



Hermetia illucens-derived chitosan as a promising sustainable biomaterial for wound healing applications: development of sponge-like scaffolds

Micaela Giani^a, Caterina Valentino^a, Barbara Vigani^a, Marco Ruggeri^a, Anna Guarnieri^b, Rosanna Salvia^{b,c}, Carmen Scieuzo^{b,c}, Patrizia Falabella^{b,c}, Giuseppina Sandri^a, Silvia Rossi^{a,*}

^a Department of Drug Sciences, University of Pavia, Viale Taramelli 12, 27100 Pavia, Italy

^b Department of Basic and Applied Sciences, University of Basilicata, Via dell'Ateneo Lucano, 10, 85100 Potenza, Italy

^c Spin off Xflies s.r.l, University of Basilicata, Via dell'Ateneo Lucano, 10, 85100 Potenza, Italy

ARTICLE INFO

Keywords:

Chitosan

Hermetia illucens

Antioxidant

Wound healing

Freeze-dried scaffolds

Circular economy

ABSTRACT

Chitosan (CS), a biopolymer known for its wound-healing properties, has garnered significant interest in biomedical research. This study explores the potential of two *Hermetia illucens*-derived CS types—unbleached CS and bleached CS—as novel biomaterials for wound-healing applications, in comparison with commercial CS derived from the shells of *Pandalus borealis* (cold-water shrimp). CS was extracted from pupal exuviae, a byproduct of insect farming, which supports a circular economy by converting waste into valuable molecules for biomedical applications. Unbleached CS exhibited viscosity and viscoelastic properties comparable to those of commercial CS. The critical entanglement concentration (CEC) of unbleached CS (1.15 ± 0.05 % w/v) was similar to that of commercial CS (1.18 ± 0.09 % w/v). Moreover, both *Hermetia illucens*-derived CS types (unbleached and bleached) demonstrated a greater capability to enhance fibroblast viability (135 ± 7 % and 123 ± 6 %, respectively) compared to commercial CS (115 ± 7 %). Further investigation revealed that unbleached CS exhibited increased antioxidant activity, reversing >30 % of the loss of viability caused by H_2O_2 treatment, and demonstrated anti-inflammatory properties, decreasing IL-6 levels by 66 ± 2 %. Sponge-like scaffolds based on unbleached CS and commercial CS were prepared via freeze-drying. When comparing structural and functional properties of commercial and unbleached CS scaffolds, the unbleached CS scaffolds exhibited higher porosity (67.9 ± 2.7 %), smaller pore size (216 ± 35 nm), higher swelling ratio (25.4 ± 1.9), greater resistance to degradation, and enhanced fibroblast proliferation. These findings underscore the potential of insect-derived CS as a sustainable and bioactive material for wound healing. However, further research is required to fully understand its interactions and mechanisms in tissue repair.

1. Introduction

Wound healing is a complex biological process involving the coordinated interaction of various cells, growth factors, and extracellular matrix components to restore tissue integrity [1]. Despite advances in medical science, chronic and non-healing wounds, such as diabetic ulcers, venous leg ulcers, and pressure sores, continue to pose significant healthcare challenges. These conditions affect millions of people worldwide and place a considerable economic burden on healthcare systems [1,2]. Conventional wound care methods, including dressings, skin grafts, and topical agents, often fall short in addressing the multifaceted nature of wound healing, particularly in chronic cases where inflammation, oxidative stress, and impaired cell proliferation hinder

effective repair [3].

Recent advances in biomaterials have introduced novel approaches to enhance wound healing by mimicking natural tissue environments, promoting cell viability, and accelerating tissue regeneration. Among these, chitosan (CS) has emerged as a promising biopolymer due to its biocompatibility, biodegradability, and intrinsic wound-healing properties, including antimicrobial, anti-inflammatory, and antioxidant activities [4–8]. CS is a biocompatible, chitin-derived cationic polysaccharide composed of $\beta(1-4)$ -D-glucosamine and N-acetyl-D-glucosamine monomers [9,10]. Traditionally, commercial CS is sourced from marine organisms, such as shrimp and crab shells; however, its reliance on seafood waste presents challenges related to seasonal availability, allergenicity, and sustainability [11–14].

* Corresponding author.

E-mail address: silvia.rossi@unipv.it (S. Rossi).

<https://doi.org/10.1016/j.ijbiomac.2025.140903>

Received 15 October 2024; Received in revised form 29 January 2025; Accepted 9 February 2025

Available online 10 February 2025

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In recent years, insect-derived materials have gained significant attention as sustainable alternatives in biomedical applications [14]. Insects, with their rapid growth rates and prolific reproductive capabilities, provide an abundant and quickly renewable source for CS production [15]. Unlike traditional marine-based sources, insect farming has a lower environmental impact, as it can be conducted in controlled environments with minimal disruption to ecosystems. Furthermore, the reduced allergen potential and minimal presence of heavy metals in insects, as opposed to crustaceans, expand the scope of potential uses for CS [15,16]. With year-round production capabilities, economic viability, and potential scalability, insect-derived CS holds promise as a sustainable and economically competitive raw material for a lot of different industries [17].

The black soldier fly (*Hermetia illucens*, HL), widely used in insect farming [18–21], consumes organic waste from diverse sources, converting it into a nutrient-rich biomass that includes fats, proteins, chitin, and antimicrobial compounds. Among the byproducts of their rearing, pupal exuviae stand out as an exceptional source of chitin, offering a valuable resource to meet the growing market demand for chitin and chitosan. Leveraging this waste stream aligns with the principles of a circular economy by converting agricultural byproducts into high-value resources while reducing environmental impact [22]. Despite its potential, the properties and performance of insect-derived CS in wound healing applications remain underexplored.

The present work aimed to characterize two HL-derived CS, unbleached (HL CS_{UBL}) and bleached (HL CS_{BL}), for their potential application in wound healing in comparison with a commercial CS (CM CS), derived from shells of *Pandalus borealis* (cold water shrimp). A schematic figure summarizing the analysis performed in the present study is reported in Fig. S1 (Supplementary Material). All the samples were characterized for their rheological properties, *in vitro* cytocompatibility, antioxidant, anti-inflammatory activities and wound healing potential. The application of insect-derived CS in biomedical formulations, particularly for wound healing, represents a novel approach in the field. A distinctive aspect of this study is its sustainability focus, utilizing pupal exuviae—a waste byproduct from the insect-rearing process—as the source for CS extraction. Another innovative feature of this research is the comparison between the properties of HL CS_{UBL} and HL CS_{BL} solutions, which highlights how different processing methods, such as bleaching to remove melanin, impact the structural and functional properties of the final material. Such insights are crucial for tailoring the material for specific biomedical applications, offering guidance on processing techniques to optimize performance.

Moreover, sponge-like scaffolds based on HL CS_{UBL} were prepared by freeze-drying and characterized in terms of morphology, hydration, bioadhesion, mechanical properties, *in vitro* degradation and cell proliferation. To investigate their potential in wound healing, CM CS based scaffolds were used as reference. To the best of our knowledge, this is the first time that scaffolds have been designed using CS from this insect source. The fabrication of sponge-like structures highlights the versatility and potential of *H. illucens* CS in creating three-dimensional biomaterials suitable for wound care. This advancement not only expands the scope of insect-derived CS but also opens new avenues for its use in advanced biomedical applications.

2. Materials and methods

2.1. Materials

Commercial chitosan (CM CS), deacetylation grade > 75 %, low Mw (80 kDa–160 kDa) (Sigma-Aldrich, Merck KGaA, Germany), acetic acid and absolute ethanol (Carlo ERBA Reagents S.A.S, Milan, Italy), Dulbecco's modified Eagle (DMEM) medium and DMEM without phenol red (Sigma Aldrich®-Merck, Milan, Italy), foetal bovine serum (FBS, Euroclone, Milan, Italy), antibiotic-antimycotic solution (Merck Life Science S.r.l., Milan, Italy), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide, dimethyl sulfoxide solution (DMSO) and lipopolysaccharide from *Escherichia coli* O111: B4 (LPS) (Sigma-Aldrich®-Merck, Milan, Italy), PBS (Phosphate Buffered Saline; VWR Chemicals, LLC, USA), gelatine (Type A, 175 g Bloom surface, Sigma Aldrich®-Merck, Milan, Italy), Carbopol® 974P NF polymer (Lubrizol, Sao Paulo, SP, Brazil).

2.2. Chitosan production

CS was obtained by heterogeneous deacetylation of bleached and unbleached chitin extracted from HL pupal exuviae as previously described by Triunfo and collaborators [22].

Briefly, pupal exuviae were collected after adult emergence, washed, oven-dried at 60 °C for 24–48 h, and ground into powder. For demineralization, the powder was stirred in 0.5 M formic acid for 1 h, filtered, washed, and dried. For deproteinization, the demineralized material was treated with 2 M NaOH at 80 °C for 2 h, filtered, washed, and dried, yielding unbleached chitin. Finally, the unbleached chitin was bleached with 5 % hydrogen peroxide solution (solid/liquid ratio 1:20) at 90 °C for 30–60 min (Fig. S2 in supplementary material). To begin with this extraction step, firstly, the H₂O₂ solution was heated to 90 °C; once the required temperature was reached (monitored with a thermometer throughout the reaction), the unbleached chitin was added. The bleached samples were then filtered using a filter paper (pore size 7–12 µm), washed with distilled water until the pH reached a value of 7 and finally dried overnight at 60 °C in an oven. After this treatment, bleached chitin was obtained [22–24]. The deacetylation degree (DD) of all CS samples was determined by potentiometric titration, according to the methodology described in Jiang et al. [25], while the viscosity average Mw [26–29] was calculated by measuring the intrinsic viscosity of the CS solutions, according to the method reported in Singh et al. [26], applying the following Mark–Houwink–Sakurada equation (eq. 1):

$$[\eta] = KMw^{-a} \quad (1)$$

where $[\eta]$ is the intrinsic viscosity, calculated by an Ostwald capillary type viscometer (Fisher Scientific, Waltham, Massachusetts, USA), and K and α are values determined by Sing et al. [26].

Commercial chitosan (CM CS), deacetylation grade > 75 %, low Mw (80 kDa–160 kDa) (Sigma-Aldrich, Merck KGaA, Germany) was used for comparison.

2.3. Preparation of HL-derived CS and CM CS solutions

Solutions of HL-derived CS (HL CS_{BL} and HL CS_{UBL}) and CM CS at increasing polymer concentrations were prepared in 1 % (v/v) acetic acid. CS samples were added to the acidic solutions and maintained overnight under gentle stirring to allow the complete hydration of the polymer.

