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Exopolysaccharides from *Porphyridium cruentum* as a reinforcing agent for chitosan biopolymer-based film

Nathana L. Cristofoli [a,b,c,*], Alexandre R. Lima [a,d], Sebastião Costa [b], Carlos Manuel Silva [b], João Varela [c,d], Margarida C. Vieira [a,e]

[a] MED–Mediterranean Institute for Agriculture, Environment and Development & CHANGE–Global and Sustainability Institute, Faculty of Science and Technology, Universidade do Algarve, Campus de Gambelas, 8005-310 Faro, Portugal
[b] CICECO–Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[c] CCMAR– Centre of Marine Sciences (CCMAR/CIMAR LA), Campus de Gambelas, Universidade do Algarve, 8005-139 Faro, Portugal
[d] GreenCoLab–Associação Oceano Verde, Campus de Gambelas, Universidade do Algarve, 8005-139 Faro, Portugal
[e] ISE–High Institute of Engineering, Department of Food Engineering, Universidade do Algarve, Campus da Penha, 8005-139 Faro, Portugal

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ABSTRACT

Microalgae exopolysaccharides (EPS), extracted from exhausted culture medium of *Porphyridium cruentum*, a rhodophyte able to grow in marine environments, may be useful as reinforcing agents in chitosan-based films produced by casting, using glycerol as a plasticizer. EPS were added at 1 and 2 % (w/v). The resulting hydrophilic films, EPS1 and EPS2, respectively, were opaquer than the control (CTR) chitosan-based films without EPS, resulting in increased thickness and solubility, and decreased moisture and water contact angle. EPS1 and EPS2 showed 1.5 and 2.0-fold higher tensile strength and a 2.9- and 16.3-fold higher Young's modulus, respectively, compared to CTR. Conversely, elongation decreased by 1.9- and 8.2-fold for EPS1 and EPS2 compared to CTR. EPS1 and EPS2 showed a homogeneous matrix surface upon SEM analysis, presenting some aggregated particles with increasing EPS concentration. FTIR histograms were not influenced by the EPS concentration used. Thermal analysis showed film degradation occurred in three phases, with no significant enhancement in thermal stability. Soil biodegradability confirmed all films degraded within 35 days. This study demonstrated the feasibility of using industrial microalgae byproducts, such as EPS, as film reinforcement agents, primarily enhancing mechanical strength, thus increasing the possibilities of using these raw materials to produce sustainable food films.

1. Introduction

The plastic packaging market from fossil resources has grown immensely due to the versatile properties presented, considerably increasing the amount of waste generated, with environmental, financial, and social impacts [1,2]. However, it is possible to use renewable and biobased polymers (biopolymers) such as polysaccharides and proteins to replace fossil resources and contribute to the development of sustainable packaging, supporting a circular economy [3]. Also, the use of by-products from the food industry has been reported in many studies with the potential to produce bio-based packaging films [4–7].

Films produced from biopolymers are a valuable alternative to common synthetic plastics due to their properties, safety, and biodegradability [8]. Chitosan is a renewable, low-cost raw material, showing promising applications in the food industry, edible films, coating, and composite films. However, it presents the disadvantage of having poor mechanical and physical properties, limiting its application as a film for food packaging. One possible approach to overcome this limitation is through the incorporation of other polymers, as reported by some studies [7,9–11].

Porphyridium cruentum excretes exopolysaccharides (EPS) into its culture medium and beyond being a valuable source of these compounds, EPS is also a byproduct of pigment extraction, often discarded after biomass recovery [12,13]. Although *P. cruentum* may not exhibit the highest EPS yields compared to other microalgal species, it was selected in this essay due to its already established cultivation for the production of high-value pigments, such as phycoerythrin [14,15]. This scenario creates an opportunity to valorise the EPS-rich culture medium,

* Corresponding author at: MED–Mediterranean Institute for Agriculture, Environment and Development & CHANGE–Global and Sustainability Institute, Faculty of Science and Technology, Universidade do Algarve, Campus de Gambelas, 8005-310 Faro, Portugal.
E-mail address: nlcristofoli@ualg.pt (N.L. Cristofoli).

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which is often discarded after pigment recovery contributing to a more sustainable process [16,17]. These polysaccharides are mainly released during the stationary phase of growth for protection against stress and harmful conditions. This rhodophyte growth can be enhanced under favourable environmental conditions (an excess of carbon source and nutrient limitation) [18]. Additionally, EPS has an average molecular weight ranging from 1.40×10^6 g/mol to 2.3×10^6 g/mol [19,20] and is composed of homo- or heteropolysaccharides with atypical sugar monomers (e.g., fucose, fructose, and rhamnose) containing valuable functional groups (e.g., methyls, sulphates and uronic acids) that are responsible for their commercial value [21,22]. EPS are biodegradable, biocompatible, non-toxic, and a functional material. Due to their rheological properties, they have diverse applications serving as thickening, gelling, stabilizing, or emulsifying agents, being also a source of new biomaterials and an alternative to non-degradable plastic, like polyethylene [7,23,24]. Because of the above-mentioned properties, it has been increasingly used in biotechnological, pharmaceutical, and biomedicine fields. Additionally, microalgae-derived EPS may confer improved or novel biological activities to food packaging, used in the formulation of composite materials by improving some polymer properties, such as mechanical and biodegradability qualities [22].

Literature has described EPS characterization, however, its application in food packaging has yet to be fully explored [12,25,26]. This study aims to develop a biodegradable film using EPS from the microalga *Porphyridium cruentum* as a reinforcing agent of chitosan-based films. To investigate their potential as a biopolymer in food packaging applications, the optical, mechanical, and structural properties, thermal stability, and their potential to be biodegradable in the soil of the reinforced films were assessed. Additionally, this study aims to promote the valorisation of EPS as a pigment extraction processes byproduct, contributing to the sustainable use of microalgal resources and advancing the development of sustainable alternatives to conventional plastic packaging.

