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**Structural features, anti-coagulant and anti-adhesive potentials of blue crab (*Portunus segnis*) chitosan derivatives: Study of the effects of acetylation degree and molecular weight**

Rim Nasri, Ikram Amor, Suming Li, Jalel Gargouri, Moncef Nasri, Murat Kaya, Marguerite Rinaudo, Marwa Hamdi

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# International Journal of Biological Macromolecules

## Structural features, anti-coagulant and anti-adhesive potentials of blue crab (*Portunus segnis*) chitosan derivatives: Study of the effects of acetylation degree and molecular weight

--Manuscript Draft--

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<b>Abstract:</b>	<p>The present study was undertaken to establish a distinct relationship between blue crab chitosan (Cs) acetylation degree (AD) and molecular weight (Mw) and its structural features, thermal properties and bioactivity. Therefore, chitosans with different AD were prepared and Cellulase was used to produce Cs derivatives with decreasing Mw. Results clearly display a decrease of the ordered structure of Cs, with the increase of AD and the decrease of Mw. Thermal stability/degradation screening disclose a greater thermal resistance for Cs with lower AD and higher Mw. The anti-adhesive potential of Cs was, additionally, studied, as function of AD and Mw. The effectiveness of Cs in preventing biofilm adhesion was strongly influenced by its AD and Mw, with the lowest inhibition values for higher AD and lower Mw. Interestingly, the effectiveness of Cs in disrupting pre-formed biofilms increased with decreasing Mw. Moreover, Cs derivatives were found to be advantageously efficient in prolonging human blood clotting times, based on data of activated partial thromboplastin time, Quick time and thrombin time assays, typically for the intrinsic coagulation pathway. Accordingly, depending on the predicted application of Cs, either in food, biomedical and pharmaceutical industries, AD and Mw are critical traits to be inevitably reflected on.</p>
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**Highlights :**

- Chitosan derivatives with diverse acetylation degree (AD) and molecular weight (Mw);
- Greater thermal resistance for chitosan derivatives with lower AD and higher Mw;
- Lowest biofilm adhesion inhibition for higher AD and lower Mw chitosan derivatives;
- Disrupting pre-formed biofilms increase with decreasing chitosan derivatives Mw;
- Derivatives efficiency in prolonging clotting, typically for the intrinsic pathway.

**Abstract**

The present study was undertaken to establish a distinct relationship between blue crab chitosan (Cs) acetylation degree (AD) and molecular weight (Mw) and its structural features, thermal properties and bioactivity. Therefore, chitosans with different AD were prepared and Cellulase was used to produce Cs derivatives with decreasing Mw. Results clearly display a decrease of the ordered structure of Cs, with the increase of AD and the decrease of Mw. Thermal stability/degradation screening disclose a greater thermal resistance for Cs with lower AD and higher Mw. The anti-adhesive potential of Cs was, additionally, studied, as function of AD and Mw. The effectiveness of Cs in preventing biofilm adhesion was strongly influenced by its AD and Mw, with the lowest inhibition values for higher AD and lower Mw. Interestingly, the effectiveness of Cs in disrupting pre-formed biofilms increased with decreasing Mw. Moreover, Cs derivatives were found to be advantageously efficient in prolonging human blood clotting times, based on data of activated partial thromboplastin time, Quick time and thrombin time assays, typically for the intrinsic coagulation pathway. Accordingly, depending on the predicted application of Cs, either in food, biomedical and pharmaceutical industries, AD and Mw are critical traits to be inevitably reflected on.

**Keywords:** Blue crab chitosan derivatives; Antiadhesive; Anticoagulant.

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2 **blue crab (*Portunus segnis*) chitosan derivatives: Study of the**  
3 **effects of acetylation degree and molecular weight**

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## 36 1. Introduction

37 Chitosan is a derived amino-polysaccharide, endowed with unique structure and highly  
38 multidimensional and sophisticated functionalities [1]. Currently, chitosan is a promising  
39 environment friendly biopolymer. Tremendous awareness of using chitosan in life science is  
40 increasing due to its advantages, such as availability from renewable agricultural or marine food  
41 resources, biocompatibility, biodegradability etc., besides, chitosan ability of being converted  
42 to a variety of chemically or enzymatically modified derivatives in a plethora of specific  
43 applications [2,3]. Food and nutrition, pharmaceuticals, biotechnology, material science,  
44 agriculture and environmental protection, are among the most concerned area [4].

