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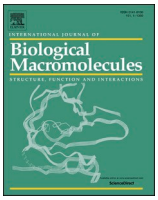

A comprehensive characterization of *Hermetia illucens* derived chitosan produced through homogeneous deacetylation

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ABSTRACT

The increasing demand for chitin and chitosan is driving research to explore alternative sources to crustaceans. Insects, particularly bioconverters as *Hermetia illucens*, are promising substitutes as they process food industry waste into valuable molecules, including chitin. Chitosan can be produced by chitin deacetylation: hot deacetylation to obtain a heterogeneous chitosan, the commonly produced, and cold deacetylation to obtain a homogeneous chitosan, not widely available. The two different treatments lead to a different arrangement of the amine and acetyl groups in the chitosan structure, affecting its molecular weight, deacetylation degree, and biological activity. This is the first report on the production and chemical-physical and biological characterization of homogenous chitosan derived from *H. illucens* larvae, pupal exuviae, and adults. This work, in addition to the report on heterogeneous chitosan by our research group, completes the overview of *H. illucens* chitosan. The yield values obtained for homogeneous chitosan from pupal exuviae (3 and 7 %) are in the range of insect (2–8 %) and crustaceans (4–15 %) chitosan. The evaluation of the antioxidant activity and antimicrobial properties against Gram-negative (*Escherichia coli*) and Gram-positive (*Micrococcus flavus*) bacteria confirmed the great versatility of *H. illucens* chitosan for biomedical and industrial applications and its suitability as an alternative source to crustaceans.

1. Introduction

In recent years, the spread of industrial-scale insect breeding has advanced further, prompted by the ongoing search for new protein sources for animal feed and, potentially, the necessity to manage the growing organic waste generated by farms [1,2]. Among these, *Hermetia illucens* rearing has emerged as a key element in this scenario; indeed, around 80 % of European insect producers engaged in its breeding. Its larvae showed versatility in growing on different organic substrates, bioconverting them efficiently into a protein-, lipid- and chitin-rich biomass that can find application in several fields [3–5]. Furthermore, *H. illucens* rearing provides several by-products, such as dead adults and pupal exuviae that represent a valuable source of chitin and chitosan [6]. Chitin stands out as one of the major natural polysaccharide, known for its structural prominence in the arthropod exoskeleton and in the fungal cell walls [6–8]. However, its hydrophobicity and limited solubility, attributed to its crystalline structure, pose challenges in processing and application, limiting the production of chitin-based products [9]. To overcome these restraints, chitin undergoes deacetylation, resulting in a more soluble derivative, chitosan [6,10]. The industrial chitin production relies on the waste from the fishing industry, particularly crustacean shells, with a chitin content ranging from 15 to 40 % [11,12]. Despite being an important source, some issues such as seasonality and sustainability related to crustaceans farming, as well as geographical limitations, have prompted the exploration of alternative sources. Insects emerge as a promising and sustainable chitin source, constituting up to 60 % of their cuticles [12–15]. The market for chitin, chitosan and their derivatives is expected to grow substantially from a value of $6.99 billion in 2021 to an estimated $23.90 billion by 2030, with a notable compound annual growth rate (CAGR) of 14.90 % from 2023 to 2030 [16]. Renowned for their biodegradable, biocompatible, non-toxic, antioxidant and antimicrobial properties, these polymers have applications in healthcare, agricultural and industrial sectors, as a sustainable alternative to synthetic materials [6,17–24]. Structurally,

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chitosan is a cationic polysaccharide, with its properties and applicability mainly depending on its chemical features. Unlike chitin, chitosan solubility is pH-dependent, facilitated by the protonation of amino groups at pH values below 6.5. The amine groups, along with the deacetylation degree (DD) and the molecular weight (Mw), influence its biological and technological features [25,26]. Chitosan has also proven to have good antimicrobial activity against both Gram-negative and Gram-positive bacteria, with varying effect on different bacterial structures [13,27]. Additionally, chitosan also demonstrates antioxidant properties, with the ability to scavenge free radicals as a consequence of the presence of hydroxyl and amine groups in its structure [28,29]. Its antioxidant activity is enhanced by some derivatives, such as chitooligosaccharides, chitosan sulfates, and N-2 carboxyethyl chitosan [28,29]. Commercially, chitosan is mainly produced through chemical deacetylation, due to its cost-effectiveness and suitability for mass production. This process involves soaking the chitin in a strong NaOH solution, with deacetylation carried out heterogeneously or homogeneously [30,31]. Heterogeneous treatment produces chitosan with a non-uniform block distribution of units, while homogeneous treatment produces a chitosan with a uniform distribution and an average DD of 50 % [31–33]. Physicochemical properties may differ between chitosan produced through these methods [34]. In insect- and crustacean-derived chitosan production, heterogeneous deacetylation is the most widely used method, yielding a polymer with high DD and readily soluble in weakly acidic solutions [35,36]. Homogeneous chitosan has not been extensively investigated because it is considered economically unfeasible for industrial production, due to low yields and long processing times. However, it is interesting to understand its potential, especially from sustainable sources such as insects. The present work aims to further deepen the knowledge on insect-derived chitosan by introducing, for the first time, the production and characterization of homogeneous chitosan from different biomasses of the insect *H. illucens*. Particularly, this study reports the methodologies and the results relating to: (i) the homogeneous deacetylation from chitin extracted from larvae, pupal exuviae and adults of *H. illucens*; (ii) the characterization of the physicochemical properties of the obtained chitosan; (iii) the evaluation of the biological properties and (iv) the comparison between homogeneous chitosan samples and those of the heterogeneous chitosan already produced from the same *H. illucens* biomasses (larvae, pupal exuviae and adults) to enhance their specific features.

2. Materials and methods

2.1. Chitin sample preparation

Chitin extraction and characterization from larvae, pupal exuviae and adults (biomasses) of *H. illucens* were performed according to the method reported by Triunfo et al. [10]. Briefly, insect samples were demineralized for 1 h at room temperature with 0.5 M HCOOH and then deproteinized for 2 h at 80 °C with 2 M NaOH. Part of chitin was decolorized at 90 °C for 60 min with 5 % H_2O_2. After washing, the resulting chitin was dried in order to be, successively, deacetylated.

2.2. Chitosan production

Chitosan was obtained by homogeneous deacetylation of both no decolorized (No Dec) and decolorized (Dec) chitin extracted from the different biomasses of *H. illucens*. Chitin was suspended in 12 M NaOH at 4 °C overnight. Ice was added and the mixture was centrifuged at 10000 rpm for 15 min at 4 °C. The supernatant was collected and incubated for 24–48 h at room temperature. Ice was added again and the solution was titrated with HCl in order to precipitate chitosan [31]. The suspension was centrifuged and the collected chitosan was neutralized with distilled water. As with the heterogeneous procedure, further solubilization in 1 % (*v*/v) CH_3COOH and precipitation of the chitosan was carried out to achieve greater purity [10]. The yield of No Dec and Dec chitosan, related to chitin and to the dry weight of the raw insect biomass were determined, according to Triunfo et al. [10]. Commercial chitosan, derived from crustaceans, was purchased from Merck KGaA (Darmstadt, Hesse, Germany).

2.3. Chitosan characterization

2.3.1. Fourier-transform infrared spectroscopy (FTIR)

The IR spectra of chitosan from *H. illucens* biomasses and commercial chitosan were recorded using a Jasco 460Plus IR spectrometer, as reported in Triunfo et al. [10]. Samples were scanned with a resolution of 4 cm^{-1} and 100 accumulations, and the transmittance values were evaluated between 4000 and 400 cm^{-1}. The spectra were processed using JASCO Spectra Manager software.

2.3.2. X-ray diffraction analysis (XRD)

The X-ray diffraction peaks of the chitosan samples were measured using an X-ray diffractometer (X'Pert PRO, Philips) at 40 kV, 32 mA and 2θ with a scan angle between 5° and 50° at a scan speed of 0.04° s^{-1}. The crystallinity index (CrI) of chitosan samples was calculated, as reported in Triunfo et al. [10]. The crystallites size of chitosan samples was also determined [37].