2.4. Characterization of HL-derived CS and CM CS solutions

2.4.1. Polymer critical entanglement concentration (CEC)

The CEC represents the lowest concentration at which the entanglement of the polymeric chains occurs; it depends on polymer Mw, chain morphology, and affinity of the solvent used. To identify the CEC, solutions of HL-derived CS (HL CS_{BL} and HL CS_{UBL}) and CM CS at increasing polymer concentrations were prepared in 1 % (v/v) acetic acid. Viscosity measurements were performed by means of a rotational rheometer (MCR 102; Anton Paar, Turin, Italy) equipped with a cone plate combination (CP50–1, diameter = 50 mm; angle = 1°) as measuring system ($\phi = 50$ mm and $\theta = 1^\circ$). Increasing shear rate values in the range 10 to 400 s^{−1} were applied. All measurements were carried out at 25 °C after a rest time of 1 min. Five replicates were performed for each analysis. Flow curves, reporting shear stress *versus* shear rate, were obtained for each solution. Viscosity values at rest (η_0) and at high shear

rates (η_{∞}) were calculated from the slopes of the straight lines that fitted the experimental data of the first and the second Newtonian zones of the flow curve. η_0 and η_{∞} were plotted *versus* the corresponding polymer concentration (% w/w) on bi-log scale. The intersection point of the two straight lines corresponded to CEC [30].

2.4.2. Viscoelastic properties

HL-derived CS and CM CS solutions containing increasing polymers concentrations (2–5 % (w/v)) were subjected to viscoelastic measurements by means of the above-mentioned rheometer, equipped with a parallel plate combination (PP25, diameter = 25 mm) as measuring system. Stress sweep and frequency sweep tests were performed at 25 °C. As for stress sweep test, increasing shear stress values were applied at a frequency of 1 Hz after a rest time of 1 min. The linear viscoelastic region (LVR), characterized by constant storage elastic modulus (G') values, on increasing shear stress, was determined.

Frequency sweep tests were also performed. A stress chosen in the LVR was applied at increasing frequencies (range of 1–20 Hz) and at constant temperature of 25 °C. G' and G'' profiles as a function of frequency were recorded. Three replicates were performed for each analysis.

2.4.3. In vitro cytocompatibility

Normal human dermal fibroblasts (NHDFs) from juvenile foreskin (PromoCell GmbH, Heidelberg, Germany) were used. Cells were between the 8th and 11th passage. NHDFs were grown in Dulbecco's modified Eagle medium (DMEM, Sigma Aldrich®-Merck, Milan, Italy), supplemented with 10 % foetal bovine serum (FBS, Euroclone, Milan, Italy) and 1 % v/v antibiotic-antimycotic solution (Merck Life Science S. r.l., Milan, Italy), kept at 37 °C in a 5 % CO₂ atmosphere with 95 % relative humidity (RH). NHDF were seeded in 96-well plates (area 0.34 cm²/well) at a density of 35,000 per well. Cells were grown 24 h to obtain sub-confluence. Then, cell substrates were washed with saline solution and put in contact with increasing concentrations of acetic acid diluted in DMEM without phenol red (0.005–0.1 % (v/v)). CS solutions in 1 % acetic acid (w/v) were diluted in DMEM without phenol red to the following concentrations: HL CS_{BL} (0.025–0.5 (v/v)), HL CS_{UBL} (0.015–0.3 % (v/v)) and CM CS (0.015–0.5 % (v/v)). Cytocompatibility was assessed after 24 h contact with NHDF cultures. Six replicates were assessed for all samples and for the negative control (NHDF cultures in pure DMEM without phenol red) and positive control (NHDF cultures in DMSO).

After the 24 h contact, growth medium and samples were withdrawn from each well, and an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test was performed. This test is based on the activity of mitochondrial dehydrogenases of vital cells that convert MTT in formazan crystals. 100 μ L of MTT (1 mg/mL) dissolution were added in each well. MTT-NHDF contact was maintained for 3 h before the whole supernatant was withdrawn and substituted by 100 μ L of dimethyl sulfoxide solution (DMSO, Sigma-Aldrich®-Merck, Milan, Italy) to dissolve formazan purple salts. The absorbance was assayed at 570 nm by means of an ELISA plate reader (Imark Absorbance Reader, Bio-rad, Hercules, CA, USA), with a reference wavelength set at 655 nm. Cell viability was calculated as % ratio of the absorbance of each sample and the absorbance of the cells kept in contact with the growth medium (control).

2.4.4. In vitro antioxidant activity

Before evaluating the antioxidant properties of CS solutions, the concentration of H₂O₂ needed to induce oxidative damage without causing cell death was determined according to Vigani and collaborators [31]. Cells were seeded at a density of 35,000 cells per well in a 96-well plate and incubated for 24 h at 37 °C with 5 % CO₂. Subsequently, various concentrations of H₂O₂ (0, 0.5, 1.25, 1.5, 2 and 3 mM), prepared in Dulbecco's modified Eagle medium without phenol red (DMEM, Sigma Aldrich®-Merck, Milan, Italy), supplemented with 10 % foetal

bovine serum (FBS, Euroclone, Milan, Italy), and 1 % v/v antibiotic-antimycotic solution (Merck Life Science S.r.l., Milan, Italy) were applied to the cells for 3 h. Untreated cells were considered as control (CM). Following the incubation period, an MTT assay was conducted on six replicates for each sample, as described in section 2.3.3. Based on the experimental results with different H₂O₂ concentrations, 1.4 mM was chosen for further investigations. Consequently, cells (35,000 cells per well) were treated with CS solutions at different concentrations (diluted in CM) for 24 h, followed by exposure to H₂O₂ for 3 h. An MTT assay was then conducted as previously described, with six replicates performed for each sample.

2.4.5. In vitro anti-inflammatory effect

The CS solutions anti-inflammatory properties were assessed using NHDF. Specifically, the levels of IL-6 in the culture medium of fibroblast cells (at a density of 35,000 cells/well), treated with CS solution diluted in CM for 24 h. CS solutions in 1 % acetic acid (w/v) were diluted in DMEM without phenol red to the following concentrations: HL CS_{BL} (0.025–0.5 (v/v)), HL CS_{UBL} (0.015–0.3 % (v/v)) and CM CS (0.015–0.5 % (v/v)). Cells were then exposed to lipopolysaccharide (LPS) at a concentration of 1 μ g/mL for an additional 24 h, were measured. IL-6 levels were detected using a Human IL-6 ELISA kit (Sigma Aldrich, St. Louis, MO, USA) following the manufacturer's instructions [32].

Untreated cells subjected to LPS-induced inflammation served as the negative control (LPS), while untreated cells not exposed to LPS-induced inflammation (CM) were used as a reference. Additionally, an MTT assay was conducted to assess whether inflammation affected cell viability percentage. Six replicates were performed for each sample.

2.4.6. In vitro wound healing properties

A wound healing live assay was conducted to evaluate the effects of CM CS and HL CS_{UBL} solutions on NHDF. NHDF cells were seeded into culture insert 2-well within a 24-well plate (Ibidi GmbH, Gräfelfing, Germany). The insert of each well comprises two chambers, each with a growth area of 0.22 cm², separated by a septum creating a cell-free gap of 500 μ m $\pm$ 50 μ m, simulating a wound. NHDF were seeded at a concentration of 10,000 cells per well. After a 24 h incubation period allowing for cell adhesion and growth to confluence, the inserts were carefully removed to initiate the wound healing process. 0.06 % (w/v) sample solutions were then applied to the wells. The choice of such concentration was based on preliminary studies, which indicated that this concentration optimally balanced efficacy and feasibility for the specific application. The plates were maintained in a humidified incubator at 37 °C with 5 % CO₂. Images of the gap area were captured every 15 min during 72 h using Confocal Laser Scanning Fluorescence Microscopy (CLSM) Leica TCS SP2 (Leica Microsystems, Milan, Italy). The wound closure rate was quantified by measuring the remaining gap area at each time point using ImageJ™ 2.0 software [33] and calculating the percentage of wound closure relative to the initial wound area at time zero. Data were analyzed to determine the time required for each sample to achieve full wound closure. Six replicates were performed for each experiment to ensure the robustness of the data.

2.4.7. Melanin content

To estimate melanin content using UV-Visible spectroscopic analysis, a modified protocol from Khairova and collaborators [34] was followed. CM and HL- CS derived (HL CS_{UBL} and HL CS_{BL}) solutions were prepared at 1 % (w/v). Baseline correction was performed using acetic acid to account for background absorption. The absorbance spectrum of each sample was measured against the solvent blank within the wavelength range of 200 nm to 800 nm. Three replicates were conducted for each measurement.

2.5. Preparation of freeze-dried scaffolds

CM CS and HL CS_{UBL} were placed in a 24-well plate (1.5 mL/well)

and subjected to freezing at -20°C for 24 h and then to sublimation for 48 h using Heto DRYWINNER (Analitica De Mori, Milan, I).

2.6. Characterization of freeze-dried scaffolds

2.6.1. Morphological analysis

The morphological assessment of each freeze-dried scaffold was conducted using a Scanning Electron Microscope (SEM) model Mira3 XMU (Tescan, Brno-Kohoutovice, Czech Republic). Samples were placed on a steel stub and rendered conductive by a layer of gold under vacuum (sputtering) before being tested. Imaging was carried out at high voltage, in high vacuum conditions, and at room temperature, employing various magnifications.

Additionally, pore size dimensional analysis was performed using the imaging analysis software ImageJ™2.0 [33]. Twenty replicates were performed to ensure reliability of the analysis.

2.6.2. Porosity analysis

The porosity of the freeze-dried scaffolds was assessed using the liquid displacement method, as described by Zeng and collaborators [35]. In this method, absolute ethanol was employed as the displacement liquid due to its ability to penetrate the porous network without altering scaffold morphology through shrinkage or swelling, as it is not a solvent for CS.

Initially, samples (designated as dry mass M_0) were immersed for 1 h in a predetermined volume (10 mL) of absolute ethanol. The process involved measuring the combined weight of the entire system (sample + ethanol) (M_a). Subsequently, after 1-h immersion period, the sample was removed, and the remaining ethanol, which was not entrapped within the sample pores, was measured (M_b). Simultaneously, a calibrated cylinder containing a known volume of ethanol (10 mL) (M_1) was measured.