45 Chitosan is characterized by its molecular weight defined as the number of sugar units  
46 per polymer molecule. The molecular weight modulates polymer physico-chemical properties,  
47 including viscosity, solubility, adsorption on solids, elasticity, tear strength and bio-  
48 functionality [5]. The acetyl content in chitosan defines its acetylation degree. The versatility  
49 of chitosan is mainly ascribed to the amino group that correlates with chitosan crystallinity and  
50 solubility in acidic solution. Moreover, charge density along the chain and flexibility decrease  
51 with the increase in the acetylation degree [6].

52 In recent years, and to increase the potential applications of chitosan, several  
53 technological approaches have been adopted to prepare chitosan chitooligosaccharides or  
54 oligomers, including acid hydrolysis [7], enzymatic method [8], ultrasonic degradation [9] and  
55 oxidative degradation [10]. Acid hydrolysis is the most commonly used to produce chitosan  
56 oligomers. Nevertheless, most of acid hydrolysis products have a low degree of  
57 depolymerization, with low production yields [11]. Thus, enzymatic production methods are of  
58 great interest in terms of the ability to minimize unwanted chemical changes and promote  
59 biological activities. Many specific enzymes, such as chitosanase [12] and non-specific, such  
60 as cellulase [13], are used in the preparation of chitosan oligomers.



61 Remarkable efforts have been made to achieve adequate comprehension of the correlation  
62 between the physical/chemical features of chitosan, as molecular weight and acetylation degree,  
63 on its reactivity and bio-functionality [6,14]. Higher viscosity and lower gelation temperatures  
64 values were displayed at lower acetylation degrees. Moreover, greater thermal resistance, in  
65 terms of higher degradation and transition temperatures were attained for chitosans with lower  
66 acetylation degrees. Chitosan-based coatings, with higher acetylation degrees, were found to be  
67 less flexible, resistant and transparent. Additionally, due to its biocompatibility and non-  
68 toxicity, chitosan has got more attention as effective anti-adhesive agent to prevent various  
69 pathogenic bacteria biofilms formation [15]. Indeed, chitosan markedly inhibited the adhesion  
70 of all microorganisms tested, and was more efficient in the disruption of pre-formed biofilms.

71 In this aspect, it is of paramount importance to shed light onto the importance of both  
72 molecular weight and polymer chemical composition on chitosan bioactivities. Accordingly,  
73 the aim of the present work was to contribute to the full understanding of the effect of chitosan  
74 acetylation degree and molecular weight on its structural properties and bioactivity, in terms of  
75 anti-adhesive and anti-coagulant potentials. To this end, different chitosan derivatives were  
76 prepared from blue crab shells chitin, with different acetylation degrees and high, medium and  
77 low molecular weights.

## 78 2. Materials and methods

### 79 2.1. Blue crab chitosan derivatives preparation

80 Chitosan (Cs) was prepared from blue crab shells chitin, by *N*-deacetylation using a 12.5  
81 N NaOH solution, at a w/v ratio of 1/10 and 140 °C, as previously described [16].  
82 Subsequently, Cs derivatives with different molecular weights (Mw) and acetylation degrees  
83 (AD) were prepared [17]. Briefly, to generate Cs with different AD, chitin was treated with  
84 NaOH 12.5 M at a w/v ratio of 1/10 at 140 °C, for 2 h, 3 h and 5 h and produced Cs were  
85 referred as CsI, CsII and CsIII, and corresponding AD were determined based on the nuclear

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86 magnetic resonance analysis. Subsequently, to produce Cs with different Mw, Cs were  
87 hydrolyzed with Cellulase (10 U/g chitosan) in 0.5 N acetate-bicarbonate buffer (pH 5.2) at 55  
88 ° C, for 1 h and 3 h, as described by Chang *et al.* [18]. For each AD group, Cs derivatives were  
89 referred as Cs-1 and Cs-3, respectively. Size exclusion chromatography was used to  
90 characterize the obtained Cs average Mw.