2.3.3. Scanning electron microscopy (SEM)

The surface morphologies of chitosan samples produced from *H. illucens* were evaluated using a FEI FEG-Quanta 450 (Thermo Fisher Scientific, Pardubice, Czech Republic). To obtain high-resolution images, all chitosan samples were coated with platinum before SEM observation.

2.3.4. Determination of chitosan deacetylation degree

The deacetylation degree (DD) of all chitosan samples was determined by potentiometric titration, according to the method of Jiang et al. [38].

2.3.5. Determination of viscosity-average molecular weight

The viscosity-average molecular weight (M_v) of each chitosan sample was calculated by measuring the intrinsic viscosity of the respective chitosan solutions, according to the method reported in Triunfo et al. [10].

2.3.6. Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The free radical scavenging activity of chitosan samples from *H. illucens* was determined according to the method of Kaya et al. [39], modified. For each chitosan, different concentrations (5–0.156 mg/mL) were tested. $6{\cdot}10^{-5}$ M DPPH was added to homogeneous chitosan samples, and they were incubated for 30 min in the dark at room temperature. The absorbance was measured at 517 nm with a spectrophotometer (Thermo Scientific Multiskan Go). The antioxidant activity was expressed as IC_{50} value (mg/mL), indicating the concentration of chitosan at which DPPH radicals were scavenged by 50 %.

2.3.7. Determination of antimicrobial properties

The antimicrobial activity of homogeneous chitosan from *H. illucens* was determined according to Guarnieri et al. [13], through agar diffusion test and microdilution assay. For each chitosan, different concentrations (1.25–0.15 mg/mL) were tested.

2.3.7.1. Agar diffusion test. No Dec and Dec chitosan and commercial chitosan were dissolved in 1 % acetic acid at 1.25 mg/mL concentration. After stirring, the solutions were filtered and stored at 4 °C. Two bacteria strains (*Escherichia coli* and *Micrococcus flavus*) were distributed on Petri dishes, each chitosan sample was spotted onto them, and the dishes were incubated at 37 °C for 24 h, according to Guarnieri et al. [13]. Acetic

acid and commercial chitosan were employed as positive controls, distilled water as negative control. The antimicrobial activity was evaluated measuring the diameter of the inhibition zones (mm).

2.3.7.2. Microdilution assay and minimum inhibitory concentration (MIC) determination. The minimum inhibitory concentration (MIC) value for each chitosan sample was determined by microdilution assay, using serial dilutions of chitosan, according to Guarnieri et al. [13]. To evaluate the solvent influence, acetic acid alone was tested at the same concentrations and each bacterial culture (*E. coli* and *M. flavus*) was used as control. After the treatment, plates were incubated at 37 °C for 24 h. The absorbance was measured at 600 nm with a spectrophotometer (Thermo Scientific Multiskan Go), detecting the bacterial concentrations. The MIC value was defined as the lowest concentration of chitosan for which no bacterial growth was observed.

2.4. Statistical analysis

All measurements were performed in triplicate and data were expressed as average ± standard deviation. Data were analyzed by one-way Anova or two-way Anova and Tukey's *post-hoc* test. Pairwise comparison of percentage data was performed with the Chi-square test with Yates' correction. Statistical analyses were performed using a GraphPad Prism version 6.0.0 for Windows (GraphPad Software, San Diego, California USA).

3. Results and discussion

3.1. Chitosan recovery and chitosan yield from H. illucens biomasses

Chitosan was produced through homogeneous deacetylation of both No Dec and Dec chitin extracted from the three biomasses of *H. illucens*, thus obtaining six different chitosan samples (Fig. 1). As expected, No Dec chitosan appeared darker than the Dec samples (Fig. 1). All homogeneous Dec chitosan appeared clearer than their respective heterogeneous chitosan reported by Triunfo et al. [10]. The low temperatures of homogeneous deacetylation prevent browning due to the high temperatures used in the heterogeneous process.

Deacetylation yields related to chitin and the dry weight of the raw insect biomass are determined for all chitosan samples from *H. illucens* and are shown in Table 1. For all insect biomasses, the deacetylation yields of the Dec chitosan were significantly higher than the respective No Dec ones (larvae $X^2 = 20.3$, $p < 0.001$; pupal exuviae $X^2 = 13$, $p < 0.001$; adults $X^2 = 11.28$, $p < 0.001$). The yield of chitosan related to the original insect biomass followed the same trend (Dec > No Dec chitosan), although no significant differences were found between the two values for each sample (larvae $X^2 = 0.107$, $p = 0.74$; pupal exuviae $X^2 = 947$, $p = 0.33$; adults $X^2 = 0$, $p = 1$). Yield of chitosan derived from *H. illucens* by homogeneous deacetylation was reported exclusively by Hahn et al. [31] for larval exoskeletons, with values of 13 % and 4 %, related to chitin and insect biomass, respectively. Comparing our results with Hahn's, they were higher, both for deacetylation yields, particularly for Dec chitosan samples (chitosan/chitin >30 %) and for final yield related to raw sample, for chitosan from pupal exuviae (3 and 7 %, No Dec and Dec, respectively).

Table 1.
Yields (%) related to chitin and raw insect biomass of the different chitosan samples homogeneously deacetylated from No Dec and Dec chitin extracted from larvae (L), pupal exuviae (PE) and adults (A) of *H. illucens*. Data are expressed as mean ± standard deviation. Different letters indicate significant differences in the yield related to chitin and raw insect among all samples ($p < 0.05$). Data were analyzed with one-way ANOVA and Tuckey *post-hoc* test. Asterisks indicate highly significant differences ($p < 0.001$) between yields of No Dec and Dec chitosan for each insect sample. Data were analyzed with Chi-Square test with Yates' correction.

Chitosan sample	Chitosan/chitin%	Chitosan/raw sample%
L No Dec	6 ± 1^{a}	0.9 ± 0.6^{b}
L Dec	$32 \pm 6.6^{a***}$	2.5 ± 0.8^{b}
PE No Dec	10.9 ± 2.4^{b}	3 ± 0.8^{b}
PE Dec	$33 \pm 7.8^{a***}$	7 ± 2.4^{a}
A No Dec	10 ± 2.3^{b}	1.0 ± 0.4^{b}
A Dec	$30 \pm 0.5^{a***}$	2.0 ± 0.1^{b}

The differences in yield values compared to chitin, between No Dec and Dec samples, demonstrated a great influence of the bleaching treatment on the ability of chitosan to deacetylate under homogeneous conditions. The pigments probably limited NaOH to the polymer chains at low temperatures, resulting in a lower efficiency in the removal of acetyl groups. Indeed, for each biomass of *H. illucens*, the deacetylation yields of No Dec homogeneous chitosan were much lower than those of No Dec chitosan obtained by heterogeneous method (hot deacetylation) (6–10 % vs 25–30 % for homogeneous and heterogeneous chitosan,

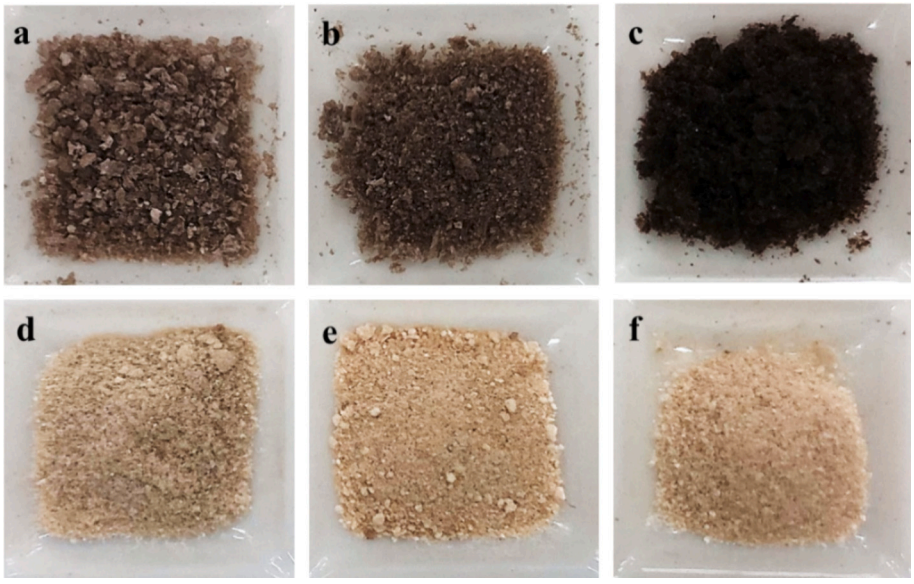

Fig. 1. Homogeneous chitosan samples produced by deacetylation of No Dec (a, b, c) and Dec (d, e, f) chitin samples from the different *H. illucens* biomasses: larvae (a,d), pupal exuviae (b,e) and adults (c, f).