Following this, the calibrated container was emptied, and the wet sample previously soaked in ethanol for 1 h was placed inside. The ethanol that had been in contact with the scaffold was then added until the container was filled to the same previously mentioned volume. Finally, the container was reweighed (M_2). Porosity (P%) was calculated according to the following equation (eq. 2):

$$P\% = \frac{M_a - M_b - M_0}{(M_a - M_b) - (M_2 - M_1)} \times 100 \quad (2)$$

Six replicates were performed for each sample.

2.6.3. Hydration properties

To evaluate the hydration properties of the freeze-dried scaffolds, a 12-well plate containing PBS (Phosphate Buffered Saline; VWR Chemicals, LLC, USA) was used. The plate was covered with a non-woven tissue membrane, and at each well, a sample was placed on a dialysis membrane, which was previously conditioned. The weight (M_1) of the sample was recorded at time 0, and the hydration properties were assessed at specified time intervals (30 min, 1, 2, 3, 4, 5, 6, and 24 h), noting the weight (M_2) of each sample. Three replicates were performed for each sample. The amount of PBS absorbed was calculated according to the following equation (eq. 3):

$$\text{Amount of PBS absorbed (mg)} = M_2 - M_1 \quad (3)$$

The swelling ratio, which represents the fractional increase in weight of the hydrogel following water absorption, was calculated at 24 h as follows (eq. 4):

$$\text{Swelling ratio} = \frac{M_2 - M_1}{M_1} \quad (4)$$

where:

M_2 = weight of the hydrated scaffold.

M_1 = weight of the dried scaffold.

2.6.4. Bioadhesion properties

The bioadhesion test was performed on the freeze-dried scaffolds using the TA.XT plus Texture Analyzer (Stable Micro Systems, UK). The matrices were attached to the P/0.5R probe ($\varnothing = 13$ mm) with a double-sided tape. A 10 % w/v gelatine (Type A, 175 g Bloom surface, Sigma Aldrich®-Merck, Milan, Italy) gel, conditioned with Wound Simulating Fluid (WSF) containing 0.68 g NaCl, 0.22 g KCl, 2.5 g NaHCO_3 , 0.35 g NaH_2PO_4 in 100 mL of MilliQ water ($\text{pH} = 8.3 \pm 0.01$), was used to simulate the wound surface. The measurements were conducted at a constant temperature of 32°C using a thermostatic water bath.

The probe with the sample was lowered to bring the sample into contact with the biological substrate; the analysis was performed with the following experimental parameters: pre-test speed of $0.5 \text{ mm}\cdot\text{s}^{-1}$; test speed of $0.5 \text{ mm}\cdot\text{s}^{-1}$; post-test speed of $1.0 \text{ mm}\cdot\text{s}^{-1}$. The analysis was carried out by applying a preload force of 0.5 N for 600 s. After 600 s, the probe was raised until the bioadhesive interface was completely separated, effectively detaching the sample from the biological substrate. 1 % (w/v) Carbopol® 974P NF polymer (Lubrizol, Sao Paulo, SP, Brazil) freeze-dried scaffolds were used as internal technical controls. Six replicates were performed for each sample. The total work of adhesion was calculated using the Texture Exponent software.

2.6.5. Mechanical properties

Freeze-dried scaffolds, as such and upon hydration in PBS for 24 h at 37°C , were subjected to a compression test by means of TA.XT plus Texture Analyzer (Stable Micro System, Godalming, UK) equipped with a 5 kg load cell. For the analysis, probe P20P was lowered into the sample at a constant speed of 0.5 mm/s to a final distance equal to 70 % of the sample height; afterwards the probe was raised at a constant speed of 0.5 mm/s. For both dried and hydrated samples three replicates were performed. Compression stress (Pa) vs strain profiles were recorded for each sample. Work of compression was also calculated as the area under the curve force vs displacement (AUC, mN·mm). Four replicates were considered for each sample.

Freeze-dried scaffolds, as such, were also subjected to dynamic mechanical analysis (DMA) in compression mode to determine the viscoelastic properties of the dry sponges, focusing on the storage modulus (E'), loss modulus (E''), and damping factor (E''/E') (MCR 702e, Dynamic Mechanical Analyzer, Anton Paar, Turin, Italy). The temperature was fixed at 25°C . A stress amplitude sweep was conducted at a fixed frequency of 0.01 Hz, applying increasing compression stresses to determine the linear viscoelastic region (LVR). Following the identification of the LVR, a frequency sweep analysis was performed using a stress value within the LVR. The frequency sweep involved the application of a sinusoidal oscillatory compression stress across a range of frequencies (0.01 Hz to 10 Hz) to investigate the frequency-dependent mechanical response of the scaffolds. Three replicates were considered for each sample.

Moreover, hydrated samples were subjected to dynamic oscillatory measurements at 37°C by means of a rotational rheometer (MCR102 Rheometer, Anton Paar, Turin, Italy), using a parallel plate combination (PP25, diameter = 25 mm) as measuring system. A stress sweep test was performed at a constant frequency (1 Hz) and the elastic response of the sample, expressed as storage modulus G' , was measured as a function of stress; such a test allowed the identification of the linear viscoelastic region, characterized by constant G' values on increasing stress. Then, samples were subjected to oscillation test: a shear stress chosen in the linear viscoelastic region previously determined, was applied at increasing frequencies (1–20 Hz) and G' (storage modulus) and G'' (loss modulus) values were recorded as a function of frequency. Loss tangent ($\tan \delta$) parameter was calculated as the ratio between G'' (loss modulus) and G' (storage modulus) at frequency values of 1, 5, 10 and 20 Hz. Four replicates were considered for each sample.

2.6.6. In vitro degradation test

A degradation test was performed on freeze-dried scaffolds based on

CM CS and HL CS_{UBL}. A wound simulating fluid (WSF), used as degradation medium, was prepared as follows: 0.68 g NaCl, 0.2281 g KCl, 2.5 g NaHCO₃, 0.35 g NaH₂PO₄ in 100 mL of MilliQ water (pH = 7.8 ± 0.01).

Dried scaffolds were placed in Eppendorf tubes, and 5 mL of the degradation medium was added. The test was conducted at 37 °C in a thermostatic oscillating water bath. At specified time points (t₀ = 0 days, t₁ = 1 day, t₃ = 3 days, t₇ = 7 days, t₁₄ = 14 days, t₂₈ = 28 days), the hydrated samples were frozen at −20 °C for 24 h, followed by sublimation for 48 h using Heto DRYWINNER (Analytica De Mori, Milan, I). Each experiment was performed in triplicate. The percentage of dry weight loss due to degradation in WSF was calculated at each time point as follows (eq. 5):

$$W\% = \left(W_f / W_i \right) \times 100 \quad (5)$$

where:

W % = percentage loss of dry weight.

W_i = weight of dry freeze-dried scaffolds before soaking.

W_f = weight of dry freeze-dried scaffold at each time point.

Morphological characterization (SEM and dimensional analysis) of each sample at each time point was performed as described.

2.6.7. In vitro cell proliferation test

To evaluate the impact of CS-based sponge-like dressings on cell proliferation, an indirect proliferation assay was conducted. HL CS_{UBL} and CM CS sponge-like dressings were immersed in DMEM F-12 culture media for 24 h to allow leaching of bioactive components. NHDF were then exposed to the conditioned media for 3 and 7 days. Cell viability was assessed at each time point using the MTT assay, which measures cellular metabolic activity as an indicator of cell proliferation. Viability data were normalized to control cells cultured in standard DMEM F-12 media without contact with the dressings. Six replicates were performed for each analysis.

2.7. Statistical analysis

All measurements were performed at least in triplicate and the data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, Boston, Massachusetts USA). One-way ANOVA followed by Dunnett's multiple comparisons test was performed to assess significance ($p < 0.05$) unless otherwise indicated. A 95 % confidence interval (CI) was considered by default for all analysis.

3. Results and discussion

3.1. Chitosan production

Bleached chitosan (HL CS_{BL}) presented a DD of 90 % whereas unbleached CS (HL CS_{UBL}) showed a DD of 85 %. Mw was lower for the bleached samples than the unbleached ones. Specifically, HL CS_{BL} had a Mw ranging between 90 and 100 kDa, while HL CS_{UBL} molecular weight reached 160–180 kDa. These results are in accordance with findings reported in the relevant literature [22–24].

Chitin bleaching has a significant impact on some parameters of solubilized CS, such as viscosity and Mw [23,24], as well as on the morphological characteristics of the resulting biopolymer. Bleaching reduces the Mw of CS due to hydrolysis and oxidative degradation of polymer chains. Additionally, the removal of impurities during bleaching increases the polymer accessibility to deacetylation reagents, further raising the DD. Triunfo and collaborators reported that an increase in the crystallinity of the final CS was observed when the bleaching process was applied, due to the decrease in CS Mw [22]. SEM analysis revealed that, in bleached samples, surface filamentous structures detected in the

unbleached biopolymer were reduced, probably due to the cleaning effect of the bleaching step.

3.2. Characterization of CM CS and HL-derived CS solutions

3.2.1. Polymer critical entanglement concentration (CEC)

Fig. 1 reports the viscosity curves of HL-derived CS (HL CS_{BL} and HL CS_{UBL}) and CM CS solutions obtained at 25 °C. All the solutions exhibit a shear-thinning behaviour, being characterized by viscosity values that decrease on increasing shear rates. The decrease of apparent viscosity with increasing shear rate is due to the polymer chains disentanglement and alignment along the flow direction caused by shear forces [36–39].

The Critical Entanglement Concentration (CEC) values of CM CS (1.18 ± 0.09 % w/v) and HL CS_{UBL} (1.15 ± 0.05 % w/v) were comparable, suggesting that the two polymers were characterized by comparable chain entanglement and interactions. In contrast, HL CS_{BL} exhibited a significantly higher CEC (1.84 ± 0.06 % w/v) compared to CM CS (**** $p < 0.0001$; Ordinary One-Way Anova and Dunnett's multiple comparisons test ($p < 0.05$)). This difference can be attributed to the bleaching process, which likely reduces the polymer Mw, making the CS molecules less likely to form entangled networks at lower concentrations.

3.2.2. Viscoelastic properties

HL-derived CS and CM CS solutions were subjected to stress sweep measurements to determine the linear viscoelastic region (LVR). In this region, G' and G'' moduli remain constant regardless of the applied stress. Stress sweep measurements provide insights into material structural strength and sample stability; stable solutions can maintain the LVR over a larger strain range compared to unstable ones. The end limit of the LVR that corresponds to the onset of non-linearity and hence structural breakdown represents the yield stress [40,41].