## 91 **2.2. Blue crab chitosan derivatives purification**

92 Cs were purified based on the method described by Qian & Glanville [19]. Thus, crude  
93 Cs (6 g) was dissolved in 600 ml of HCl 0.1 M under stirring overnight at 40 °C, and  
94 subsequently, vacuum filtered. Cs was, thereafter, precipitated with NaOH 0.5 M under  
95 continuous stirring until ~pH 8.5. Afterward, a volume (6 ml) of sodium dodecyl sulfate (10%,  
96 w/v) was added to the mixture and heated at 95 °C for 5 min. After cooling, NaOH 0.5 M was  
97 used to adjust the pH to 10.0. The mixture was vacuum filtered and the hydrated Cs was washed  
98 5 times with 600 ml of deionized water at 40 °C, lyophilized, milled to powder and then sieved.

## 99 **2.3. Chitosan derivatives characterization**

100 Chitosans ADs were determined by using solid-state nuclear magnetic resonance (<sup>13</sup>C  
101 CP/MAS NMR) spectroscopy (Varian VMMRS300 spectrometer). A frequency of 300 MHz,  
102 an acquisition time of 40 ms, a contact time of 1 ms and a 4 s repetition time were applied.

103 Based on the area of the resonance of the methyl group carbon compared to the average  
104 area of the resonances of the carbon atoms of glycosyl ring, ADs of the samples were calculated  
105 using the following relationship [20]:

$$106 \quad \text{AD (\%)} = \frac{I_{\text{CH}_3}}{(I[\text{C1}] + I[\text{C2}] + I[\text{C3}] + I[\text{C4}] + I[\text{C5}] + I[\text{C6}])/6} \times 100 \quad \text{Eq (1)}$$

107 where *I* is the area of the particular resonance peaks allowing to get AD % with 5% precision.

108 Steric Exclusion Chromatography (SEC) was performed using multi-detector equipment  
109 with a differential refractometer, a multiangle laser light scattering detector and a viscometer  
110 from WYATT Technology (DAWN DSP-F). Acetic acid 0.3 M/sodium acetate 0.2 M (pH =  
111 4.5) was adopted as solvent (at  $25 \pm 2$  °C). TSK Gel GMPWXL column type was used and a  
112 flow of 0.4 ml/min was adopted. The increment of refractive index  $dn/dc$ , determined for the  
113 different AD values in the same solvent, was 0.190 [21]. Resulted chromatograms were  
114 analyzed with ASTRA 6.1.2 (WYATT Technology) software and weight-average molecular  
115 weights ( $\text{g mol}^{-1}$ ), were determined.

## 116 2.4. Spectroscopic analysis

117 To investigate the structural characteristics of the prepared Cs, X-ray diffraction (XRD)  
118 patterns were recorded using an X-ray diffractometer (D8, Advance Bruker XRD  
119 diffractometer, Germany). Ni-filtered Cu  $K\alpha$  radiation ( $k = 1.5406$  °Å) was used to record the  
120 X-ray powder patterns. The relative intensity was recorded in the scattering range  $2\theta$  of  $5\text{--}50^\circ$   
121 with a step size of  $0.02^\circ$  and a counting time of 5 s/step, with an error of  $\pm 1^\circ$ . The crystalline  
122 index value (CrI) was calculated according to the following formula [22]:

$$123 \text{CrI}_{110}(\%) = \frac{I_{110} - I_{\text{am}}}{I_{110}} \times 100 \quad \text{Eq (2)}$$

124 where  $I_{110}$  is the maximum intensity at  $2\theta \approx 20^\circ$  and  $I_{\text{am}}$  is the intensity of amorphous diffraction  
125 at  $2\theta \approx 10^\circ$ .