2. Material and methods

2.1. Materials

Chitosan with a high molecular weight (310,000–375,000 g/mol), and a deacetylation degree of >75 g/kg, was purchased from Sigma Aldrich (Darmstadt, Germany). Glycerol was obtained from Panreac (Barcelona, Spain), and glacial acetic acid from Fisher Scientific (Leics, UK). The EPS used was extracted from *Porphyridium cruentum* as described in section 2.2. The total carbohydrate content was 237.8 mg/L, with sulphated carbohydrates accounting for 102.7 mg/L. The monosaccharide composition of the EPS includes xylose (60.76 %), galactose (20.13 %), glucose (7.79 %), fucose (5.38 %), rhamnose (3.12 %), and mannose (2.82 %). EPS exhibits shear-thinning properties, a characteristic of polymer solutions where viscosity steadily decreases as the shear rate increases. EPS2 demonstrates consistently higher viscosity across all shear rates when compared to EPS1 (Supplementary data Fig. S1). The soil (Eco Grow) was purchased from AKI (Faro, Portugal).

2.2. Exopolysaccharide extraction

The extraction of exopolysaccharides (EPS) from *P. cruentum* exhausted culture medium (ECM) was performed according to the previous study by Cristofoli et al. (2023) [25]. The EPS was first precipitated by adding cold absolute ethanol (1:1, *v*/v), stored at −18 °C (overnight) followed by filtration of the precipitate. Next, the precipitate was dissolved in distilled water and treated with trichloroacetic acid 5 %, homogenized at 37 °C for 40 min, centrifugated at 7871 ×*g* for 5 min to remove the proteins and re-precipitated using ethanol. Then, the precipitate was washed with distilled water and dialyzed against distilled water under mild stirring using a 12–14 kDa cut-off membrane (Medicell) for two days and four water renewals. The EPS was re-precipitated using ethanol, washed, and freeze-dried. The EPS extractions flowchart is represented in Fig. 1.

2.3. Preparation of the films

The films were produced by the solvent-casting evaporation method. The chitosan-based solution was prepared by adding 2 % (w/v) of chitosan in acetic acid aqueous solution 1 % (*v*/v), stirring until complete dissolution in Ultra-Turrax (Ultra Turrax IKA T25, Germany) at 9500 rpm for 5 min. The solution was filtered, glycerol was added at 1 % (v/v) and submitted to an ultrasound bath at 25 °C for 15 min to remove any existing air bubbles. Then, 22 g of the mixed solutions were spread on a polypropylene Petri dish (9 cm diameter) and dried at 25 °C for 48 h. This control film was named CTR.

Reinforced chitosan-based films were prepared with the addition of 1 and 2 % (w/v) EPS. Concentrations above 2 % (w/v) were not explored due to the potential drawbacks of reduced flexibility and enhanced solubility, as noted in similar systems where high filler content compromised film integrity [7]. The EPS was first dissolved in acetic acid solution and the preparation of the films followed the same steps as described above. The samples were named EPS1 and EPS2. The flowchart of film preparation is represented in Fig. 2.

2.4. Characterization of films

2.4.1. Film colour and opacity

Colour measurements and opacity of the films were determined by a spectrophotometer (PCE Instruments, PCE-CSM10), according to the CIE LAB colour system. The parameters L* (light-dark), a* (green-red), and b* (blue-yellow) were used to calculate the total colour difference (ΔE) using the following equation:

$$\Delta E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad (1)$$

in which $\Delta L^* = L_2^* - L_1^*$, $\Delta a^* = a_2^* - a_1^*$, and $\Delta b^* = b_2^* - b_1^*$, presents the differences observed in the colour of the EPS1 and EPS2 films when compared to the control. The ΔE rating was assessed according to Zachary Schuessler's theory [27].

Measurements were carried out in triplicate for each film.

2.4.2. Moisture content, solubility and water contact angle

The film moisture content was determined by drying about 100 mg of the film at 105 °C for 24 h [28]. The weight loss of the films was determined, and moisture content was calculated as follows:

$$\text{Moisture content } (\%) = \frac{(M_i - M_f)}{M_i} \text{ x } 100 \quad (2)$$

Where M_i is the initial mass, and M_f is the dried mass of the films. The analysis was performed in triplicate and expressed in terms of % of dry mass.

To determine the solubility in water, the films were first dried at 105 °C until constant weight. Film disks of 2 cm in diameter were weighed (M_i), immersed in 50 mL of distilled water and gently shaken. After 24 h, the remaining films were filtered and dried to constant

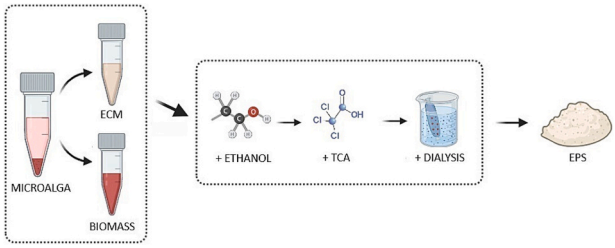

Fig. 1. Flowchart of EPS extraction.

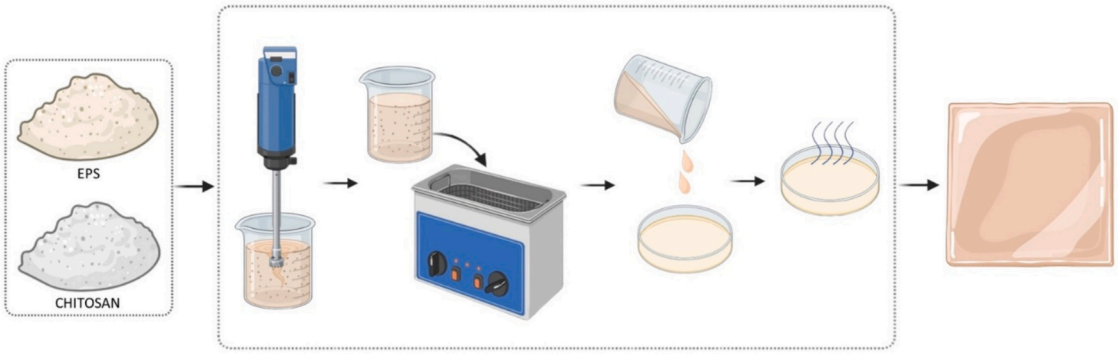

Fig. 2. Flowchart of film preparation.

weight (M_f) in an oven at 105 °C, upon which the results were calculated as follows [29]:

$$\text{Solubility in water (\%)} = \frac{(M_i - M_f)}{M_i} \text{ x } 100 \quad (3)$$

Where M_i is the initial mass, and M_f is the final mass of the sample. The analysis was performed in triplicate.