HL CS_{BL} solutions consistently exhibited lower yield stress values compared to both CM CS and HL CS_{UBL}, particularly at higher concentrations. At 5 % concentration, HL CS_{BL} showed a significantly lower yield stress of 3.2 ± 0.4 Pa ($p < 0.0001$), compared to 76.5 ± 4.4 Pa for CM CS and 94.6 ± 2.2 Pa for HL CS_{UBL} ($p < 0.0001$). Similarly, at 4 %, HL CS_{BL} had a yield stress of 1.7 ± 0.4 Pa ($p < 0.0001$), while CM CS and HL CS_{UBL} had 17.2 ± 1.6 Pa and 21.0 ± 0.6 Pa, respectively ($p < 0.001$ for HL CS_{UBL}). These results highlight the distinct structural behaviour of HL CS_{BL}, which forms a weaker network. In contrast, HL CS_{UBL} solutions show higher yield stress values than CM CS at concentrations above 3 % (w/v), indicating the formation of a stronger structural network despite having a similar CEC.

The loss tangent ($\tan\delta = G''/G'$) is a crucial parameter in oscillation analysis as it indicates the balance between the viscous and elastic components of the sample structure. Samples with $\tan\delta$ values >1 ($G'' > G'$) are characterized by a prevalence of the viscous contribution to the elastic one, *vice versa* for the samples with $\tan\delta$ values lower than 1 ($G' > G''$) the elastic behaviour prevails on the elastic one. The highest values of loss tangent calculated in the LVR region were detected for HL CS_{BL}, indicating that such sample, independently of concentration, is the least elastic (Fig. 2A). At the highest concentration tested (5 % w/v), both HL CS_{UBL} and CM CS solutions demonstrated a loss tangent below 1, indicating a prevalence of the elastic contribution on the viscous one.

Both CM CS and HL CS_{UBL} at the highest concentration tested (5 % w/v) are characterized by an increase in G' on increasing frequencies (Hz), a typical behaviour of polymer solutions (Fig. 2B). Up to 4 % (w/v) concentration HL CS_{UBL} and CM CS solutions showed comparable G' values (measured at 5 Hz) (Fig. 2C). At 5 % w/v concentration, the significantly higher G' value observed for HL CS_{UBL} suggests that such solution possesses a stronger structural network with respect to CM CS (Fig. 2C).

3.2.3. In vitro cytocompatibility of HL-derived CS and CM CS solutions

CS has been widely reported as compatible with fibroblasts, making

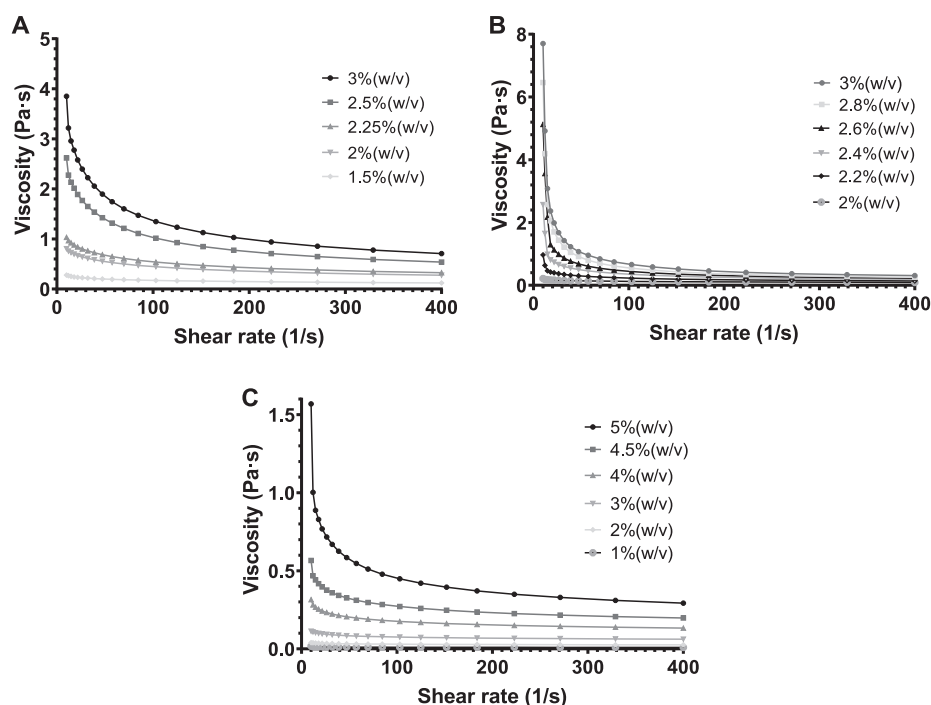


Fig. 1. Apparent viscosity (Pa·s) (mean values $\pm$ SD; $n = 5$) vs shear rate (s^{-1}) curves (viscosity curves) of CM CS (A) and HL-derived CS, HL CS_{UBL} (B) and HL CS_{BL} (C), solutions in 1 % (v/v) acetic acid at 25 °C (mean values $\pm$ SD; $n = 5$).

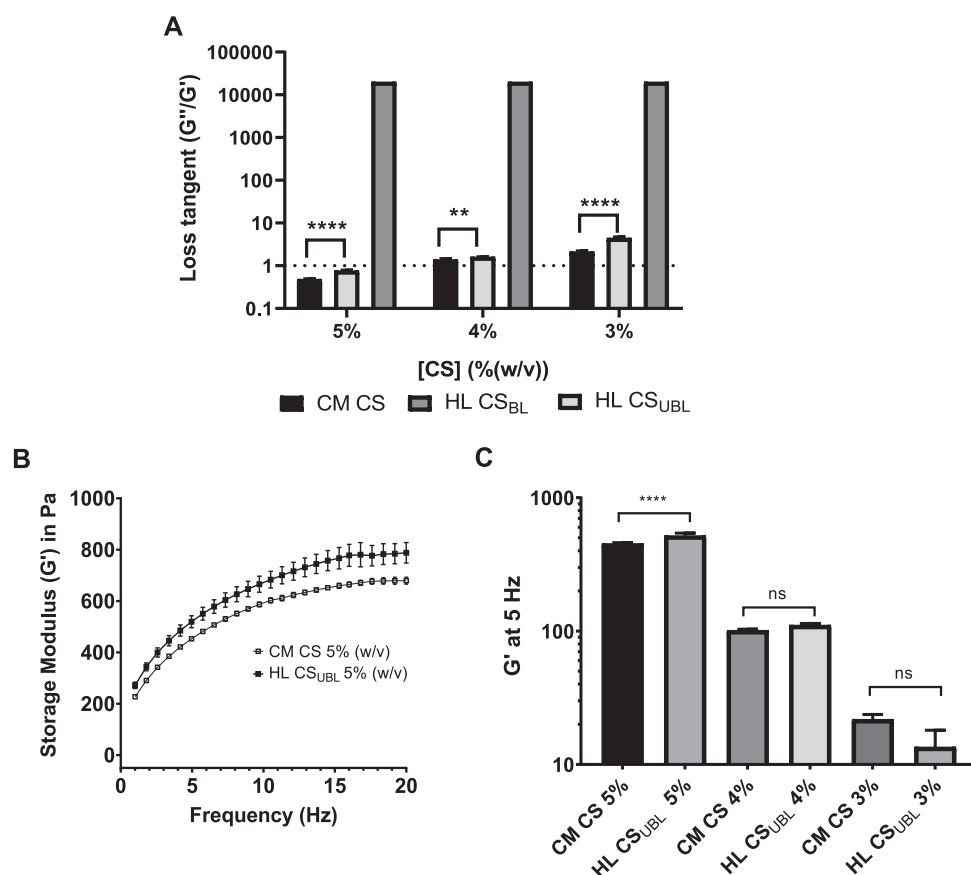


Fig. 2. (A) Loss tangent values (G''/G') (mean values $\pm$ SD; $n = 5$) for CM CS and HL-derived CS (HL CS_{UBL} and HL CS_{BL}) solutions in 1 % (v/v) acetic acid) at 25 °C. (B) Storage modulus (G') (Pa) of CM CS and HL CS_{UBL} at increasing frequencies (Hz). (C) G' at 5 Hz of CM CS and HL CS_{UBL} at different concentrations. Ordinary One-Way Anova and Dunnett's multiple comparisons test ($p < 0.05$) were carried out. **** $p < 0.0001$, ns: not statistically significant.

it a promising material for wound healing applications [42–45]. Its biocompatibility and biodegradability ensure that it integrates well with biological tissues, promoting cell proliferation and tissue regeneration. Studies have shown that CS supports the attachment and growth of fibroblasts, which are crucial for wound healing due to their role in

collagen production and tissue repair [43–45].

First, the biocompatibility of acetic acid, CS solvent, was assessed. Specifically, human fibroblasts were exposed to different concentrations of acetic acid solutions to mimic the conditions adopted for CS dissolution. Notably, none of the concentrations tested led to a decrease in