## 126 2.5. Chitosan derivatives thermal properties analyses

### 127 2.5.1. Thermogravimetric analysis

128 Thermal stability of Cs powders was studied using thermogravimetric analysis (TGA  
129 Q500 High Resolution, TA Instruments), operating under nitrogen flow. The thermogravimetric  
130 analysis is based on the mass change of a sample as a function of temperature augmentation  
131 and a thermogram, showing the progressive change in mass in percentage (%) as a function of

132 temperature, is recorded. Cs powders were heated from 25 to 800 °C at a heating rate of 20  
133 °C/min. The weight of Cs powder samples, initially about 4 mg, was constantly measured with  
134 an accuracy of 0.01 mg.

### 135 **2.5.2. Differential scanning calorimetry**

136 Differential Scanning Calorimeter (Modulated DSC Q20, TA Instruments), equipped  
137 with a liquid nitrogen cooling system, was used to further investigate the thermal properties of  
138 Cs, allowing the estimation of melting and crystallization point, as well as the glass transition  
139 of the macromolecular materials. Cs samples were accurately weighed into sealed aluminum  
140 pans. An empty capsule serves as an inert reference and the apparatus was calibrated using  
141 indium. The thermal profile was analyzed in a temperature range of 0-225 °C, at a heating rate  
142 scan of 10 °C/min, under nitrogen flow rate of 50 ml/min. Thermograms were then analyzed  
143 by using TA Universal V4.5A software.

### 144 **2.6. Influence of AD and Mw on blue crab chitosan derivatives anti-adhesive potential**

145 The ability of Cs derivatives, with different AD and Mw, to hinder microbial adhesion **or**  
146 **to remove pre-formed biofilms**, through surface pre-treatment and post-treatment, **respectively**,  
147 **were** tested according to **the modified method of O'Toole** [23].

148 Regarding surface pre-treatment, 200 µl of Cs (0.01 to 12.5 mg/ml dissolved in **phosphate**  
149 **buffered saline (PBS)** pH 7.2) were transferred into the wells of a microplate. After incubation  
150 for 6 h at room temperature (25 °C), plates were washed twice with PBS. For biofilm formation,  
151 *Escherichia coli* (ATCC 25922) was chosen as microorganism model [16] and strains were  
152 grown overnight in **Muller Hinton broth (MHB)**. A volume of 200 µl of 1/50 dilution in the  
153 medium proposed by O'Toole [23] (g/l: glucose, 2; casamino acids, 5; KH<sub>2</sub>PO<sub>4</sub>, 3; K<sub>2</sub>HPO<sub>4</sub>, 7;  
154 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.12) were putted in the microplate wells, and incubated for 20 h  
155 at 37 °C. Wells were washed three times with distilled water to remove unattached cells and the

156 adherent microorganisms were fixed for 15 min with pure methanol (150 µl), and stained for  
157 20 min with crystal violet (1%). After washing with water and drying, the stains in the wells  
158 were diluted with 200 µl of acetic acid (33%). Control wells contained only PBS or Cs solution.  
159 Subsequently, the absorbance was determined at 595 nm, and percentages of microbial adhesion  
160 inhibition were estimated as follow:

$$\text{Adhesion inhibition (\%)} = \left(1 - \frac{A_C}{A_O}\right) \times 100 \quad \text{Eq (3)}$$

162 where  $A_C$  corresponds to the absorbance of the well with Cs at concentration  $C$ , and  $A_O$  the  
163 absorbance of the control well (without Cs).

164 For the post-treatment with Cs, wells were first incubated, with 200 µl of bacterial  
165 suspension prepared as mentioned above, for 20 h at 37 °C. Afterward, wells were washed three  
166 times with distilled water to remove the unattached microbial cells. Then, 200 µl of Cs solution  
167 were added to each well and incubated at 25 °C for 6 h. After incubation, wells were treated as  
168 above mentioned and adhesion inhibition was calculated.

## 2.7. Anticoagulant behavior of **chitosan derivatives** as affected by its AD and Mw

170 Plasma-based coagulation time was studied based on the activated partial thromboplastin  
171 time (APTT), the Quick time (QT) and the thrombin time (TT) *in vitro* assays, using a semi-  
172 automatic line STA (Diagnostica Stago).