The water contact angle (WCA) was measured with Attension Theta of Biolin Scientific. The wettability was evaluated from water drop (3 μL) contact angles with their surface. The result is expressed in degrees.

2.4.3. Film thickness and mechanical properties

The thickness was determined by a digital micrometer (MTS Adamel Lhomargy model MI21, France) with a precision of 0.1 μm. The mechanical properties of the films were measured using a CTX texture analyser (Ametek Brookfield, Middleborough, USA) equipped with tensile grips, through a tensile strength, elongation at break, and young's modulus values, according to the ASTM D882 [30] standard method. The measurements were performed in triplicate, with the films cut into strips (10 mm width x 80 mm length) and placed between the grips. The traction speeds were set at 0.5 mm/s. Data processing was through the software Texture Pro V 1.0 Build 19 (AMETEK Brookfield, USA).

2.4.4. Thermal properties

Thermal properties of the films were performed according to ASTM E2550–21 [31] determined by thermogravimetric analysis (TGA, Setsys Evolution 16, Setaram, Lyon, France). About 3–18 mg of films were weighed for each sample, sealed in a platinum pan and heated from 10 to 700 °C at a heating rate of 10 °Cmin^{-1}, under nitrogen atmosphere.

2.4.5. Fourier transform infrared spectroscopy (FTIR)

The Fourier Transform Infrared Spectroscopy (Nicolet iN10, Thermo Fisher Scientific, Waltham, MA, USA) were analysed using attenuated total reflectance mode (ATR). The samples were scanned at room temperature in a spectral range from 400 to 4000 cm^{-1} at 4 cm^{-1} resolution. The results were obtained using the eFTIR software (Essential FTIR®, USA).

2.4.6. Scanning electron microscopy (SEM)

Film surface morphology was analysed by scanning electron microscopy (Hitachi, model SU-70, Japan) with an accelerating voltage of 15 kV. The samples were attached to carbon tape and covered with gold.

2.4.7. Soil biodegradation tests

The soil biodegradation test was performed according to ASTM D5988–12 [32] with some modifications. The films were cut into rectangles (2 cm × 3 cm) and placed inside a perforated polyethylene net (mesh opening 4 mm) and placed in soil (pH: 5.5–6.5; Humidity: 57.7 %; Conductivity: 50–100 μS cm^{-1}; Mineral fertilizer (NPK) = 380 mg/L; Nitrogen: 200 mgL^{-1}; Phosphorus: 200 mgL^{-1}; Potassium and organic matter: >70 %). EPS reinforced chitosan and control films were buried at a depth of 10 cm with 5 cm between each film, in three rectangular vases (60x18x15 cm), filled with soil and watered every seven days with 400 mL of water at 25 °C.

The test was carried out in triplicate for each sample. The aspect was photographed, and the biodegradation percentage was calculated by estimating the remaining film area relative to the initial area. This was done by counting the number of 4 mm × 4 mm mesh squares (from the perforated net) still covered by the film, including partial coverage, and comparing it to the initial coverage.

2.5. Statistical analysis

Statistical analysis was performed using Statistica 7.0 software (Statsoft Inc., USA) with significant differences of variance (ANOVA) and Tukey HSD (honestly significant differences) test at 5 % significance ($p < 0.05$). The experiments were processed in triplicate and results were expressed as mean ± standard error deviation.

3. Results and discussion

3.1. Optical properties

The films' L*, a*, b* parameters, ΔE and opacity, were measured as described in 2.4.1, and are shown in Table 1 and Fig. 3. According to [27], a ΔE <1 reflects that the colour difference is not perceptible by human eyes, while a ΔE between 1 and 2 signifies that the colour difference is perceptible through close observation. A ΔE from 2 to 10 signifies a colour difference perceptible at first glance, values between 11 and 49 reflects colours with slight similarity, and a ΔE exceeding 49 indicates a complete contrast between the colours.

The incorporation of EPS into chitosan-based films significantly influenced their aspect, becoming opaquer. EPS1 and EPS2 showed higher a* and b* values with increasing EPS concentrations when compared with the control chitosan-based films. Therefore, adding 2 % (w/v) of EPS (EPS2) provided films with the highest red-yellowish coloration. The presence of more EPS also changed the opacity and total colour variation of the films, with ΔE values of 2.63 ± 0.69 for EPS1 and 6.43 ± 1.67 for EPS2, indicating that the colour change was perceptible at first sight. Therefore, the EPS concentrations significantly influence the optical properties of the chitosan-based films. This observation aligns with Kim et al. [33], who reported a reduced transparency with increased pullulan content in tapioca starch/pullulan composite films. As noted by the authors, the films's formulation solids content is a major factor affecting their transparency and thickness (results presented in Table 2, section 3.3), suggesting that the increased solids content resulting from EPS incorporation is the principal factor of changes in optical properties.

Table 1
Colour, opacity, and colour variation of the EPS films when compared to the control (CTR) chitosan-based films.