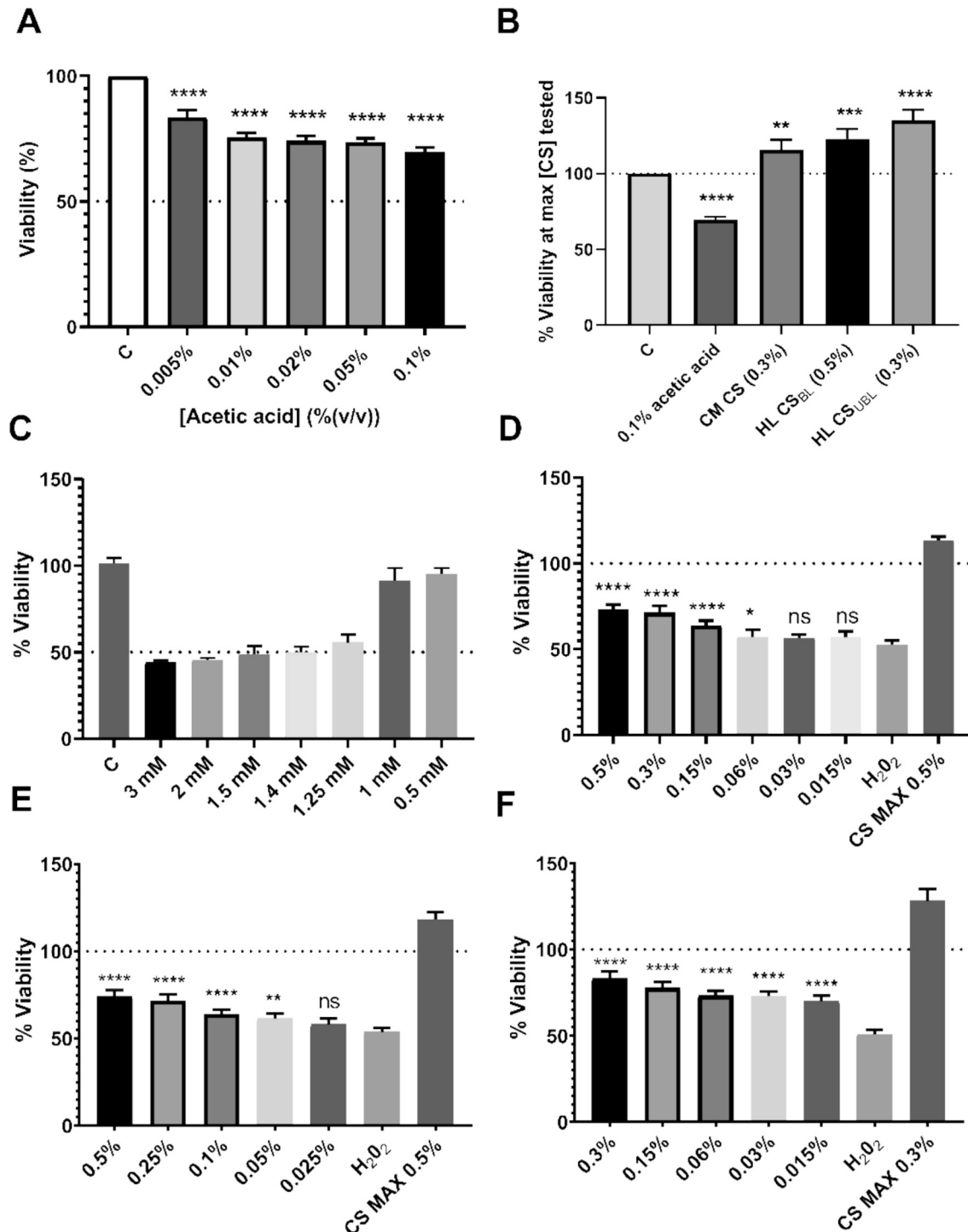


Fig. 3. Biocompatibility tests (contact time 24 h) towards NHDF. (A) Cell viability (%) vs acetic acid concentrations. (B) Cell viability (%) upon contact with CS solutions. C stands for negative control test (no treatment). Cell viability % calculated: C) after cell treatment with increasing H₂O₂ concentrations (mM) for 3 h. (D–F) after 24 h treatment with different concentrations of the samples: (B) CM CS, (C) HL CS_{UBL} and (D) HL CS_{BL} for 24 h and, then, with H₂O₂ (1.4 mM) for 3 h. Cells exposed to the oxidative stress in absence of the samples were considered as positive control (H₂O₂), whereas treatment with only the sample at the maximum tested concentration, without oxidative stress was considered negative control (CS MAX) (mean values $\pm$ SD; $n = 6$). Dot line indicates 50 % (A) and 100 % (B) viability. Ordinary One-Way Anova and Dunnett's multiple comparisons test ($p < 0.05$) were carried out. All samples were compared with the control with H₂O₂ without any CS treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0005$, **** $p < 0.0001$, ** $p < 0.01$, *** $p < 0.0005$, **** $p < 0.0001$, ns: not statistically significant.

viability below 70 %, indicating that even at the highest concentration tested, the acetic acid did not induce a significant cytotoxicity (Fig. 3A).

The results demonstrate that HL-derived CS solutions, particularly HL CS_{UBL}, exert a markedly greater effect on enhancing human fibroblast viability when compared to CM CS. Furthermore, when CS solutions were placed in contact with cells, they not only reversed the cytotoxic effect of acetic acid but also increased cell viability to levels exceeding 100 %. These findings suggest that all tested CS solutions have a protective effect on cellular viability, potentially promoting fibroblast proliferation. This probably correlates directly with the source used to produce the biopolymer, *i.e.* insects, as their exoskeleton is rich in lipids, chitin fibers and several pigments. Among these, one of the most abundant is surely melanin, which has an intrinsic protective effect on skin cells [46]. The presence of melanin is confirmed by the results described in section 3.1.7 “Melanin content”. Recent studies have reported the cytocompatibility of other insect-derived CS solutions [47] and formulations [42,48] on fibroblasts. It is known that Mw, DD, pH, and chitin source significantly influence CS activity [49]. In the present study, HL CS_{UBL} exhibited a similar DD and Mw of CM CS. Yielded results showed that *H. illucens* CS solutions, particularly HL CS_{UBL} (that has a higher pigmentation and thus probably a higher melanin content) exert a significantly greater effect on increasing the viability of human fibroblasts than the commercial biopolymer, which highlights the potential advantages of utilizing CS sourced from *H. illucens*, particularly in their unbleached state, for biomedical applications. It is worth highlighting that the extraction of HL CS_{UBL} and HL CS_{BL} from the pupal exuviae of *H. illucens* offers significant environmental advantages over conventional CS production. Furthermore, the HL CS_{UBL} requires fewer chemical inputs, reducing the environmental footprint of the production process. In contrast, conventional CS production from crustacean shells often involves intensive chemical treatments, which can generate harmful waste [50]. Additionally, the use of insect-based CS mitigates the risks of allergenic reactions associated with crustacean-derived CS, expanding its potential applications [51].

3.2.4. *In vitro* antioxidant activity

The addition of hydrogen peroxide (H₂O₂) to fibroblast cell cultures induces oxidative stress, leading to a decrease of cell viability (Fig. 3). The reactive oxygen species (ROS) generated by H₂O₂ can cause damage to cellular components such as DNA, proteins, and lipids, ultimately compromising cell function and viability [52]. The addition of HL CS_{UBL}, HL CS_{BL} and CM CS to NHDF cell cultures has been found to mitigate this oxidative damage, thereby preserving cell viability. The antioxidant effect of CS has been previously reported; however, in most cases, it was evaluated using methods like 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays rather than on cells as in the present study [42,53,54]. A similar restoration of viability was also reported in RAW264.7 macrophages after exposure to CS and H₂O₂ [55]. Since CS chains have active hydroxyl and amino groups that can react with free radicals, its scavenging activity is likely due to the reaction between free radicals and protonated amino groups. One of the proposed scavenging mechanisms of CS involves hydroxyl and superoxide anion radicals reacting with active hydrogen atoms in CS, resulting in the formation of a stable macromolecule radical [56]. In the present study HL CS_{UBL} exerted a particularly effective protection against H₂O₂-induced oxidative stress. The enhanced protective effect is hypothesized to be attributed to the presence of CS-melanin complexes in the HL CS_{UBL} [34,46]. Melanin, known for its antioxidant properties [57–59], may synergistically interact with CS to scavenge ROS and reduce oxidative damage within the cells. This suggests that the combined antioxidant activity of CS and melanin within HL CS_{UBL} plays a crucial role in preventing oxidative stress and preserving cell viability in presence of H₂O₂-induced damage. These findings highlight the potential of HL CS_{UBL} as a promising agent for mitigating oxidative stress-related pathologies and promoting cellular health in various biomedical applications.

3.2.5. *In vitro* anti-inflammatory effect

The anti-inflammatory activity of CM CS, HL CS_{UBL}, HL CS_{BL} was evaluated on NHDF treated with LPS, a known pro-inflammatory agent [60]. IL-6 levels were measured as an indicator of inflammation.

In Fig. 4 the percentage of IL-6 release is reported in the presence of different concentrations of CM CS, HL CS_{UBL} and HL CS_{BL} solutions. When NHDF cells were pre-treated for 24 h with concentrations over 0.03 % (w/v) for all the three CS samples, a lower IL-6 release was observed, indicating a significant reduction of LPS-induced inflammation.

Both CM CS and HL CS_{BL} treatments resulted in a notable reduction in IL-6 levels, with no significant differences observed between the two samples. However, HL CS_{UBL} treatment led to a significant reduction in IL-6 levels in comparison to CM CS at concentrations higher than 0.03 % (w/v), demonstrating a stronger anti-inflammatory activity. The greater anti-inflammatory activity of HL CS_{UBL} might be attributed to the retention of bioactive compounds such as natural pigments and polyphenols [61,62], which are removed during the bleaching process.

While this study demonstrates the potential of *Hermetia illucens*-derived CS for wound healing applications, further investigations are needed to fully understand its anti-inflammatory properties. Although IL-6 is a key interleukin involved in the inflammatory response during wound healing [60], other inflammatory mediators, such as IL-8 and TNF- α , also play crucial roles in the process [63]. Future studies will focus on evaluating these additional factors in order to provide a more comprehensive understanding of the anti-inflammatory effects of insect-derived CS and its potential therapeutic applications in wound healing.

3.2.6. *In vitro* wound healing properties

A wound healing live assay was performed on fibroblasts using plates with inserts that were removed prior to treatment to ensure the formation of a gap between cells. CM CS and HL CS_{UBL} solutions at 0.06 % (w/v) were tested for their efficacy in promoting cell migration and gap closure. Observations after 24 h revealed a significant difference in the percentage of wound closure: HL CS_{UBL} treated cells showed 82.9 ± 5.5 % closure, while CM CS treated cells showed 71.3 ± 7.2 % closure (*; *p*-value < 0.05; Ordinary One-Way Anova and Dunnett's multiple comparisons test (*p* < 0.05)) (Fig. 5). NHDF cells treated with HL CS_{UBL} reached full closure after 39 h, whereas those treated with CM CS required 58 h to achieve the same result (**; *p*-value < 0.01; Ordinary One-Way Anova and Dunnett's multiple comparisons test (*p* < 0.05)). This suggests that HL CS_{UBL} has a greater capability of facilitating fibroblast migration and proliferation. These findings highlight HL CS_{UBL} as a more effective agent for promoting rapid wound closure, potentially offering improved therapeutic benefits in clinical applications, in line with recent research [64].