### 2.7.1. Activated partial thromboplastin time

174 The APTT was estimated on a mixture of 45 µl of normal citrated platelet poor plasma  
175 (healthy adult subjects not receiving drugs that interfere with coagulation), 5 µl of Cs and Cs  
176 derivatives -solutions at different concentrations and 50 µl of the APTT reagent (CK-PREST).  
177 The mixture was pre-incubated at 37 °C for 3 min, then the reaction was initiated by the addition  
178 of 100 µl of  $\text{CaCl}_2$  (0.025 M), previously heated to 37 °C (coagulation intrinsic path). The

179 coagulation time was expressed in seconds, based on the method reported by Langdell, Wagner  
180 & Brinkhous [24].

### 181 **2.7.2. Quick time**

182 For the QT, the test consists of the comparison of the coagulation time of citrated plasma  
183 sample to be studied with a normal reference plasma), in the presence of an excess of calcified  
184 thromboplastin [25]. Thus, in a microplate reader, at 37 ° C, a reaction mixture was prepared  
185 containing 45 µl of plasma, 5 µl of the Cs and Cs derivatives solutions at different  
186 concentrations and a volume of 100 µl of calcified thromboplastin (Neoplastin® Diagnostica  
187 stago, tissue activating factor of the extrinsic path). The coagulation time, subsequently to  
188 mixing, was expressed in seconds.

### 189 **2.7.3. Thrombin time**

190 The TT allows to explore the common path of coagulation, by measuring the coagulation  
191 time of a citrated plasma in the presence of a known amount of thrombin and calcium. Thus, 5  
192 µl of Cs and Cs derivatives solutions at different concentrations were mixed with 45 µl of  
193 platelet depleted and decalcified plasma, previously heated to 37 °C for 3 min (or 0.4%  
194 fibrinogen solution in 0.05 mM of Tris-HCl buffer (pH 7.2) containing 0.12 mM of NaCl).  
195 After addition of 100 µl of calcified thrombin (12 UI) prepared in the same buffer (initiation of  
196 the reaction), the coagulation time was measured and expressed in seconds [25].

## 197 **2.8. Statistical analysis**

198 All experiments were carried out in triplicate, and average values with standard deviation  
199 are reported. Mean separation and significance were analyzed using the SPSS software package  
200 ver. 17.0 professional edition (SPSS, Inc., Chicago, IL, USA) using ANOVA analysis.  
201 Differences were considered significant at  $p < 0.05$ .

### 3. Results and Discussion

#### 3.1. Blue crab chitosan derivatives preparation and characterization

Cs was initially prepared by *N*-deacetylation of blue crab shells chitin, with an average extraction yield of approximately 70% (chitin dry weight basis) and typically characteristic of  $\alpha$ -chitosan [16]. Accordingly, Cs derivatives with different AD and Mw were prepared by increasing *N*-deacetylation reaction times followed by enzymatic hydrolysis with Cellulase.

Considering the obtained  $^{13}\text{C}$  NMR spectra (**Fig. 1**), AD of prepared Cs derivatives was determined and results are reported in **Table 1**. As the duration of *N*-deacetylation increased, the AD significantly decreased, as evidenced by a gradually negligible detected signals for - $\text{CH}_3$  and  $\text{C}=\text{O}$  groups. AD of the prepared Cs derivatives was found to be ranging from 17% (undigested CsI) to 8% (undigested CsIII). Furthermore, the increase in the enzymatic depolymerization duration was found not significantly affecting the AD of blue crab Cs derivatives.

The estimation of average Mw by steric exclusion chromatography (**Table 1**) reveal the effectiveness of Cs depolymerization using Cellulase to produce Cs derivatives with decreasing average Mw. Results reveal that Cs derivatives, recovered at different hydrolysis incubation times, exhibited an average Mw of 17 800, 59 270 and 78 430  $\text{g mol}^{-1}$ , for CsI-1, CsII-1 and CsIII-1, respectively, and 10 440, 18 540 and 16 040  $\text{g mol}^{-1}$  for CsI-3, CsII-3 and CsIII-3, respectively (**Table 1**). Average Mw of 125 600, 118 900 and 115 000  $\text{g mol}^{-1}$  were reached for undigested CsI, undigested CsII and undigested CsIII, respectively.