Films	L*	a*	b*	Opacity	ΔE*
CTR	34.76 ± 1.03a	−0.48 ± 0.05a	2.85 ± 0.23a	8.48 ± 0.36a	
EPS1	36.92 ± 1.54a	−0.33 ± 0.15a	3.26 ± 0.06b	11.79 ± 0.23b	2.63 ± 0.69a
EPS2	40.75 ± 1.58b	0.25 ± 0.16b	4.76 ± 0.27c	13.64 ± 0.02b	6.43 ± 1.67b

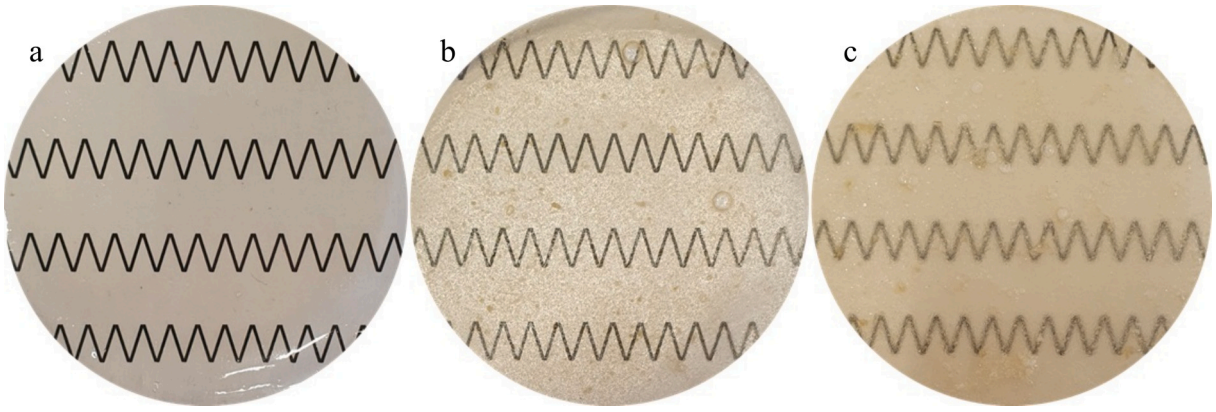

Fig. 3. Visual aspects of (a) chitosan-based film, (b) chitosan-based film + EPS 1 % (w/v), (c) chitosan-based film + EPS 2 % (w/v).

Table 2
Physical and mechanical properties of the films.

Films	Thickness (mm)	Moisture (%)	Solubility (%)	Tensile strength (MPa)	Elongation at break (%)	Young modulus (MPa)
CTR	0.10 ± 0.01a	20.07 ± 0.08a	20.32 ± 0.74a	38.1 ± 4.46a	23.5 ± 2.15a	20.3 ± 2.44a
EPS1	0.16 ± 0.02b	15.52 ± 0.02b	36.59 ± 0.14b	55.5 ± 9.84ab	12.3 ± 1.73b	58.5 ± 18.3a
EPS2	0.25 ± 0.02c	14.43 ± 0.02c	45.36 ± 4.25b	75.5 ± 14.5b	2.87 ± 0.31c	331.3 ± 69.2b

The observed increase in opacity and colour variation with higher EPS concentrations can be attributed to the increased solids content and the intrinsic properties of the EPS. Specifically, the addition of EPS introduces more light-scattering centers within the film matrix due to the presence of polysaccharide aggregates and the heterogeneous distribution of EPS particles, as also observed in the SEM analysis. This results in reduced light transmittance and increased opacity. Furthermore, the natural coloration of the EPS itself contributes to the shift toward red-yellowish hues in the films. Thus, the changes in optical properties are primarily caused by the higher content and distribution of EPS within the chitosan matrix, which affects both the film's structure and its interaction with light.

While transparency is an important factor for the packaging of certain foods, where visual appearance is a crucial indicator of freshness and quality for the consumer, it is less relevant for other products. Furthermore, opacity can be beneficial for protecting light-sensitive foods and increasing their shelf life [34–36]. Therefore, the choice between a transparent or opaque film should be based on the specific needs of the product. In the context of this study, the reduction in transparency caused by the addition of EPS can be offset by improvements in the mechanical and barrier properties of the film (as discussed in section 3.3 and 3.4), making it a promising alternative for sustainable food packaging where protection and durability are prioritized.

3.2. Morphology and FTIR analyses of the films

The microstructure of chitosan-based films and reinforced films observed by SEM are shown in Fig. 4. As shown in Fig. 4a, the control film displayed a smooth surface without agglomeration. The incorporation of EPS provided a surface with some aggregated EPS particles and an irregular distribution of these particles was visible, more noticeable when the EPS concentration in films was increased to 2 % (w/v). Other studies also reported a non-uniform distribution to higher concentrations of the reinforcement agent, which is related to poor dispersion within film matrices [37,38]. In addition, the EPS incorporation provided a matrix surface without any crack or fracture and the interaction with chitosan can be proved by the reduction of contact angle values.

The FTIR spectra of the films are presented in Fig. 5. The main absorption band at 3300 cm^{-1} corresponds to −OH stretching. The split peaks at 2900 and 2800 cm^{-1} are attributed to the C—H stretching vibrations of CH_3 and CH_2 groups when glycerol was added to chitosan also reported by [39]. Signals at 1639 cm^{-1} and 1500 cm^{-1} indicated the presence of amide I (C═O stretching), and amide II (N−H bending and the C−N stretching). The peak at 1407 cm^{-1} corresponds to C−H stretching, while the bands 1376 cm^{-1} and 1319 cm^{-1} indicate a C−H deformation oscillation [40,41].

The intense absorption region between 1180 and 944 cm^{-1} is attributed to C−O−C stretching vibrations related to the glycoside bonds in chitosan arrangement [39,42,43], with the peak around 1033 cm^{-1} for all films. The shoulders at 1150 and 925 cm^{-1} can be assigned to the C−OH side groups and C−C stretching vibrations of the pyranose ring, and the weak peak around 900 indicates low content of nucleic acids in EPS, as reported by [44]. Overall, all the histograms have the typical peaks of chitosan, whereas for films with EPS, a slight modification was noted.