3.2.7. Melanin content

To explore the hypothesis of a synergistic effect between CS and melanin, UV-Vis spectroscopy was employed to analyse solutions of CM CS, HL CS_{UBL}, and HL CS_{BL} at 1 % (w/w) concentration. This analysis aimed to estimate the levels of melanin present in the CS solutions and investigate any potential absorbance patterns indicative of melanin content. The UV-Vis spectra revealed distinct absorbance profiles among the different CS samples. Fig. 6 reports the spectrophotometric data of CM CS, HL CS_{UBL} and HL CS_{BL} in the UV region, showing that CM CS has minimal light absorption in this wavelength range. The absorption values of CM-melanin complex are higher than those of the individual biopolymers, indicating their synergistic activity and enhanced absorption in the UV region [34,46]. Specifically, HL CS_{UBL} exhibited a synergistic absorbance peak at 280 nm, suggesting the presence of CS-melanin complexes. Additionally, a specific absorbance at 500 nm was observed, indicative of melanin absorption. In contrast, CM CS solution did not display any significant absorbance at 500 nm, confirming the lack of melanin content [65]. In this study, melanin content was determined using UV-Vis spectroscopy as previously reported by Khairova

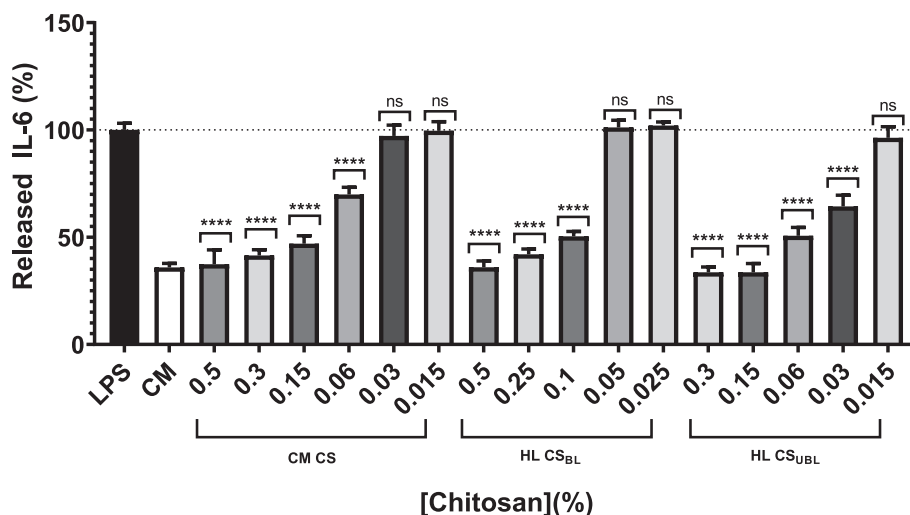


Fig. 4. IL-6 release values (%) calculated after cell treatment with CM CS, HL CS_{UBL} and HL CS_{BL} solutions for 24 h followed by LPS solution (1 µg/mL) for other 24 h. Cells subjected to LPS-inflammation in absence of samples were considered as negative control (LPS) and correspond to 100 %; cells not subjected to LPS-inflammation (CM) were considered as positive control. Ordinary One-Way Anova and Dunnett's multiple comparisons test ($p < 0.05$) comparing HL CS_{UBL}, HL CS_{BL} and CM CS to the negative control (mean values $\pm$ SD; $n = 6$). **** $p < 0.0001$, ns: not significant.

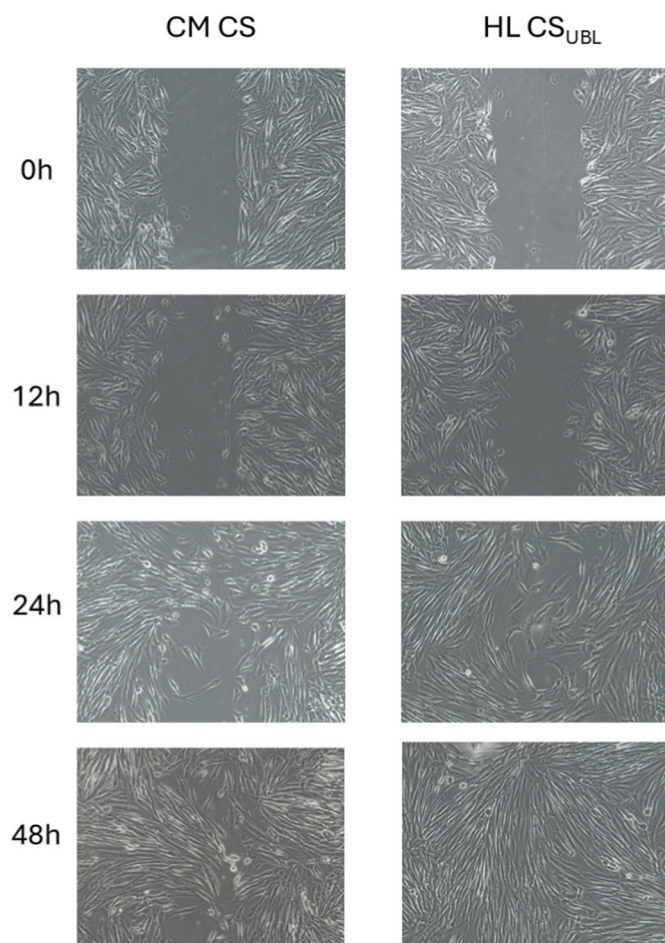


Fig. 5. Wound healing assay showing the effects of CM CS and HL CS_{UBL} on NHDF cell migration over time (0, 12, 24, and 48 h post-treatment).

and colleagues [34]; however, this method can be limited in terms of sensitivity and its ability to provide detailed structural information about melanin. Therefore, further analyses, such as FTIR or HPLC-MS, will be helpful in obtaining a more accurate and comprehensive

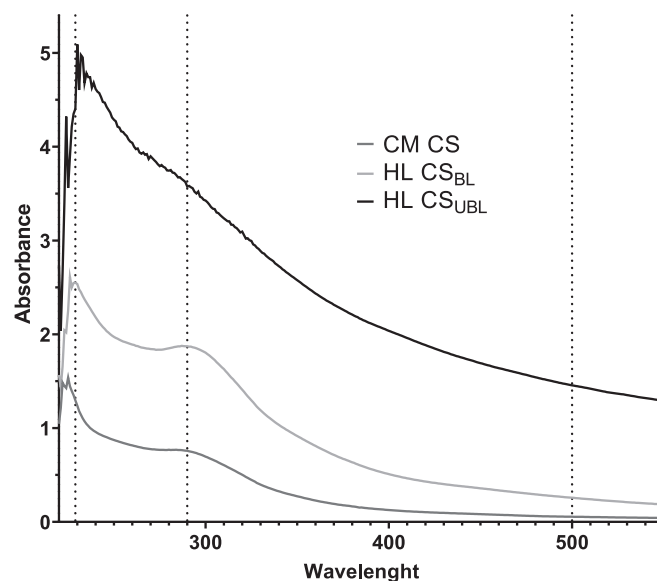


Fig. 6. UV-Visible spectra of CM and HL-derived CS (HL CS_{UBL} and HL CS_{BL}). Dotted lines indicate from left to right the following wavelengths: 230 nm, 280 nm and 500 nm ($n = 3$).

assessment of melanin content in the CS samples. These advanced methods will help to confirm the findings from UV-Vis spectroscopy and provide deeper insights into the interaction between CS and melanin. Future studies will focus on investigating this interaction to better understand how melanin influences the properties and bioactivity of insect-derived CS.

3.3. Characterization of freeze-dried scaffolds

Based on the results so far obtained, HL CS_{UBL} was chosen as the most promising HL-CS derivatives. HL CS_{UBL} and CM CS based scaffolds were prepared by freeze-drying and compared for functional and biological properties.

3.3.1. Morphological analysis

Figs. 7 (A-B) report the SEM images and pore sizes of CM CS and HL

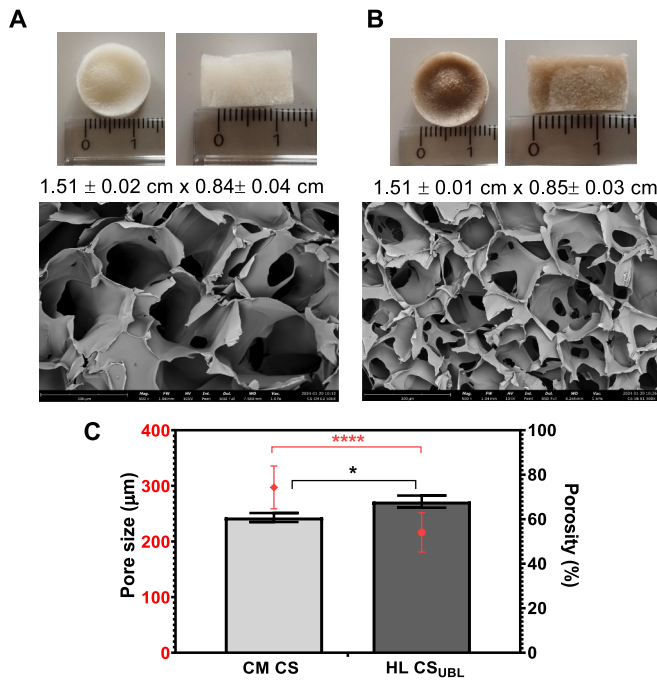


Fig. 7. A) CM CS and B) HL CS_{UBL} sponge-like scaffolds images. C) Porosity (%) and pore size (μm) of CM and HL CS_{UBL} scaffolds. Pore measurements were performed with ImageJ™ software [33]. Unpaired *t*-test with Welch's correction (*p* value ≤ 0.05) (mean values ± SD; *n* = 20 (pore size), *n* = 6 (porosity)). * *p* < 0.05, **** *p* < 0.0001.

CS_{UBL} freeze-dried scaffolds. Both CM CS and HL CS_{UBL} scaffolds present a porous organized internal structure (Figs. 7A e 7B). Fig. 7C shows the porosity (histogram) and pore size (red points) of the two scaffolds. According to the results reported in Fig. 7C, HL CS_{UBL} presents the smallest pore size. Regarding porosity percentages, HL CS_{UBL} displays the highest mean porosity value (67.9 ± 2.7 %) with respect to CM CS (60.8 ± 2.0 %). The differences observed might be due to the presence of CS-melanin complexes which leads to a higher crosslinking of CS chains in the scaffold structure.

The pore structure and porosity of scaffolds play a crucial role in influencing their biological performance, particularly in wound healing and tissue regeneration [66]. Highly porous scaffolds, such as those obtained in this study, are beneficial for facilitating cell proliferation and migration by providing an interconnected network that mimics the extracellular matrix (ECM) [67,68].

In the context of wound healing, porous structures can also promote angiogenesis by allowing endothelial cells to migrate and form capillary networks [68]. An optimal balance between porosity and mechanical strength is essential, as excessive porosity can compromise the scaffold's structural stability [66]. These considerations underscore the importance of tailoring pore characteristics for specific biomedical applications, including wound healing and tissue repair.