respectively). However, for Dec chitosan samples the differences with heterogeneous ones are less pronounced (30–33 % vs 33–42 % for homogeneous and heterogeneous chitosan, respectively) [10]. As for heterogeneous chitosan, the highest final yields were achieved from *H. illucens* pupal exuviae (3 and 7 %, for No Dec and Dec, respectively). Results obtained suggest the influence on the yields of the experimental conditions of the deacetylation method adopted. This can be explained by the homogeneous deacetylation occurring selectively in chitin amorphous region, leaving the crystalline fraction unaffected [31,32]. Therefore, homogeneous deacetylation could be considered economically viable for industrial production only for samples further purified by bleaching. Homogeneous deacetylation was also experimented for crustacean chitin in order to obtain a chitosan with a regular distribution of the residual acetamide groups, compared to heterogeneous deacetylation [32,40]. Indeed, by applying high temperatures, deacetylation proceeded rapidly towards the crystalline portion of the chitin, producing a block distribution of units on the chitosan chain. Incubation of chitin in alkali at low temperature, on the other hand, induced swelling and activation of the polymer, which became amorphous, allowing similar deacetylation along the chitin chain [40]. However, no information is available on the yields of chitosan obtained by this method. The yields obtained in the present work were in the range of heterogeneously produced chitosan from insects (2–8 %) and are slightly lower than those of crustacean-derived chitosan (4–15 %) [30,41–45]. The differences can be explained considering that insect biomass has a higher protein and lipid content than crustaceans, which may lower the final polymer yield [46]. However, as reported for chitin, chitosan yield can be affected by various factors, including the source, the purification methods and the deacetylation treatments applied to chitin [32].

3.2. FTIR analysis

Spectra obtained from FTIR analysis of homogeneous chitosan, both No Dec and Dec, derived from *H. illucens*, are shown in Fig. 2(a–b), along with a reference sample of commercial chitosan.

The characteristic peaks confirming the identity of chitosan were

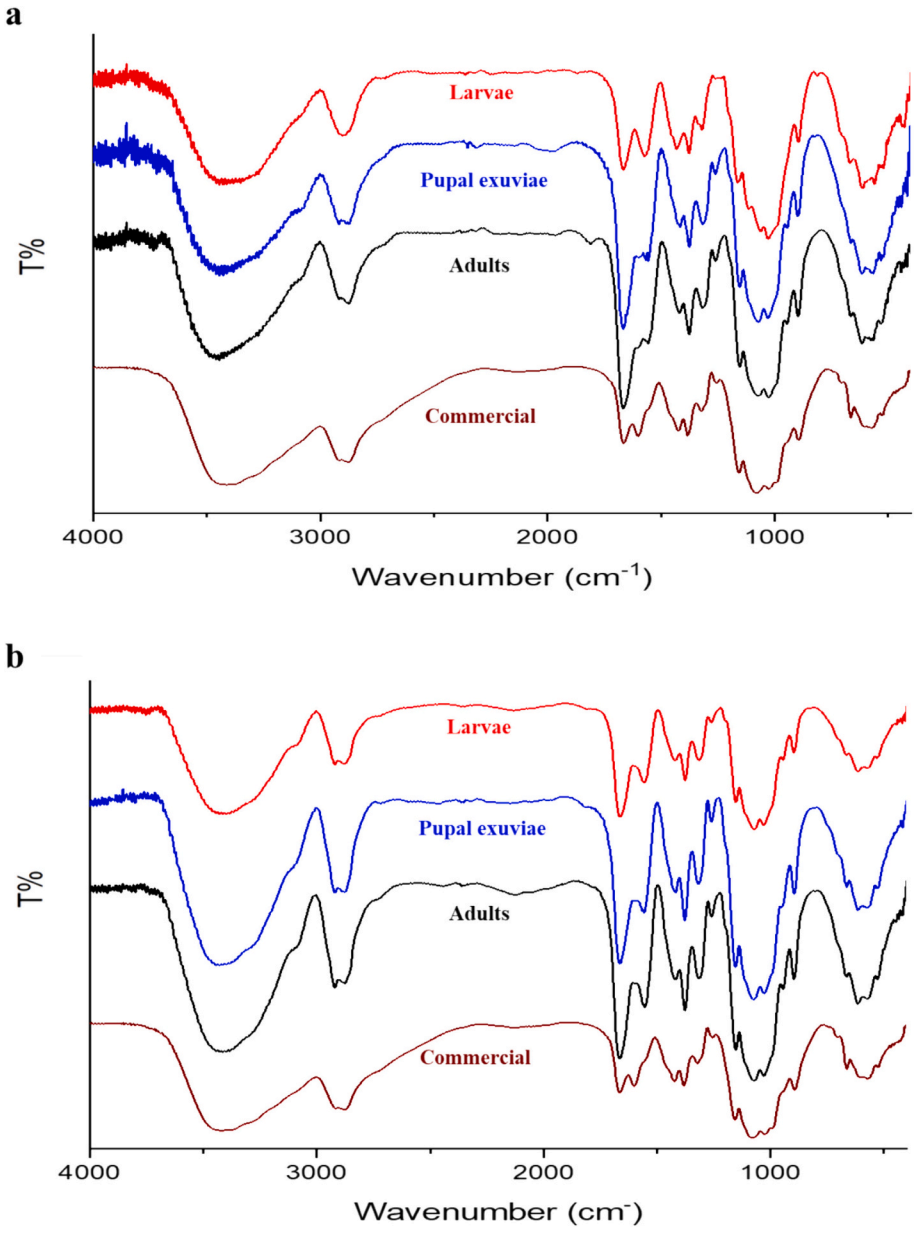

Fig. 2. FTIR spectra of No Dec (a) and Dec (b) homogeneous chitosan samples produced from *H. illucens* larvae (red line), pupal exuviae (blue line) and adults (black line). Commercial chitosan (wine lines) derived from crustaceans is also reported.

identified, specifically the NH-bending (amide II) and CO-stretching (amide I) bands around 1590 cm^{-1} and 1655, respectively [10,43,47–49]. The spectra analysis revealed no significant distinctions between the chitosan produced from different insect biomasses; similarly, within each sample, there were no observable differences between the spectra of the Dec and respective No Dec chitosan (Fig. 2a–b). However, some distorted or extraneous signals, noticed in the spectra of No Dec samples, were attributed to residual chitin portions that were not completely deacetylated. Particularly, differences between homogeneous and heterogeneous samples were identified around peaks at 1655 and 1590 cm^{-1}, attributed to the distinct deacetylation methods employed. Indeed, as expected, the homogeneous chitosan samples, both Dec and No Dec, appeared less deacetylated than the respective heterogeneous samples [10].

3.3. X-ray diffraction

XRD patterns, representing No Dec and Dec homogeneously deacetylated chitosan obtained from larvae, pupal exuviae and adults of *H. illucens,* are reported in Fig. 3(a–b).