As reported by Wang et al. [44], studying the exopolysaccharides from a thermophilic bacterium *Geobacillus* sp. there is no strong peak found around 1200 cm^{-1} indicating the absence of sulphate groups (S═O or C−O−S). Since the amount of EPS did not exceed 2 wt%, it proved challenging to identify their characteristic signals, which is consistent with previous studies [11]. Therefore, it is possible to confirm that the concentration used in EPS reinforcement does not affect the macroscopic aspects of the films.

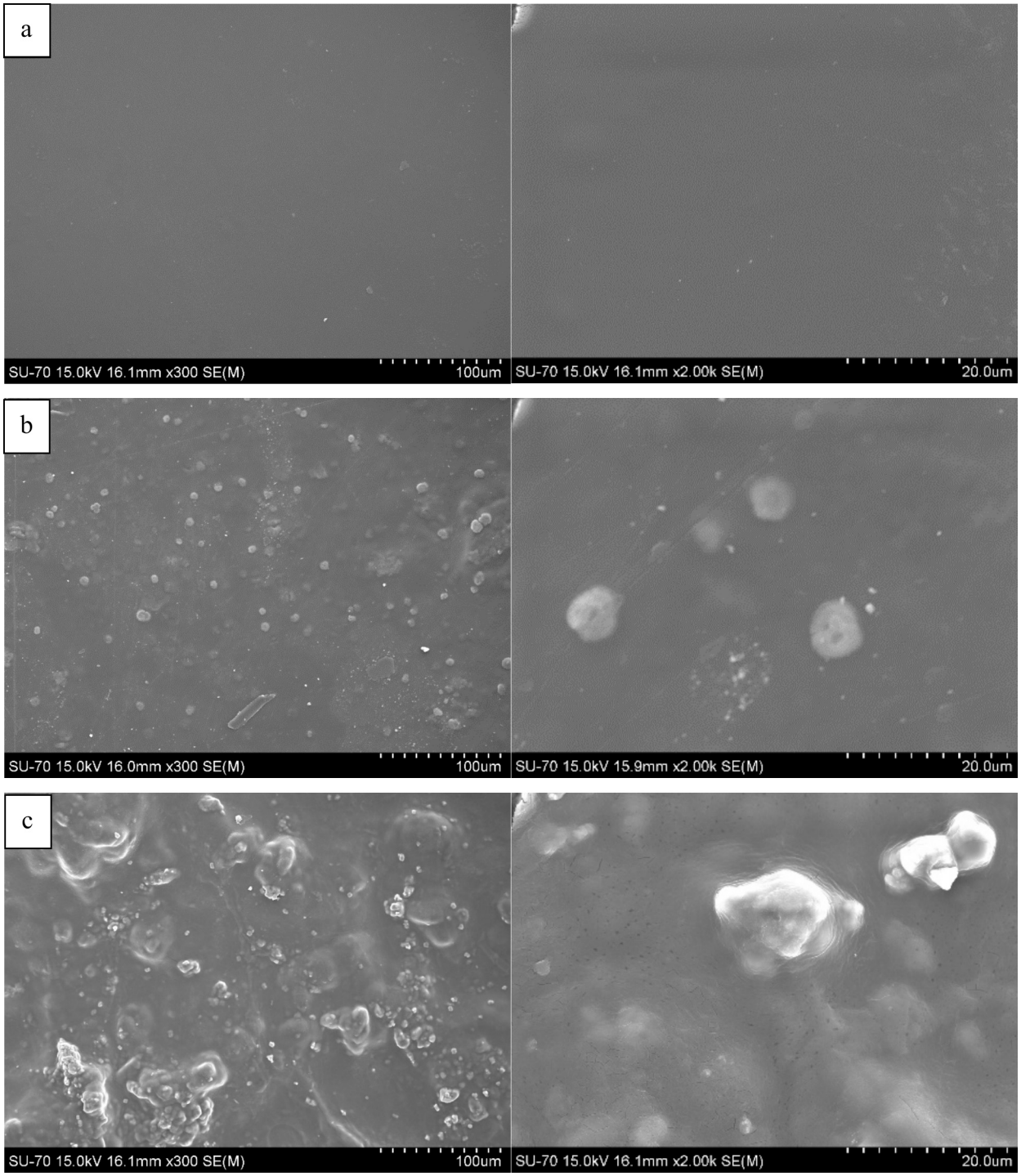

Fig. 4. Surface morphology of (a) chitosan-based film, (b) chitosan-based film + EPS1% (w/v), (c) chitosan-based film + EPS 2 % (w/v).

3.3. Physical and mechanical properties

The thickness of chitosan-based film was 0.10 ± 0.01 mm, while the incorporation of EPS significantly increased the film thickness to 0.16 ± 0.02 and 0.25 ± 0.02 mm (EPS1 and EPS2, respectively). The increase can be attributed to the content of solids in the solution, providing thicker dry films. These values are higher than those reported by Vivek et al. [40] that produced chitosan and exopolysaccharide (from food-grade lactic acid bacterium) composite films with thickness of 0.05 mm. However, this value falls within the range observed in commercial films (up to 0.03 mm) [45]. The water contact angle (WCA) was analysed to characterize the hydrophobicity of the films (Fig. 6). The chitosan-based films showed a WCA of 88.64°, indicating a hydrophilic material, since the value was lower than 90°. The incorporation of EPS decreases the WCA, achieving values of 82.77°, and 77.00° to EPS1 and EPS2, respectively. According to Fig. 6, the EPS addition of 1 and 2 % (w/v) decreases the WCA of approximately 6 and 13 %, respectively, and may be associated with the hydrophilic nature of the microalgae exopolysaccharide. This was due to the presence of polar functional groups, such as hydroxyl and carboxyl groups in polysaccharides, leading to a decrease in the contact angle. Similar results were observed in other study when a polysaccharide-based extract was added to