3.3.2. Hydration properties

The hydration properties of CM CS and HL CS_{UBL} scaffolds were evaluated to determine their effectiveness in fluid absorption and retention. Understanding these characteristics is crucial for optimizing scaffold performance in wound care applications, where fluid management is essential for promoting healing. Fig. 8 shows the amount of PBS absorbed over time by the two different scaffolds. HL CS_{UBL} and CM CS scaffolds exhibit similar absorption levels for up to 1 h, with no statistically significant difference. After 2 h of hydration, HL CS_{UBL} scaffold absorbed more PBS than CM CS, likely due to its higher porosity.

Table I compares the swelling ratio values of HL CS_{UBL} and CM CS scaffolds after 24 h of hydration in PBS medium at pH 7.4. HL CS_{UBL}

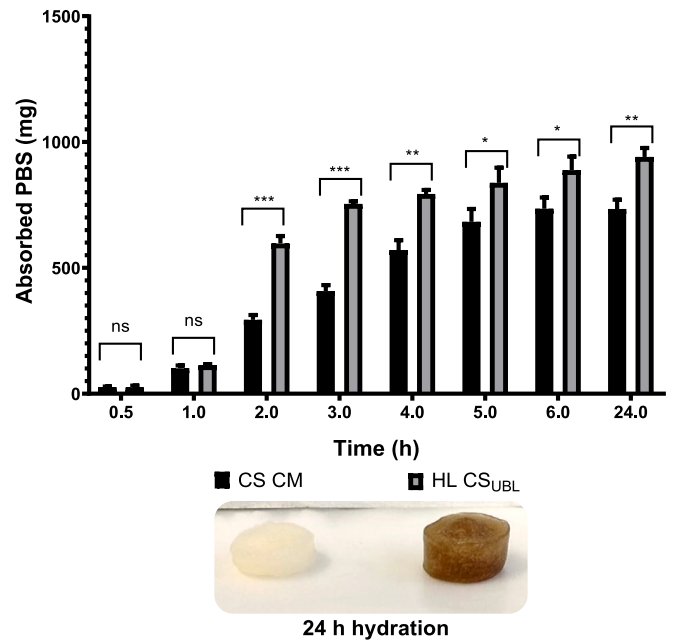


Fig. 8. Amount of PBS absorbed by CM CS and HL CS_{UBL} scaffolds as a function of time (30 min, 1, 2, 3, 6 and 24 h). Unpaired *t*-test with Welch's correction (*p* value ≤ 0.05) (mean values ± SD; *n* = 3). ns: not significant, * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.0005.

Table I

Swelling ratio (%) of HL CS_{UBL} and CM CS freeze-dried scaffolds. Unpaired *t*-test with Welch's correction (*p* value ≤ 0.05) (mean values ± SD; *n* = 3). * *p* < 0.05.

Sample	Swelling ratio
CM CS	20.0 ± 1.1
HL CS _{UBL}	25.4 ± 1.9*

scaffold showed the highest swelling ratio, significantly greater than that of CM CS one. This difference can be attributed to the presence of CS-melanin complexes in HL CS_{UBL} structure, which allows the scaffold hydration without losing their integrity. HL CS_{UBL} scaffolds displayed higher swelling properties than similar CS-based freeze-dried scaffolds reported in the literature [69–71].

Both samples demonstrated a high absorption capacity, with swelling ratios ranging from 20.0 ± 1.1 to 25.4 ± 1.9. This indicates that the samples can absorb PBS to reach weights approximately 20 to 25-fold greater than their initial weight. When applied to an injury site, a highly porous structure is necessary to absorb and drain wound exudate efficiently. This absorption leads to the formation of a gel that closely resembles the hydrated extracellular matrix (ECM) environment, thereby providing an optimal structure for cell proliferation, and accelerating re-epithelialization and promoting faster wound healing [72].

3.3.3. Bioadhesion properties

Freeze-dried scaffolds obtained from 2 % (w/v) CM CS, 2 % (w/v) HL CS_{UBL} and 1 % (w/v) Carbopol® 974P NF solutions were subjected to bioadhesion studies to evaluate the matrix ability to interact with a substrate simulating the wound bed, specifically a 10 % w/v gelatine gel conditioned with Simulated Wound Fluid (SWF). The concentration of Carbopol® 974P NF was carefully selected based on the viscosity of the initial solution, ensuring that all the three polymer solutions (CM CS, HL CS_{UBL} and Carbopol® 974P NF) had similar viscosities before undergoing the freeze-drying process. This methodological consideration is

crucial as it allows for a comparison of bioadhesive properties on homogeneous basis. Carbopol® 974P NF serves as positive control in this study due to its well-documented bioadhesive properties [73–75].

In Fig. 9, the bioadhesion potential of the scaffolds is expressed as the work of adhesion, calculated as the area under the curve of the detachment force *versus* distance. This represents the work needed to overcome the attractive forces between the freeze-dried scaffold and the substrate. The phenomenon of bioadhesion can be facilitated by the establishment of electrostatic interactions, hydrogen bonds and Van der Waals forces between the polymer matrix and the substrate [76]. HL CS_{UBL} scaffold exhibits lower adhesive capability compared to CM CS scaffold, but not significant differences when compared to Carbopol® 974P NF. The relatively lower adhesion with respect to CM CS could be advantageous to avoid damage during dressing changes. These properties could be particularly advantageous for sensitive skin or wounds that require frequent dressing changes. One possible explanation for the differences observed could be the presence of CS-melanin complexes in HL CS_{UBL} scaffolds, since they could alter surface properties, interfering with the binding sites of CS, reducing the overall adhesion strength. Moreover, the higher porosity in HL CS_{UBL} scaffolds (Section 3.2.1) could result in a rougher surface texture, leading to a reduced contact area and a weaker adhesion. The porous structure, enhancing fluid absorption and retention, also contributes to lower detachment forces.

3.3.4. Mechanical properties

Mechanical properties of the scaffolds as such and upon hydration were investigated. Dried HL CS_{UBL} scaffolds are characterized by lower compression work values compared to CM CS (Table IIa). This could be due to the higher scaffold porosity. Lower compression work values indicate a softer and more compliant scaffold, which can better conform to wound shapes, enhancing patient compliance.

The DMA results revealed that both scaffolds had damping factor values much lower than 1. Since the damping factor is calculated as ratio between E'' (loss modulus) and E' (storage modulus), this indicates that for both scaffolds the elastic mechanisms have a greater influence on the final properties of the material. In general, no statistical differences were observed between the values measured for the two samples, except for the values at 10 Hz, where the HL CS_{UBL} scaffold exhibited a lower value compared to the CM CS one.

The results from the compression test on the scaffolds upon 24 h hydration revealed significant differences in the mechanical properties between CM CS and HL CS_{UBL} scaffolds (Table IIa, IIb). The force required to compress the CM CS scaffold to 70 % of its original height is

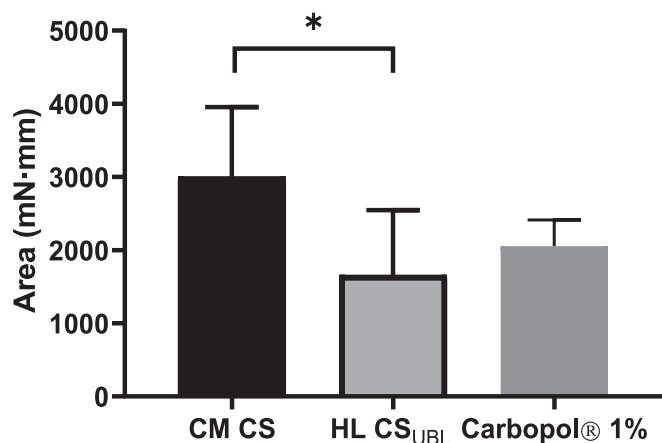


Fig. 9. Adhesion work value calculated as the area under the curve of maximum detachment force *versus* distance (mN-mm) for CM CS, HL CS_{UBL} and Carbopol® scaffolds on a gelatine substrate conditioned with WSF. Ordinary One-Way Anova and Dunnett's multiple comparisons test ($p < 0.05$) (mean values $\pm$ SD; $n = 6$). * $p < 0.05$, *** $p < 0.0005$.

Table IIa

Compression work (AUC, mN-mm) and ultimate strength (mN) values of freeze-dried CM CS and HL CS_{UBL} scaffolds as such and upon hydration in PBS. Unpaired t -test with Welch's correction (p value ≤ 0.05) (mean values $\pm$ SD; $n = 4$). ** $p < 0.01$, **** $p < 0.0001$.

Sample	AUC (mN-mm)		Ultimate strength (mN)	
	Dried	Hydrated	Dried	Hydrated
CM CS	68,715 $\pm$ 5052	3625 $\pm$ 446	30,865 $\pm$ 3040	14,563 $\pm$ 1921
HL CS _{UBL}	54,497 $\pm$	1104 $\pm$	24,204 $\pm$	557 $\pm$ 74
	3292**	91****	2700**	****

Table IIb

Damping factor (E''/E') values calculated at different frequency (10, 1, 0.1 and 0.01 Hz) of freeze-dried CM CS and HL CS_{UBL} scaffolds as such. Unpaired t -test with Welch's correction (p value ≤ 0.05) (mean values $\pm$ SD; $n = 3$). ** $p < 0.01$, ns: not significant.

Frequency (Hz)	Damping factor	
	CM CS	HL CS _{UBL}
10	0.1533 $\pm$ 0.0095	0.0994 $\pm$ 0.0146**
1	0.1357 $\pm$ 0.0136	0.1115 $\pm$ 0.0117 ^{ns}
0.1	0.0932 $\pm$ 0.0243	0.0923 $\pm$ 0.0101 ^{ns}
0.01	0.1200 $\pm$ 0.0035	0.1024 $\pm$ 0.0055 ^{ns}

considerably higher (14,563 $\pm$ 1921 mN) compared to the force required for the HL CS_{UBL} one (557 $\pm$ 74 mN). This behaviour is probably due to the greater HL CS_{UBL} scaffold hydration properties. As already mentioned, a softer scaffold should conform more closely to the wound bed, reducing discomfort and potential damage to newly formed tissue during the healing process [77].