The XRD analysis of homogeneous chitosan samples revealed two sharp peaks around 10° and 20°, confirming the identity of the polymer. However, notable differences in intensity were observed compared to the heterogeneous samples reported by Triunfo et al. [10]. Unlike the latter, the homogeneous samples exhibited inverted intensity peaks, with the peak at 10° being more pronounced than the one at 20°. This intensity discrepancy is probably due to the experimental conditions of the two deacetylation methods. Indeed, the use of low temperature NaOH induced chitin amorphization before deacetylation, resulting in a reduction of crystalline portions [40]; upon recrystallization, a redistribution of crystals may occur in a different manner. As reported for chitin and for heterogeneous chitosan from *H. illucens* [10], no significant differences were identified in the spectra between No Dec and Dec chitosan, except for the No Dec chitosan derived from larvae, which displayed peaks with more similar intensities. The CrI values for the chitosan samples were found similar, ranging from 50 to 68 % (Table 2), as well as the crystallite size (around 2 nm), both of which were lower than those obtained for commercial chitosan through the heterogeneous process (Table 2). As for heterogeneous chitosan, there is a trend for Dec samples to exhibit slightly higher crystallinity compared to the No Dec ones, although not significantly, except for adult chitosan, in which No Dec chitosan preserves the crystalline structure of chitin more effectively. The comparative analysis of the effect of bleaching and deacetylation methods on the crystallinity of chitosan remains limited due to the absence of relevant studies in the literature. Notably, within the limited literature reporting the production of chitosan from *H. illucens*, the degree of crystallinity was never calculated. The crystallinity of homogeneous chitosan derived from *H. illucens* was similar to that reported for the other insects (33–69 %) [43,50–52]. In contrast, compared to heterogeneous chitosan obtained from the same biomass of *H. illucens* (74–86 %), it was lower [10].

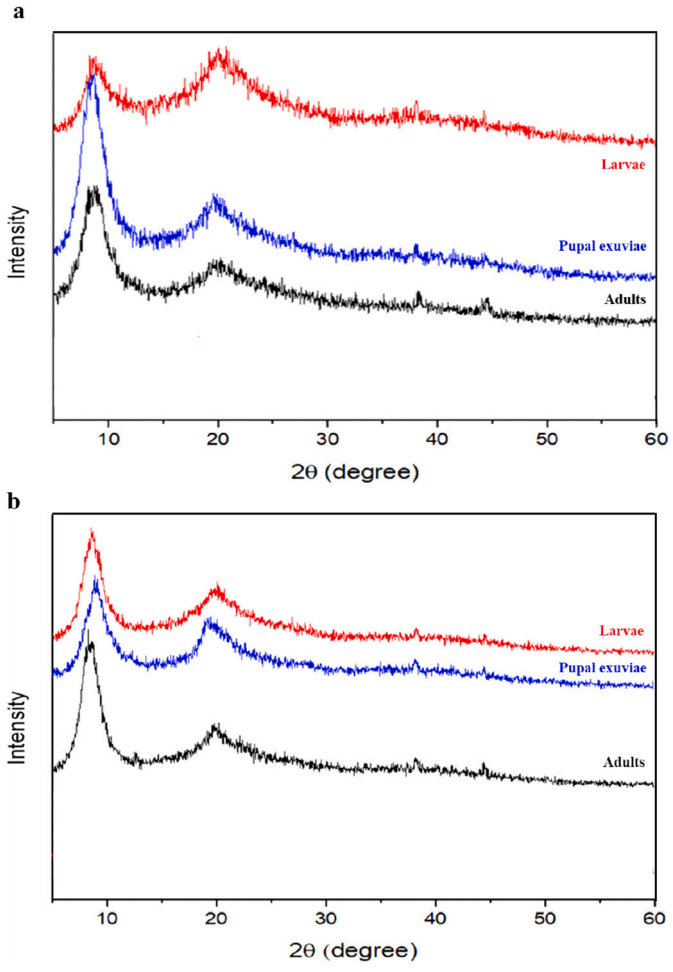

Fig. 3. XRD spectra of both No Dec (a) and Dec (b) homogeneous chitosan samples produced from *H. illucens* larvae (red line), pupal exuviae (blue line) and adults (black line).

3.4. SEM analysis

Surface morphologies of homogeneous chitosan, both No Dec and Dec, derived from *H. illucens* biomasses are represented in Fig. 4.

All chitosan samples were shown to partially preserve chitin cell structure, but no particular differences were noted. Indeed, in contrast to heterogeneous chitosan reported in Triunfo et al. [10], homogeneous samples, especially No Dec, exhibited filamentous, spider web-like structures with a less pronounced fibrillar arrangement, typical of the chitin structural morphology. Furthermore, the Dec samples showed a reduction in surface filamentous structures, indicating a potential cleaning effect of the bleaching process. This alteration in morphology can be attributed to the different deacetylation conditions applied, as already stated for crystalline properties. Analogously to chitin, also chitosan had pores on their surface. Due to the missing relevant studies on the surface morphology of chitosan from *H. illucens*, a direct comparison with the existing literature is not possible. The morphology of insect chitosan, as for chitin, revealed the presence of nanofibers with a regular or random distribution and the possible existence of pores. The surface morphology of chitosan was affected not only by deacetylation conditions, but also by the features of the original chitin, including species, developmental stage, genus and body part of the insect

Table 2.
Crystallinity index (CrI%), crystallite size (nm-D_{100}), deacetylation degree (DD) and viscosity-average molecular weight (M_v) of homogeneous chitosan obtained from both No Dec and Dec chitin from *H. illucens* larvae (L), pupal exuviae (PE) and adults (A) and a commercial chitosan derived from crustaceans. Data are expressed as mean ± standard deviation. Different letters in a column indicate significant differences ($p < 0.05$) in the CrI, DD and M_v, among the samples. Data were analyzed with one-way ANOVA and Tuckey *post-hoc* test.

Chitosan sample	CrI (%)	Crystallite size (nm)	DD (%)	M_v (kDa)
L No Dec	60^{d}	1.3	56 ± 1.9^{c}	195 ± 35^{c}
L Dec	64^{c}	1.5	60 ± 3.6^{c}	97 ± 9^{d}
PE No Dec	51^{e}	1.6	62 ± 3.5^{c}	285 ± 29^{b}
PE Dec	68^{b}	2	72 ± 3.6^{b}	115 ± 16^{d}
A No Dec	58^{d}	1.4	59 ± 2.9^{c}	258 ± 28^{b}
A Dec	52^{e}	1.7	61 ± 2.3^{c}	89 ± 6^{d}
Commercial	79^{a}	4	92 ± 0.7^{a}	370 ± 13^{a}