X-ray diffraction patterns for the prepared Cs, investigated between 5 and 50° of  $2\theta$ , display typical fingerprints of semi-crystalline chitosan, with strong reflections at around  $2\theta = 10.8^\circ$  and  $2\theta = 20.1^\circ$  (**Fig. 2**), due to the crystalline chitosan structure [16]. Based on the X-ray diffractograms, the crystallinity index (CrI) were further determined and results show an increase in Cs crystallinity with the decrease of its AD. In fact, CrI of 23% and 29% were

227 obtained with ADs of 17% (undigested CsI) and 8% (undigested CsIII), respectively. Moreover,  
228 the decrease of Cs Mw was reflected by a decrease of its ordered structure, as evidenced by a  
229 drop in the reflection around  $2\theta = 20.1^\circ$ . For example, with an AD of 8% (undigested CsIII),  
230 after digestion with Cellulase, reached CrI values were of 20% and 1% for Mws of 78 000 g  
231  $\text{mol}^{-1}$  (CsIII-1) and 16 000  $\text{g mol}^{-1}$  (CsIII-3) vs. 29% for 115 000  $\text{g mol}^{-1}$  (undigested CsIII),  
232 respectively. This increase in the disordered structure with the increase of the AD and the  
233 decrease of the Mw could be explained by a transition from a crystalline structure to an  
234 amorphous state during the enzymatic depolymerization process, due to the arrangement of the  
235 polysaccharide chain as well as the intermolecular hydrogen bonds, compared to the free amine  
236 forms (low acetylation degree and high molecular weight) [26,27].

### 237 3.2. Thermal behavior and stability of blue crab chitosans

238 Thermal stability/degradation behavior of Cs, with respect to its Mw, was further studied,  
239 in the present work, and results in terms of TGA and DSC thermograms, are reported. Thermal  
240 features, in terms of degradation and glass transition ( $T_g$ ) temperatures, of chitosan were  
241 previously studied and found to be strongly dependent on its AD, where greater thermal  
242 resistance was noted for Cs with lower AD, in terms of significantly higher degradation  
243 temperature and improved  $T_g$  values [28]. Consequently, it seems to be very interesting to  
244 further verify the combined effects of Cs AD and Mw on its thermal degradation/stability  
245 behavior.

246 Based on data from the obtained TGA thermograms (Fig. 3A), the thermal decomposition  
247 profiles of the overall Cs exhibited a similar weight loss process in the temperature range of  
248 20–800 °C, indicating the polymer pyrolysis, and characterized by two major phases, typical  
249 fingerprint of Cs thermal decomposition [14]. The thermal decomposition data, considering the  
250 derivate (DTG) thermograms (Fig. 3B), in terms of the corresponding degradation temperatures  
251 (**T<sub>d</sub>**: onset temperature of degradation, **T<sub>max</sub>**: maximum degradation temperature and **T<sub>f</sub>**:



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2 252 temperature of the end of degradation), the weight loss ( $\Delta w$ ) and the residue (**R**), were estimated  
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4 253 (**Table 2**).

