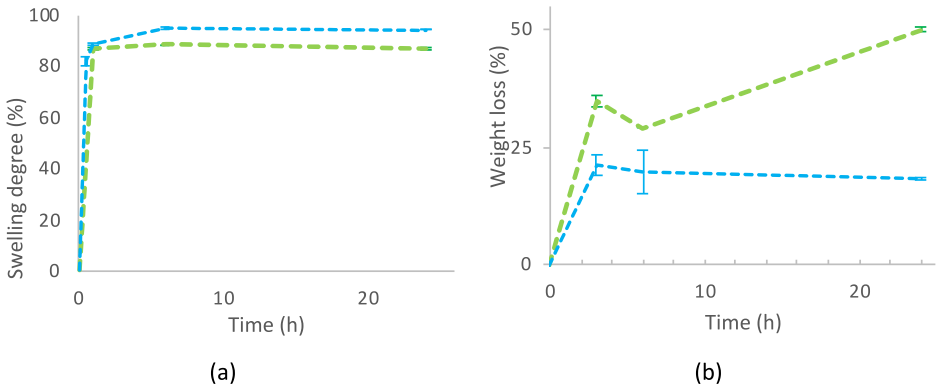

Fig. 7. (a) Swelling degree (%) and (b) degradation behaviour of the chitosan films: CS-E (—) and CS-C (—) after contact with the simulated vaginal fluid.

simulating vaginal fluid, and using films with 3 % (*w*/*v*) chitosan, the degradation was delayed in about 20 % after 24 h. [61] Our films degrade a bit more at 24 h, meaning that there may be a need of adjustment on the formulation according to the application. The antimicrobial activity was evaluated after the degradation of films in the simulated vaginal fluid. The effectiveness of the activity was assessed by determining whether an inhibitory action was noticed throughout various time periods of contact between the film and the simulated vaginal fluid (Table 4).

Chitosan films, when submerged in the simulated vaginal fluid, started to break into its free form inhibiting bacteria and yeast growth. The antimicrobial action of CS-C films begun after 3 h of contact, which corresponds to a film degradation of 21 ± 2 %, while for the CS-E films, microorganisms growth inhibition occurred after 1 h, with a membrane degradation of 45.4 ± 0.3 %. However, both chitosan films showed antimicrobial activity against *Lactobacillus* spp., which is not desirable. Nevertheless, this issue can be overcome by introducing a prebiotic into the film formulation. [62,63] Considering all the evidences, the chitosan extracted from crayfish shells could be a good option to be used as a prophylactic or preventive agent for vaginal infections, either as a gel or a membrane.

Table 4
Results of antimicrobial activity of CS-C and CS-E films after immersion in the simulated vaginal fluid. Antimicrobial activity evaluated by the observation of an inhibitory halo (+), non-observation of the halo (−).

	CS-E				CS-C			
Time (h) Microorganism	1	3	6	24	1	3	6	24
E. coli	+	+	+	+	−	+	+	+
C. albicans	+	+	+	+	−	+	+	+
MSSA	−	+	+	+	−	−	+	+
MRSA	+	+	+	+	−	−	−	+
L. acidophilus	+	+	+	+	+	+	+	+

4. Conclusions

This is aimed at converting unavoidable waste into a valuable resource for our daily life. Crayfish can be found all over the world, and while it is considered an invasive species in some places like Europe, it is a staple food in other regions like USA and China. Although crayfish shell are often considered waste, they have commercial potential. In this study, crayfish waste was used as raw material to extract chitin and transform it into chitosan for the creation of vaginal films. Various tests were conducted to analyse the extracted polymers, including FTIR, DSC, XRD, and Zeta-potential analysis. The results showed that the chitosan extracted had excellent antimicrobial properties against MSSA, MRSA, *E. coli*, and *C. albicans*. Both chitosan samples inhibited the growth of *Lactobacillus* spp., which is a beneficial microorganism to the microbiota. Future studies may include adding prebiotics to the film formulation, testing its degradation over a longer time, and evaluating the behaviour and storage conditions of these films.

CRediT authorship contribution statement

Alexandra Conde: Writing – original draft, Validation, Methodology. **Sandra Borges:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Sara Baptista-Silva:** Writing – original draft, Validation, Supervision, Methodology. **Telma Veloso:**

Writing – original draft, Validation, Methodology. **Joana L. Pereira:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Sónia P.M. Ventura:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Manuela M.E. Pintado:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors do not have any interests to declare.

Data availability

Data will be made available on request.

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