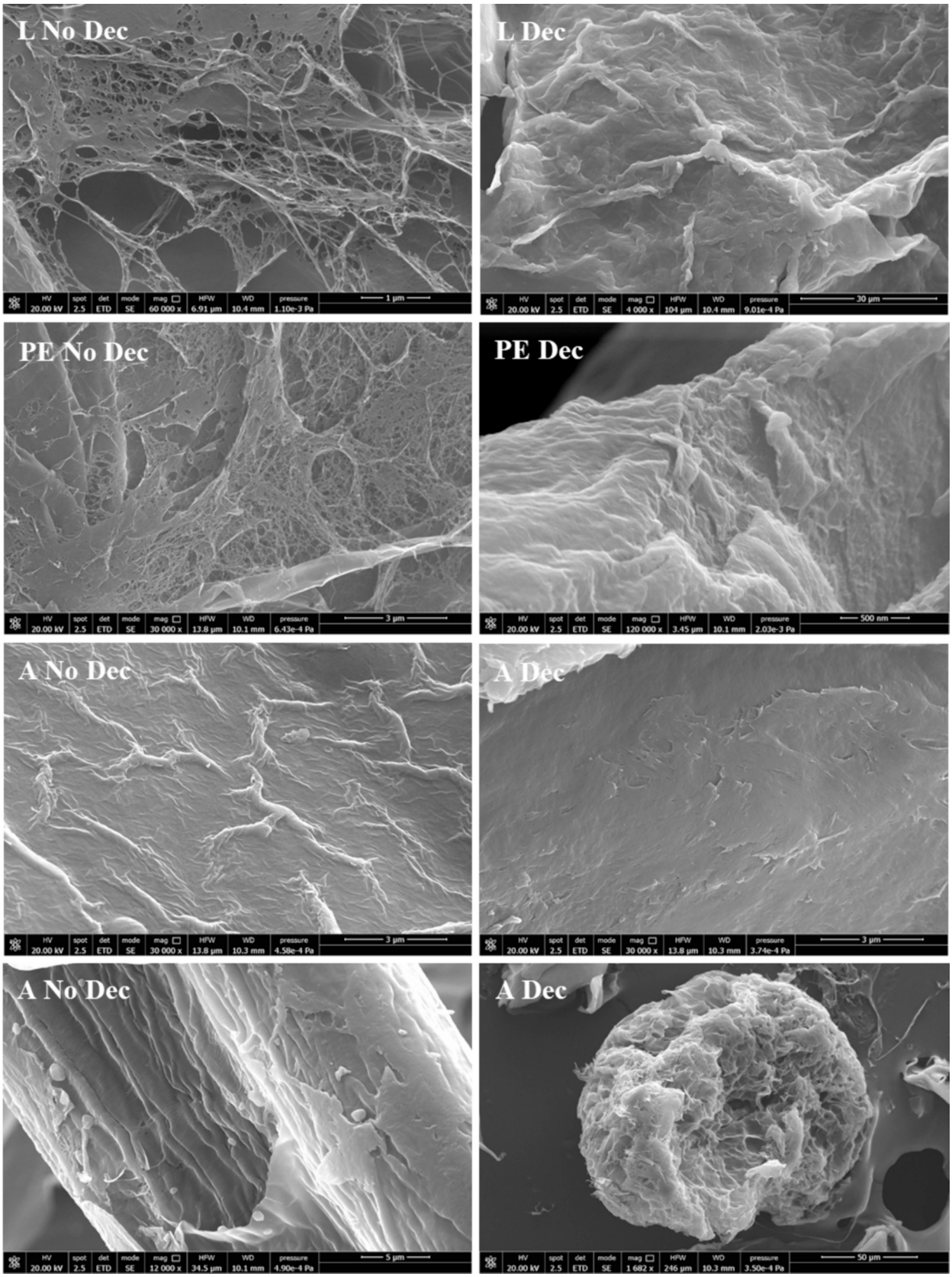

Fig. 4. SEM images of homogeneous chitosan from *H. illucens* larvae (L), pupal exuviae (PE) and adults (A) before (No Dec) and after (Dec) bleaching treatment. Bars in (L): 1 μm; bars in (PE): 500 and 3 μm; bars in (A): 1, 40 and 500 μm.

[43,50,52,53]. When trying to establish a correlation between the final physicochemical properties of chitosan and the structure of the initial chitin, the relevance of the biomass of *H. illucens* became evident.

The study found different morphologies in various biomasses. The findings suggested that deacetylation process itself played a significant role in modelling both the morphological structure and the chemical properties of the obtained chitosan samples.

3.5. Deacetylation degree (DD) and Viscosity-average molecular weight (M_v)

Chitosan DD was determined by potentiometric titration. DD values of homogeneous chitosan samples produced from larvae, pupal exuviae and adults of *H. illucens* are presented in Table 2. DD values approximately ranged from 55 to 60 %, with the Dec chitosan from pupal exuviae being significantly the most deacetylated (around 70 %) among all chitosan samples. Since no significant differences were found between the No Dec and the respective Dec samples, except for chitosan from pupal exuviae, the results suggest an influence of the deacetylation method on the DD value. Therefore, treatment with concentrated NaOH at low temperatures appears to be less effective in removing acetyl groups from the chitin chain than hot deacetylation, the commonly used method. Indeed, Triunfo et al. [10] reported DD values around 90 % for heterogeneous chitosan obtained from the same biomasses. The same effect was observed by Hahn et al. [31], who obtained chitosan from larval exoskeletons with 34 % DD through homogeneous deacetylation, in contrast to the 72 % measured with heterogeneous deacetylation. Also for crustacean-derived chitosan, the average DD obtained by homogeneous deacetylation is low (about 55 %) [33]. DD is dependent on the deacetylation conditions applied, in terms of temperature, reaction time and NaOH concentration. Generally, in the heterogeneous deacetylation, higher temperatures can increase the DD [35].

In addition to DD, another chemical parameter affecting chitosan properties is its Mw. Mw for all chitosan samples was measured via viscosimetry (M_v), with the determination of intrinsic viscosity (η). Results are reported in Table 2. The M_v values of all chitosan samples derived from *H. illucens* ranged widely from 90 to 285 kDa, with significantly lower values compared to the commercial chitosan (370 kDa), used as reference. Particularly, the M_v of homogeneous chitosan samples obtained from Dec chitin exhibited lower values than their respective No Dec samples (Table 2). These results indicate an observable impact of the chitin bleaching process on the M_v of the resulting chitosan, suggesting a reduction in the Mw of the starting chitin due to induced polymer chain scissions [10,35]. Comparing the M_v values of homogeneous with heterogeneous chitosan, especially with regard to No Dec samples, the first ones showed the highest values [10]. This difference is attributed to the presence of chitinous portions not fully deacetylated, as confirmed by SEM analysis, leading to increased viscosity and, subsequently higher Mw. Generally, under homogeneous conditions these higher Mw values can be expected as attributed to the deacetylation effect only on the amorphous region of polymer [31]. Our M_v values, aligned with Triunfo et al. [10], are in the range reported for insect-derived chitosan (400–450 kDa) [52,54], although heterogeneous, and those for commercial chitosan (100–1000 kDa) [42,55].

It is known how DD and Mw affect physicochemical properties and biological activity of chitosan. It was generally reported that low and medium Mw chitosan has better antibacterial properties than high Mw ones, as it can more easily pass through the bacterial cell wall, disrupting its metabolism [56,57]. Other papers reported that high Mw creates an external barrier inhibiting nutrient uptake [58]. For DD, higher values may enhance the biological capacities of the polymer [59,60].

3.6. Antioxidant properties

The free radical scavenging activity of homogeneous chitosan produced from *H. illucens* was assessed. Results are shown in Table 3 and Fig. 5. The radical scavenging capacity increased proportionally with rising concentrations for all chitosan samples. At 5 mg/mL concentration, this activity ranged from 31 to 40 % for Dec and commercial samples, while slightly lower values (24–31 %) were observed for No Dec ones (Fig. 5). Generally, homogeneous chitosan demonstrated notable free radical scavenging activity, with IC_{50} values ranging from 7.5 to 18 mg/mL (Table 3). Notably, the Dec samples (IC_{50} 7.5–8 mg/mL) exhibited significantly similar antioxidant activity compared to commercial chitosan, in contrast to the No Dec samples (IC_{50} 10.7–18 mg/mL), with the lowest activity observed for No Dec chitosan from adults. This difference confirms the influence of the bleaching step on the antioxidant activity, as demonstrated for the other analyzed parameters, especially evident in samples derived from adults, the highest pigment content biomass. In general, this is the first report on the antioxidant activity of chitosan produced through a homogeneous method and, specifically, as a product derived from *H. illucens*. As for the other parameters, the comparison was made with chitosan produced by the heterogeneous method. Notably, with these IC_{50} values, the antioxidant activity of all homogeneous chitosan was similar (No Dec samples) or higher (Dec samples) than that of chitosan samples derived from shiitake fungi and crab shells (9–16 mg/mL) [61,62], pupal exuviae of *H. illucens* (9–10 mg/mL) [23], grasshoppers (11 mg/mL) [63] and larvae of *Leptinotarsa decemlineata* (10 mg/mL) [48]. Furthermore, our chitosan samples showed better activity than chitosan from *Zophobas morio* (66–141 mg/mL) [64], and worse activity than chitosan derived from L. *decemlineata* adults and *Musca domestica* larvae (2–4 mg/mL) [48,54].

Table 3.
Scavenging ability (IC_{50}) and minimum inhibitory concentration (MIC) of homogeneous chitosan obtained from both No Dec and Dec chitin from *H. illucens* larvae (L), pupal exuviae (PE) and adults (A) and a commercial chitosan derived from crustaceans. Data are expressed as mean ± standard deviation. Different letters in a column indicate significant differences ($p < 0.05$) in the IC_{50} and MIC among the samples. Data were analyzed with one-way ANOVA and Tuckey *post-hoc* test).