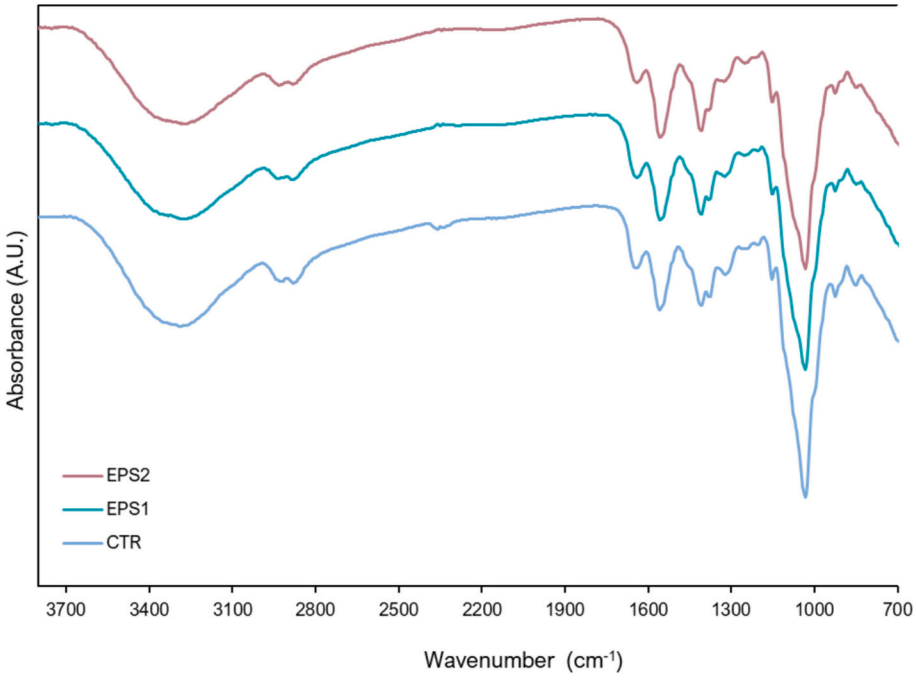

Fig. 5. FTIR spectrum from 400 to 4000 cm^{-1} of chitosan-based films, control (CTR), 1 and 2 % (w/v) of EPS.

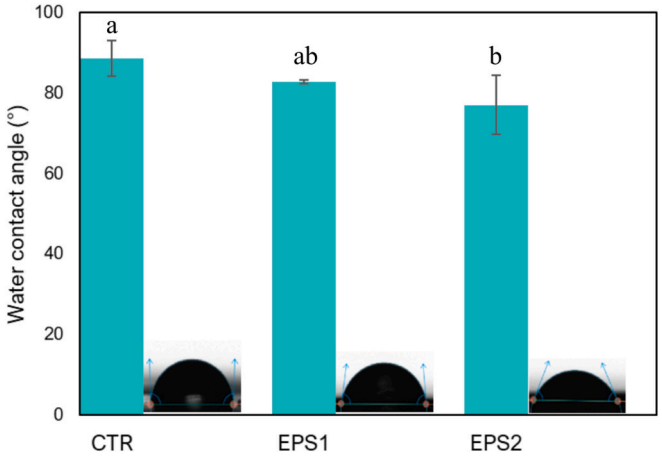

Fig. 6. Water contact angle of chitosan-based films, control (CTR), 1 and 2 % (w/v) of EPS incorporation and representative images of water droplets on the film surfaces used in the measurements are presented.

chitosan [46].

The solubility in aqueous solution of a control film was 20.32 ± 0.74 %, while the incorporation of EPS significantly increased the values to 36.59 ± 0.14 % and 45.36 ± 4.25 % (EPS1 and EPS2, respectively). Vivek et al. [40] reported that the addition of EPS to chitosan increased the film solubility from 19.15 to 51.48 %. Higher values of solubility are related to the hydrophilic groups of EPS that easily interact with water molecules [47]. Since there is no chemical reaction between the polymers, the free hydroxyl groups and amines present in exopolysaccharides are available for hydrogen bonding [40]. Thus, when the material is immersed in water, the polymers absorb the water molecules, resulting in the loosening of a compact polymer matrix, indicating an excellent option as a biodegradable material. These results agree with the solubility analysis, where films with EPS incorporation had a significant increase in solubility when compared with the control film, while reducing the moisture content absorbed in the matrix.

The mechanical properties of the films were conducted to assess the effect of EPS incorporation on tensile strength (TS), elongation at break (EA), and Young's modulus (YM), being presented in Table 2. The tensile strength of chitosan-based control films was 38.1 ± 4.46 MPa. Films with 1 % (w/v) EPS incorporation showed an increase in tensile strength to 55.5 ± 9.84 MPa, though this increase was not statistically significant ($p > 0.05$) compared to the control. However, increasing the EPS concentration to 2 % (w/v) resulted in a significant increase in tensile strength to 75.5 ± 14.5 MPa ($p < 0.05$). All obtained values were in the range of common biological films (10–100 MPa) [48]. These values were in agreement with those reported by Vivek et al. [40] for a chitosan exopolysaccharide film, in which the addition of EPS and plasticizer increased the tensile strength from 31.13 to 43.44 MPa. Another study showed similar values with 41.6 MPa for pure chitosan films and 42.7 and 39.5 MPa when EPS were added [11]. This increase is probably due to the interaction between chitosan and EPS polymers, improving the tensile strength of the films.

Regarding elongation at break, which evaluate the film's ability to stretch before breakage (flexibility), significant differences were found between all samples. CTR films reached 23.5 ± 2.15 %. However, when EPS were added at 2 % (w/v) resulted in a decrease in this parameter, leading to weaker films. The EA of EPS1 (12.3 %) was higher than those obtained by Gongi et al. [10], for a film with 0.9 % of EPS extracted from the microalga *Graesiella* sp. that exhibited EA of 4.98 %, indicating a relative mechanical fragility of EPS-containing films.