Upon 24 h hydration, the viscoelastic properties of the two freeze-dried scaffolds, CM CS and HL CS_{UBL}, were evaluated. HL CS_{UBL} scaffold was characterized by a higher storage modulus (G'), indicating a superior elastic behaviour compared to CM CS scaffold (Fig. 10A), suggesting a greater protective action of the wound bed. Both scaffolds were characterized by similar loss tangent ($\tan \delta$) lower than 1, indicating a prevalence of the elastic contribution on the viscous one (Fig. 10B).

3.3.5. In vitro degradation test

A degradation test was performed on HL CS_{UBL} and CM CS scaffolds to evaluate their stability in PBS medium at 37 °C for 28 days. Fig. 11 shows the comparison of the weight loss (%), for each sample, at different time points. HL CS_{UBL} scaffold was significantly more resistant to degradation preserving 75.5 $\pm$ 5.1 % ($p < 0.0001$) of their initial weight after 28 days of immersion in WSF medium with respect to CM CS scaffold and other CS-based formulations reported in literature [70]. This behaviour can be attributed to the different HL CS_{UBL} structure, due to the presence of melanin-CS complexes.

After 7 days of immersion in WSF, as shown in Fig. S3 (Supplementary Material), many of the pores in the sponge-like dressings had collapsed. However, the SEM images confirmed that the overall structure was better preserved in the case of HL CS_{UBL} compared to CM CS. The HL CS_{UBL} dressing retained a more defined porous architecture, indicating greater structural stability after prolonged exposure to the fluid.

Although the biocompatibility of the solutions prior to lyophilization was tested and shown to be compatible, and the scaffolds promoted fibroblasts proliferation over 7 days (See Section 3.3.6), the degradation products in simulated wound fluid were not specifically analyzed. However, given the positive cell response, it can be concluded that these degradation products are not toxic. Nonetheless, the exact degradation behaviour remains a limitation for understanding long-term performance.

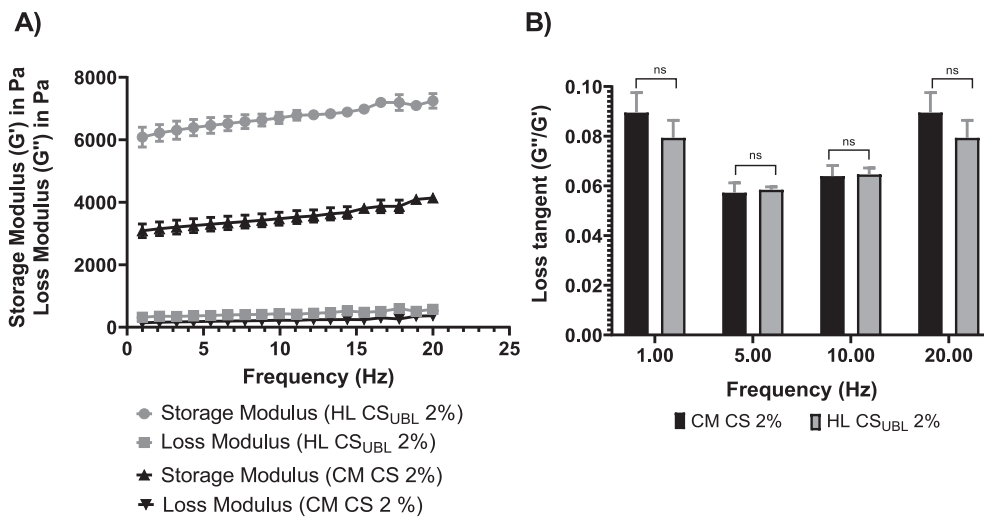


Fig. 10. Viscoelastic properties of CM CS and HL CS_{UBL} scaffolds hydrated for 24 h: A) Storage modulus (G') (Pa) and loss modulus (G'') (Pa) at increasing frequencies (Hz); B) Loss tangent (tang δ) at increasing frequencies (Hz). Unpaired *t*-test with Welch's correction (*p* value ≤ 0.05) (mean values $\pm$ SD; *n* = 4).

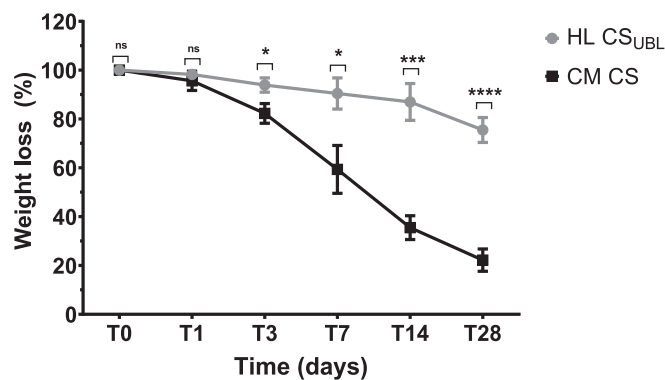


Fig. 11. % loss on dry weight (%) calculated for each sample (HL CS_{UBL} and CM CS) at different time points (days) (mean values $\pm$ SD; *n* = 3). Statistical significance was determined using multiple *t*-test using the Holm-Sidak method. ns: not significant, * *p* < 0.05, *** *p* < 0.0005, **** *p* < 0.0001.

3.3.6. In vitro cell proliferation test

After 3 and 7 days of exposure, NHDF cultured in media conditioned by CM CS and HL CS_{UBL} scaffolds showed significantly enhanced proliferation compared to the control, with absorbance values (570 nm) approximately 2-fold greater than the control (complete medium) (Fig. 12). Higher absorbance values are observed for HL CS_{UBL} scaffold indicating a higher cell proliferation.

4. Conclusions

This study comprehensively characterizes HL-derived CS for their potential application in wound healing. Between the two HL-derived CS, HL CS_{UBL} is characterized by the best functional properties in comparison to HL CS_{BL} and a CM CS characterized by similar Mw and DD. The presence of melanin-CS complexes in HL CS_{UBL} could be responsible for the enhanced rheological, antioxidant and anti-inflammatory properties, making it a promising biomaterial for wound healing treatment.

HL CS_{UBL} freeze-dried scaffolds show different morphological, hydration and mechanical properties with respect to CM CS ones. In particular, the combination of softer mechanical properties, higher porosity and hydration properties, slower degradation and higher capability of enhancing fibroblast proliferation indicates the high potential of such scaffolds in the treatment of wounds. HL CS_{UBL} scaffolds provide a balance between an optimal structural support and a

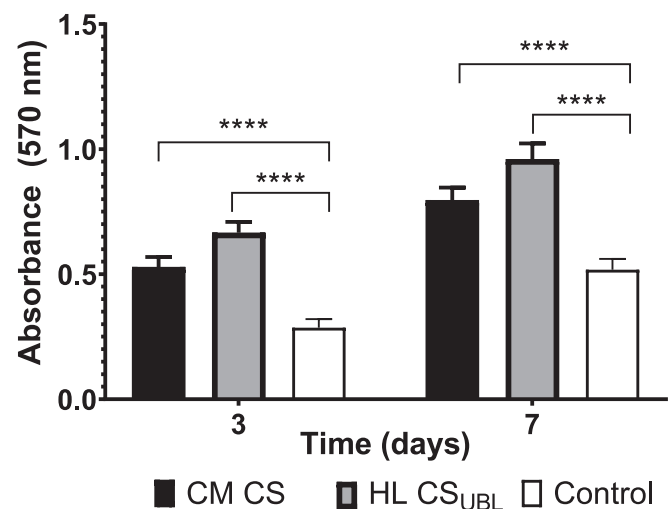


Fig. 12. Absorbance (570 nm) of NHDF after indirect exposure to CS-based sponge-like dressings. Ordinary One-Way Anova and Dunnett's multiple comparisons test (*p* < 0.05) comparing both HL CS_{UBL} and CM CS to the control (mean values $\pm$ SD; *n* = 6). **** *p* < 0.0001.

favourable environment for tissue regeneration over extended periods of time.

Moreover, the use of HL CS_{UBL} brings undoubted benefits such as reduced processing costs and environmental impact, thus presenting a promising avenue for a sustainable material development. Future research efforts will focus on elucidating the role of CS-melanin complexes on the physico-chemical and biological properties of CS-based scaffolds and on the validation of the efficacy of HL CS_{UBL} scaffolds on an animal model.

This study presents several notable advancements in the field of biomaterials. The use of *Hermetia illucens*-derived CS as a bioactive material for wound healing formulations introduces a novel and underexplored approach in biomedical applications. More specifically, repurposing pupal exuviae—a waste byproduct from insect farming—as a source for CS highlights the sustainability aspect of this research, aligning with circular economy principles. The comparison of HL CS_{UBL} and HL CS_{BL} solutions provides valuable insights into how processing methods, such as melanin removal, influence the structural and functional properties, offering a framework for optimizing CS-based

biomaterials for targeted applications. Finally, the development of sponge-like scaffolds using this insect-derived CS represents another significant contribution, demonstrating the versatility of this material. While this study demonstrates the potential of *Hermetia illucens*-derived CS for wound healing applications, further investigations are required to fully elucidate the role of melanin and other bioactive compounds, such as polyphenols, to the unique properties of *Hermetia illucens*-derived CS. Addressing these aspects in future research will provide a deeper understanding of how these components influence the functional properties of insect-derived CS and its suitability for biomedical applications.

Additionally, preclinical validation using an animal model should be considered to further evaluate the efficacy, safety, and long-term performance of this CS in wound healing applications, providing valuable insights for its potential clinical use.

CRediT authorship contribution statement

Micaela Giani: Writing – original draft, Visualization, Software, Investigation, Data curation, Conceptualization. **Caterina Valentino:** Validation, Software, Investigation, Conceptualization. **Barbara Viganani:** Writing – review & editing, Methodology, Conceptualization. **Marco Ruggeri:** Writing – review & editing, Validation. **Anna Guarnieri:** Resources, Investigation. **Rosanna Salvia:** Resources, Investigation. **Carmen Scieuzo:** Writing – review & editing, Resources, Investigation. **Patrizia Falabella:** Writing – review & editing, Resources, Investigation. **Giuseppina Sandri:** Writing – review & editing, Project administration, Funding acquisition. **Silvia Rossi:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Acknowledgements

This study has been funded by EU funding within the NextGenerationEU-MUR PNRR,

Extended Partnership initiative on Emerging Infectious Diseases (Project no. PE00000007, INF-ACT). EMBO Post-Doctoral Fellowship (EMBO ALTF 189-2023) for M.G.

Moreover, University of Pavia (Fondo Ricerca Giovani, FRG) is kindly acknowledged for C.V.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2025.140903>.

Data availability

All data were submitted in the manuscript.

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