Chitosan sample	DPPH IC_{50} (mg/mL)	MIC (mg/mL)
L No Dec	10.7 ± 0.6^{b}	0.15 (*E. coli*) 0.15 (*M. flavus*)
L Dec	7.8 ± 0.2^{a}	0.15 (*E. coli*) 0.15 (*M. flavus*)
PE No Dec	12.1 ± 1.0^{c}	0.15 (*E. coli*) 0.3 (*M. flavus*)
PE Dec	7.9 ± 0.3^{a}	0.15 (*E. coli*) 0.15 (*M. flavus*)
A No Dec	17.8 ± 0.2^{d}	0.15 (*E. coli*) 0.15 (*M. flavus*)
A Dec	7.5 ± 0.5^{a}	0.15 (*E. coli*) 0.15 (*M. flavus*)
Commercial	7.1 ± 0.1^{a}	–

In literature, it was reported that properties such as Mw and DD influence the antioxidant capacity of chitosan. Particularly, DD showed to promote it at high values; unlike Mw, which has greater activity at lower values, explainable by the shorter size of the polymer chains and fewer intramolecular bonds releasing the reactive groups now operating to stop ROS activity [60,65]. This is in agreement with our results, reporting higher antioxidant activity for Dec chitosan samples, lighter in Mw than the No Dec ones. For commercial chitosan, characterized by high Mw, the antioxidant activity, comparable to that of our Dec chitosan, could be related to the higher DD. Considering the chemical peculiarities of chitosan produced from *H. illucens* using the two deacetylation methods, homogeneous samples derived from pupal exuviae showed a scavenging activity no different from that demonstrated for heterogeneous chitosan samples from the same biomass [10,23].

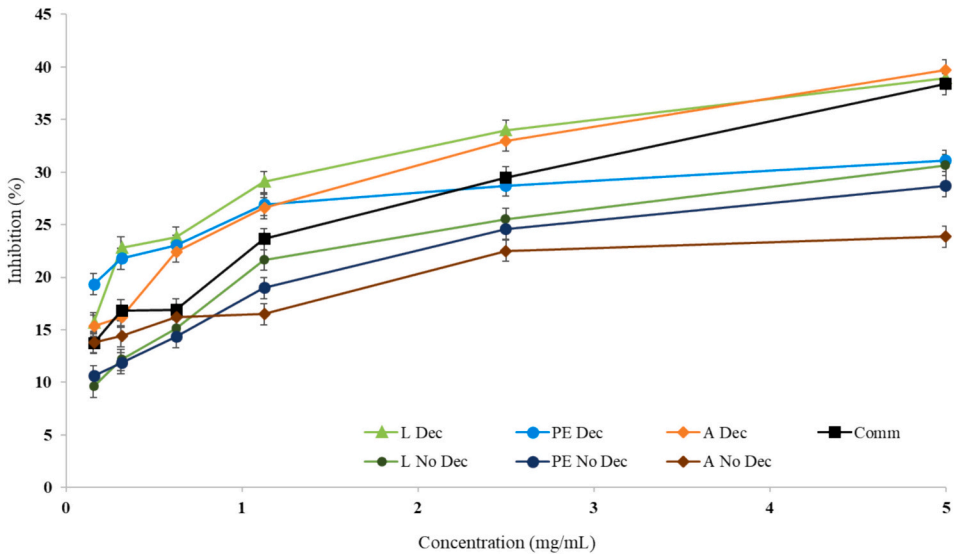

Fig. 5. Free radical scavenging activity of No Dec and Dec chitosan samples obtained from *H. illucens* larvae (L), pupal exuviae (PE) and adults (A) and commercial one derived from crustaceans (Comm). Data are expressed as mean ± standard deviation.

3.7. Antimicrobial properties

The antimicrobial activity of homogeneous chitosan produced from *H. illucens* was assessed with agar diffusion test and microdilution assay. This is the first report investigating the antimicrobial activity of chitosan homogeneously produced from *H. illucens*, both qualitatively and quantitatively. Therefore, as for the other studied parameters, the comparison was made with commonly produced chitosan where not possible with homogeneous one.

3.7.1. Agar diffusion test

Homogeneous chitosan produced from *H. illucens* induced the formation of inhibition zones on both the bacteria, *E. coli* and *M. flavus*, at all tested concentrations (1.25–0.15 mg/mL). Results are reported in Table 4 and Fig. 6.

Commercial chitosan, used as positive control, showed inhibition zones, in contrast to distilled water, used as negative control, which did not produce inhibitions; acetic acid, on the other hand, had a slight effect, showing non-determinable inhibition zones, confirming what has already been shown in Guarnieri et al. [13], for heterogeneous chitosan.

Homogeneous chitosan samples from *H. illucens* produced significant inhibition zones compared to commercial chitosan, for all tested concentrations. From comparison with heterogeneous chitosan from *H. illucens* characterized by Guarnieri et al. [13], homogeneous chitosan produced on *E. coli* inhibition zones within the same range. At all concentrations, chitosan from *H. illucens* produced inhibition zones (10–7 mm) statistically significant compared to the commercial sample/positive control (6 mm). At 1.25, 0.6 and 0.3 mg/mL concentrations, No Dec chitosan showed better activity than Dec samples, with the largest inhibition diameters obtained by chitosan from pupal exuviae and adults (10 mm). At the lowest concentration, 0.15 mg/mL, only No Dec samples from adults (9 mm) showed on *E. coli* significant differences with commercial chitosan (Table 4). There is no other work reporting antimicrobial activity of homogeneous chitosan for valid comparison besides that of Younes et al. [32] from crustaceans, who obtained similar inhibition zones on *E. coli*.

When the inhibitory activity against *M. flavus* was evaluated, at the highest concentrations, 1.25 and 0.6 mg/mL, chitosan from *H. illucens* was statistically more effective than commercial chitosan, with the Dec samples showing the greatest inhibition (8–10 mm). Particularly, Dec chitosan from pupal exuviae was the best for all concentrations tested (Table 4). These differences are probably related to the higher

Table 4.
Diameters (mm) of inhibition zones formed by No Dec and Dec homogeneous chitosan samples produced from *H. illucens* larvae (L), pupal exuviae (PE) and dead adults (A), commercial chitosan and acetic acid at four different concentrations (1.25, 0.6, 0.3 and 0.15 mg/mL) on *E. coli* and *M. flavus*. Distilled water was tested as negative control. Results are expressed as mean ± standard deviation of diameters measured. For each bacterium, different letters indicate significant differences ($p < 0.05$) among treatments for the same concentration (lowercase letters) and among different concentrations in the same treatment (capital letters). Data were analyzed with two-way ANOVA and Tuckey *post-hoc* test.

Bacterial species	Sample	1.25 mg/mL	0.6 mg/mL	0.3 mg/mL	0.15 mg/mL
E. coli	L No Dec	10 ± 0.5^{aA}	9 ± 0.3^{abAB}	8 ± 0.2^{aBC}	7 ± 0.4^{bC}
	L Dec	9 ± 0.5^{aA}	8 ± 0.8^{bAB}	8 ± 0.5^{aAB}	7 ± 0.5^{bB}
	PE No Dec	10 ± 0.3^{aA}	10 ± 0.4^{aA}	9 ± 0.2^{aB}	7 ± 0.2^{bC}
	PE Dec	10 ± 0.4^{aA}	9 ± 0.4^{abAB}	8 ± 0.5^{aBC}	7 ± 0.5^{bC}
	A No Dec	10 ± 0.3^{aA}	10 ± 0.5^{aA}	9 ± 0.6^{aA}	9 ± 0.6^{aA}
	A Dec	9 ± 0.5^{aA}	8 ± 0.8^{bAB}	8 ± 0.8^{aAB}	7 ± 0.5^{bB}
	Commercial	6 ± 0.5^{bA}	6 ± 0.8^{cA}	6 ± 0.8^{bA}	6 ± 1^{bA}
	Acetic acid	–	–	–	–
	Distilled water	–	–	–	–
M. flavus	L No Dec	7 ± 0.2^{cdA}	6 ± 0.4^{cAB}	6 ± 0.6^{cAB}	5 ± 0.3^{dB}
	L Dec	9 ± 0.3^{abA}	8 ± 0.5^{bAB}	7 ± 0.5^{bcBC}	6 ± 0.5^{bcC}
	PE No Dec	8 ± 0.2^{bcA}	8 ± 0.5^{bA}	7 ± 0.5^{bcA}	7 ± 0.8^{abcA}
	PE Dec	10 ± 0.4^{aA}	10 ± 0.4^{aA}	9 ± 0.5^{aAB}	8 ± 0.4^{aB}
	A No Dec	9 ± 0.4^{abA}	9 ± 0.2^{abA}	8 ± 0.3^{abA}	8 ± 0.6^{aA}
	A Dec	8 ± 0.5^{bcA}	8 ± 0.7^{bA}	7 ± 0.7^{bcA}	7 ± 0.5^{abcA}
	Commercial	7 ± 0.5^{dA}	7 ± 0.5^{cA}	7 ± 0.5^{bcA}	6 ± 0.5^{cA}
	Acetic acid	–	–	–	–
	Distilled water	–	–	–	–