Young's modulus is a measure of the stiffness of the film. An increase in this parameter was observed in all samples, in which a significant increase to 331.3 MPa was noticed in EPS2 compared to 20.3 MPa for the control chitosan film without EPS. This behaviour indicates that all the films became more rigid, probably due to the nature of EPS and the strong bonds formed between the polymers. The mechanical reinforcement observed in EPS-containing films is likely due to the presence of sulphate and uronic acid groups in the EPS, which can form ionic and hydrogen bonds with the amino and hydroxyl groups of chitosan. These interactions contribute to a more cohesive and rigid polymer network, as reflected in the increased tensile strength and Young's modulus (Table 2). A similar observation was made by Concórdio-Reis et al. [24] to some of the films based on exopolysaccharides from *Alteromonas* sp., which rigid films were characterized by higher TS, lower EA, and higher

YM. Therefore, the EPS can be considered a good additive as a reinforcing agent to chitosan-based films developing a resistant and flexible film at 1 %, whereas 2 % produces more rigid and less extensible films.

3.4. Thermal stability

The TGA and DTG (first TGA derivative) curves represented the thermogravimetric results of the films (Fig. 7). The weight loss of the films was analysed and could be divided into three major phases: dehydration, degradation, and decomposition. The first weight loss phase started at 30 °C to 136 °C, which can be attributed to the solvent evaporation in the films, probably due to the evaporation of water molecules linked with carboxylic acid groups (COOH) of the EPS [49–51]. The second phase between 137 and 220 °C can be associated with structural breakdown and pyrolysis of saccharide rings causing the depolymerization of EPS [50,52,53], as well as the decomposition of residual glycerol in the films [54]. The third weight loss phase showed at 221 °C to 350 °C corresponds to the major loss, mainly due to the thermal decomposition of chitosan-EPS polymeric organization, breaking the C−O, and C−C bonds, followed by C−O, CO_2, and H_2O evaporation [50,55].

In general, the incorporation of EPS into chitosan-based films did not significantly affect the thermal stability of the films, as evidenced by the TGA results. This is consistent with previous findings by Kaya et al. [56] who reported that the addition of plant extracts to chitosan films did not improve thermal stability. The FTIR spectra (Fig. 5) further support this result, as no new peaks or significant shifts were observed after EPS addition, suggesting that no strong covalent or ionic interactions were formed between chitosan and EPS at the concentrations tested. Thus, EPS appears to act mainly as a physical filler within the polymer matrix rather than as a crosslinking agent, which explains the lack of improvement in thermal stability.

Overall, the results indicate that while EPS incorporation modifies some physical and mechanical properties of chitosan-based films, it does not substantially alter their thermal degradation behaviour. Further studies could explore additional thermal analysis techniques to provide a more comprehensive understanding of the impact of EPS on the films' thermal behaviour.

3.5. Biodegradability tests

The results of biodegradability tests of chitosan-based films are shown in Fig. 8. The modifications in the films, such as the formation of holes and cracks indicate that biodegradation is occurring. After 3 days it is possible to note the films absorbed water from the soil, and from day 14 began to show changes in their structure. The higher degradation rate was noted after 28 days, where the control film degraded at 86.64 %, and EPS1 and EPS2 at 77.28 %. All films can be considered biodegradable for up to 35 days, having >90 % of loss. Thus, this result agrees with the European standard EN 13432 which considers biodegradable packaging when the material is degraded to CO_2, H_2O, and biomass by at least 90 % in 6 months [57]. Additionally, the naturally available microorganisms present in soil are responsible for breakdown the polymers into smaller fragments, in which enzymatic degradation is one of the first responsible for biodegradation [58,59].

The slower degradation rate observed for EPS1 and EPS2 compared to CTR can be attributed to the high molecular weight of EPS, as reported in literature (1.40×10^6 g/mol [19]) and the higher solids content in EPS1 and EPS2. The incorporation of EPS increased the films' hydrophilicity, as shown by the reduced water contact angle and higher solubility compared to pure chitosan films. At the same time, EPS promotes stronger intermolecular interactions, particularly hydrogen bonding within the chitosan matrix, resulting in a denser polymer network [24]. This denser structure may slow water diffusion through the film, while also enhancing the film's mechanical stability. However, these enhanced interactions can also make the material less accessible to microorganisms and their enzymes, thereby reducing the biodegradation rate [60].

These results are consistent with previous studies. For instance, Wrońska et al. [61] reported a total decomposition in soil environment to pure chitosan film after 2 weeks, whereas Nakashima et al. [62] reported a total biodegradability after 1.5 months to chitosan films, with the same behaviour for the outdoor tests. A study of films based on chitosan and chitosan exopolysaccharide composite films shows a biodegradation time of one week when the soil compost was prepared using vermicompost soil and dried biogas digested [40]. In this study, although EPS addition slightly reduced the degradation rate, both CTR and EPS-reinforced films meet biodegradability standards within a practical timeframe for sustainable packaging applications.

4. Conclusion

In this study, chitosan-based films reinforced with EPS extracted from *P. cruentum* were developed and characterized. An increase in the EPS concentrations made the films less transparent and thicker resulting in an improvement of its mechanical properties, with higher tensile strength and stiffness, while reducing the elongation at break. The hydrophilic properties of the chitosan films were also higher, as shown by