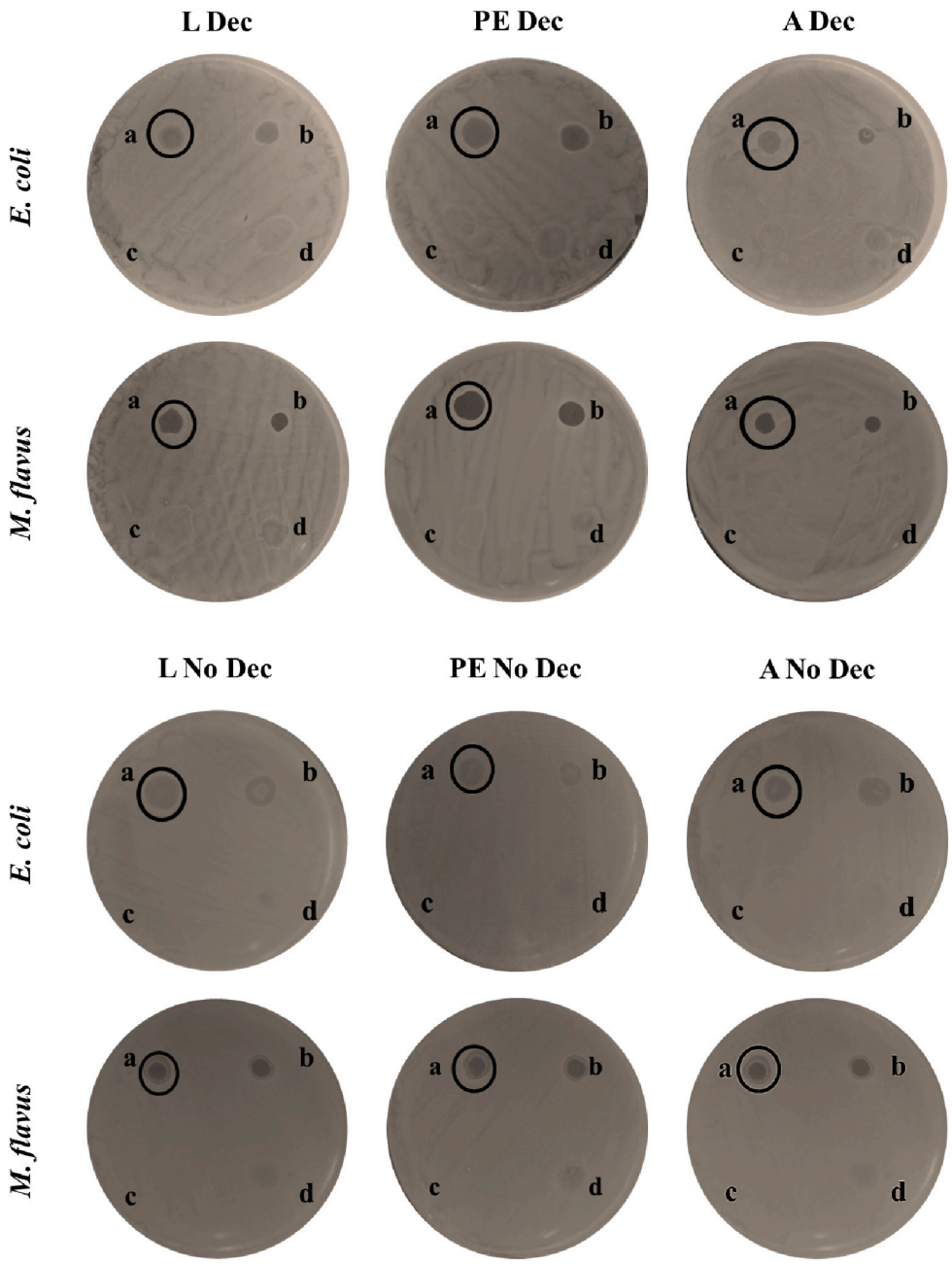

Fig. 6. Inhibition zones of Dec and No Dec homogeneous chitosan samples produced from *H. illucens* larvae (L), pupal exuviae (PE) and adults (A). Chitosan samples from *H. illucens* (circle *a*), commercial chitosan (circle *b*), distilled water (circle *c*) and acetic acid (circle *d*) on *E. coli* and *M. flavus* resulting from the agar diffusion test are reported.

purification of Dec samples, compared to the No Dec ones. These findings are consistent with those reported for heterogeneous chitosan from the same biomass [13]. Among the No Dec samples, inhibition zones comparable to Dec samples were measured for chitosan from dead adults (8–9 mm), especially at the highest concentrations. Other studies conducted on chitosan heterogeneously produced from pupal exuviae of *H. illucens*, showed higher inhibition zones than those measured for our homogeneous chitosan, but at higher concentrations tested [66]. As with heterogeneous chitosan from *H. illucens*, homogeneous chitosan, at the same concentrations tested, exhibited inhibition zones similar to those measured by Lagat et al. [67]. The results showed higher antimicrobial activity of homogeneous chitosan from *H. illucens*, compared to heterogeneous chitosan from *Tenebrio molitor* and *Z. morio* [51].

3.7.2. Microdilution assay

The results of microdilution assay on homogeneous chitosan samples from *H. illucens* are shown in Fig. 7. As already reported by Guarnieri et al. [13] for chitosan produced by heterogeneous deacetylation, the main problem in assessing the antimicrobial activity was to determine the concentration at which the inhibitory activity could be exclusively attributed to chitosan alone, without being masked by acetic acid. Indeed, at 1.25 and 0.6 mg/mL, although both insect and commercial chitosan showed significant inhibition of both bacterial strains tested, the antimicrobial effect could be ascribed to the acetic acid. Thus, also for homogeneous chitosan from *H. illucens*, as was already proved for the heterogeneous one [13], MIC values were always equal to or lower than 0.3 mg/mL, the point where the solvent used (0.06 %) lost its activity (Table 3).

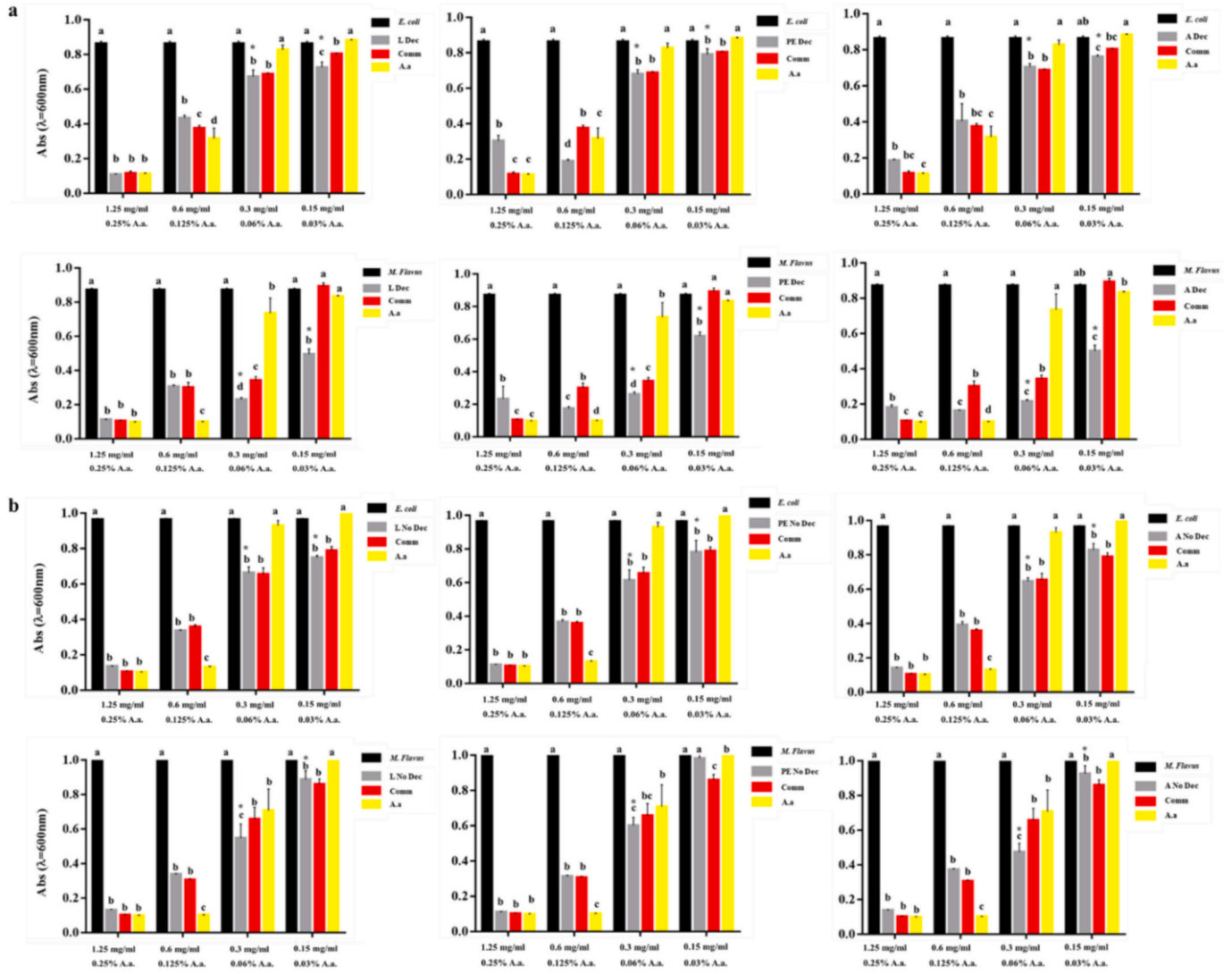

Fig. 7. Results of microdilution assay for (a) Dec and (b) No Dec homogeneous chitosan from larvae (L), pupal exuviae (PE) and adults (A) of *H. illucens*, commercial (Comm) chitosan and acetic acid (A.a) at the four concentrations of 1.25, 0.6, 0.3, 0.15 mg/mL against *E. coli* and *M. flavus*. Bars indicate the absorbance of the bacterial culture (black bars), the culture treated with *H. illucens* chitosan samples (gray bars), commercial chitosan (red bars) and acetic acid (yellow bars). Data are expressed as mean ± standard deviation. Different letters indicate significant differences ($p < 0.05$) between absorbance values of the bacterial culture alone and that of bacteria treated with the different concentrations of each treatment. Asterisks indicate significant differences ($p < 0.05$) among treatments for the same concentration. Data were analyzed with two-way ANOVA and Tuckey *post-hoc* test.

On *E. coli*, MIC values of our chitosan samples are directly comparable with those calculated by Younes et al. [32], for different homogeneous chitosan obtained from crustaceans. In contrast, the MIC values of homogeneous chitosan from larvae, both Dec and No Dec, are better than those reported by Khayrova et al. [68] (0.15 vs 0.5 mg/mL). On *M. flavus*, the MIC value identified, proved to be better than that obtained by Lin et al. [69] (0.15 vs 0.6 mg/mL), who tested heterogeneous chitosan produced from pupal exuviae of *H. illucens*. Literature research also showed that the identified MIC values are better than those obtained for chitosan from crustaceans and fungi in other studies [70,71].

Chitosan, with its positive charge, interacts differently with the bacterial walls of *E. coli* and *M. flavus,* due to differences in their structure. The efficacy of chitosan on one bacterial strain rather than another is somewhat controversial. Some authors claimed that chitosan is more effective on Gram-positive than Gram-negative bacteria, while others demonstrated the opposite [27]. However, homogeneous chitosan produced from *H. illucens* proved to be effective against both types of bacteria, with equivalent MIC values. As already explained by Guarnieri et al. [13], this suggests that the antimicrobial efficacy of chitosan depends not only on its action mechanism but also on its chemical-physical and morphological properties. Furthermore, unlike heterogeneous chitosan, it was possible to detect the MIC values for all homogeneous samples, both Dec and No Dec, generally at the lowest concentration tested (0.15 mg/mL). The discriminating factor between the two chitosan types could be the distribution of acetyl groups along the polymer chain. It can be hypothesized that the more regular distribution of functional groups in the homogeneous chitosan could result in a better and more uniform electrostatic interaction with bacteria cell wall, with the consequent loss of protein and DNA, resulting in an antimicrobial activity already at low concentrations [72]. Moreover, the higher Mw compared to the heterogeneous one [10], is a factor to be considered for the different antimicrobial action. Generally, it was also reported that chitosan with low- and medium- Mw had better antibacterial properties than high Mw chitosan [56,57]. This is supported by our results, reporting a greater efficacy for homogeneous and heterogeneous chitosan from *H. illucens* than commercial chitosan.

4. Conclusions

The present study significantly contributes to the understanding of chitosan production from different biomasses of the insect *H. illucens* via homogeneous method and their physical-chemical and biological characterization. The production of homogeneous chitosan is more effective for the Dec samples than No Dec ones, suggesting the effect of the bleaching step on cold deacetylation. As with heterogeneous deacetylation, pupal exuviae confirmed to have the highest yields and the better features indicating the potential economic feasibility for industrial-scale production. An effect of deacetylation method on the final properties of chitosan was observed, generally leading to a reduction in DD, crystallinity and fibrillary state at the morphological level compared to chitosan obtained by heterogeneous procedure. Homogeneous chitosan from *H. illucens* also exhibited notable free radical scavenging activity, particularly for the Dec samples, with pupal exuviae-derived chitosan showing the better results. Furthermore, as already demonstrated for the heterogeneous, the homogeneous chitosan from *H. illucens* also proved to be a valid antimicrobial agent against both Gram-negative and Gram-positive bacteria, compared to the commercial sample.

In conclusion, the homogeneous chitosan derived from *H. illucens*, as already demonstrated from the heterogeneous one, exhibits promising characteristics from the point of view of sustainable production of biomaterials, proving to be an alternative source to crustaceans for various applications in areas such as healthcare, agriculture and industry. The next steps will be to further investigate the economic aspects and the industrial-scale production of this biopolymer. The results represent a starting point for further investigations exploring the potential applications of both heterogeneous and homogeneous chitosan types, in relation to their specific chemical-physical and morphological characteristics.

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CRediT authorship contribution statement

Micaela Triunfo: Writing – review & editing, Writing – original draft, Methodology, Data curation. **Anna Guarnieri:** Writing – review & editing, Methodology, Data curation. **Dolores Ianniciello:** Writing – review & editing, Methodology. **Maria Beatrice Coltelli:** Writing – review & editing, Methodology. **Rosanna Salvia:** Writing – review & editing, Data curation. **Carmen Scieuzo:** Writing – review & editing, Writing – original draft, Supervision, Data curation. **Angela De Bonis:** Writing – review & editing, Methodology. **Patrizia Falabella:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Data curation, 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.

Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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