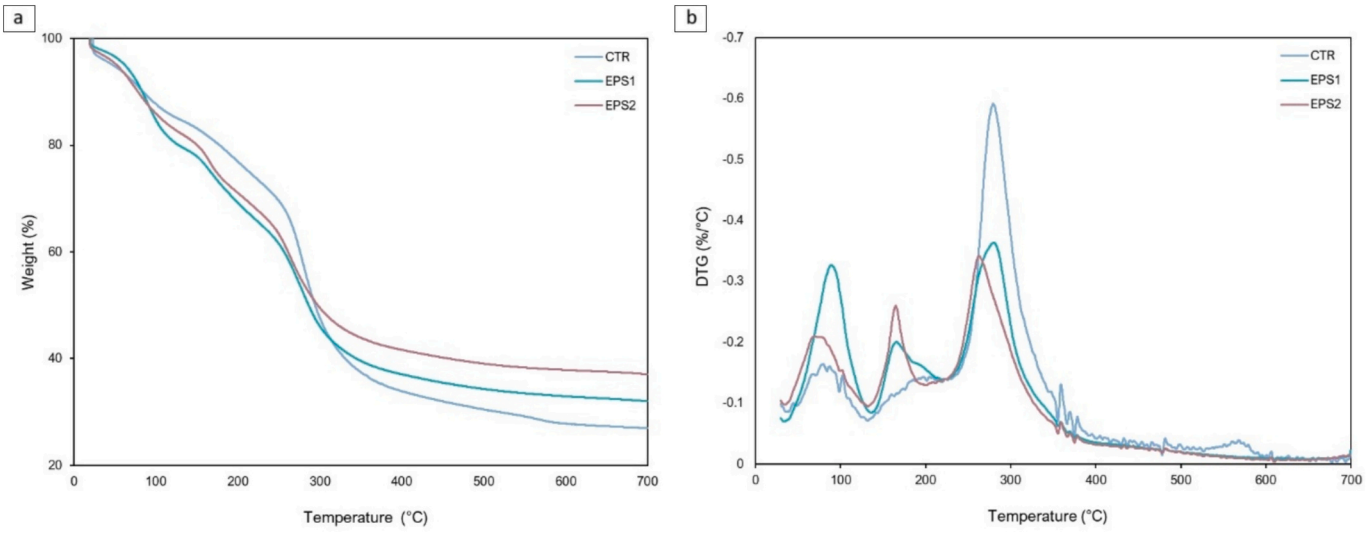

Fig. 7. TGA (a) and (b) DTG of chitosan-based films, control (CTR), 1 and 2 % (w/v) of EPS.

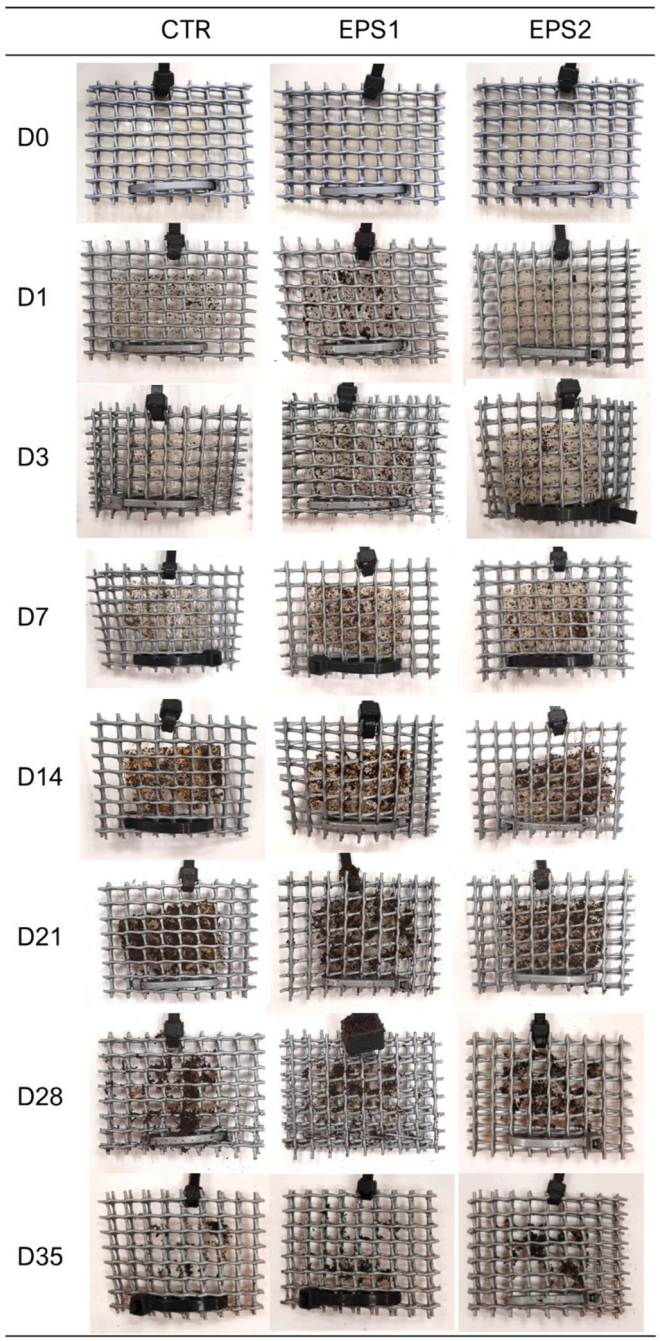

Fig. 8. Soil biodegradation of chitosan-based films, control (CTR), 1 and 2 % (w/v) of EPS. The images show the visual appearance of the films after removal from the soil at different time points.

WCA values, and agreed with the aqueous solubility values. In addition, the structural analyses revealed a homogeneous structure of the films, without cracks, despite rugous surfaces at upon EPS addition. Furthermore, EPS films exhibited a good soil biodegradability rate by fully degrading within 35 days.

The development of films with EPS has the potential for the sustainable production of packaging films with lower environmental impact as this material is generally discarded upon the harvest of microalgal biomass. However, it is important to acknowledge both the strengths and limitations of this approach. The films exhibit enhanced mechanical properties, making them suitable for application where transparency is not a critical factor. Nonetheless, limitations such as reduced flexibility and increased solubility at higher EPS concentrations highlight the need for further optimization. Future research should focus on optimizing EPS recovery on a large scale to make the algae industry more profitable and exploring intermediate EPS concentrations to balance film properties. Overall, this study contributes to ongoing efforts in developing environmentally friendly packaging solutions, offering a promising foundation for future research and development in sustainable food packaging.

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CRediT authorship contribution statement

Nathana L. Cristofoli: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Alexandre R. Lima:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Sebastião Costa:** Methodology, Investigation. **Carlos Manuel Silva:** Writing – review & editing, Supervision, Resources, Funding acquisition. **João Varela:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Margarida C. Vieira:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

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

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