5 254 The first phase, apparently resulted from the evaporation of adsorbed water by Cs,  
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7 255 corresponded to a weight loss of 6-8% at a Td 1 range from 41-58 °C to Tf 1 range of 142-267  
8  
9 256 °C, reaching its maximum mainly below 150 °C, depending on Cs derivatives Mw. For  
10  
11 257 example, regarding an AD of 8%, Tmax 1 values dropped to 143 °C and 123 °C for CsIII-1 and  
12  
13 258 CsIII-3, respectively, vs. 267 °C for undigested CsIII. The same tendency was noted with CsII  
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15 259 (AD of 13%) and CsI (AD of 17%).  
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19 260 In the range of the second phase of Cs pyrolysis process, corresponding to the major loss  
20  
21 261 of weight, three major steps could be defined: until 300 °C (ascribed to the decomposition of  
22  
23 262 the D-glucosamine units), from 300 °C to ~500 °C, (attributed to the N-acetyl-D-glucosamine  
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25 263 units depolymerization) and above ~600 °C (due to residual decomposition reactions). Peaks  
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27 264 located at Tmax 2 of 460, 306 and 295 °C, were found for the CsI-0, CsI-1 and CsI-3,  
28  
29 265 respectively, whereas those found at 474, 402 and 355 °C were related to the CsIII-0, CsIII-1  
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31 266 and CsIII-3, respectively (**Table 2**). Moreover, the highest weight loss ( $\Delta w$ ) of 64% was  
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33 267 detected with CsI-3, exhibiting the highest AD of 17% and the lowest average Mw of 10 440 g  
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35 268 mol<sup>-1</sup>. Nonetheless, the lowest  $\Delta w$  of 56% was revealed by the undigested CsIII (AD of 8% and  
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37 269 Mw of 115 000 g mol<sup>-1</sup>) and 57% with the undigested CsII (AD of 13% and Mw of 118 900 g  
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39 270 mol<sup>-1</sup>). Polymers pyrolysis temperature differences were mainly assigned to the  
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41 271 macromolecular interaction, in line with the crystallinity index values [6].  
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48 272 Additionally, residual mass (R) was found to decrease with the decrease of the Cs Mw,  
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50 273 with values of 28% (CsI-3), 30% (CsI-1) and 34% (undigested CsI). Considering CsII, R values  
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52 274 of 29%, 31% and 36% were reached for the CsII-3, CsII-1 and undigested CsII, respectively. R  
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54 275 values of 38%, 33% and 31% were noted for undigested CsIII, CsIII-1 and CsIII-3, respectively  
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56 276 (**Table 2**). Accordingly, it could be concluded that the thermal stability of Cs derivatives was  
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277 disproportionate with its AD and positively correlated to its Mw. Therefore, AD and Mw could  
278 be considered as extremely important Cs's structural traits, modulating its macromolecular  
279 chains properties and thereby, Cs solubility, macromolecular chains flexibility and  
280 conformation [29].

281 In another aspect of the study of Cs thermal properties dependence on its Mw, considering  
282 DSC analysis, the glass transition temperature (T<sub>g</sub>) was determined (**Table 2**). It was found that  
283 the more the Cs Mw was higher, the more was its thermal stability. In this context, T<sub>g</sub> values  
284 of 202 °C, 188 °C, 173 °C, 191 °C, 172 °C, and 157 °C were noted for undigested CsIII, CsIII-  
285 1, CsIII-3, undigested CsII, CsII-1 and CsII-3, respectively. Nevertheless, for CsI group, T<sub>g</sub>  
286 values decreased further to 166 °C and 142 °C, for CsI-1 and CsI-3, respectively, vs. 188 °C for  
287 undigested CsI. Accordingly, besides Cs AD [28], Mw affected strongly its glass transition  
288 temperature, and subsequently, Cs thermal resistance, in the same line of the TGA findings.

### 289 **3.3. Blue crab chitosans anti-adhesive behavior as influenced by its AD and Mw**

290 Among the most challenging problems in food industries and hospitals, finding an  
291 alternative and effective strategy to hinder or to remove biofilm formation, is a public concern  
292 and has broadly attracted wide attention [30]. Microbial adhesion could be considered as the  
293 optimal time for anti-adhesive compounds action. In fact, in utmost environments, the prevail  
294 mode of growth for microbes is living as biofilm, defined as biotic or abiotic surfaces adhering  
295 communities of microbes. Individual bacteria adhesion to a surface is the initial phase of biofilm  
296 development to establish the complex community architecture of a biofilm [31]. Biosurfactants  
297 have been reported to be powerful inhibitors and disruptors of microbial adhesion and biofilm  
298 formation [32]. Moreover, the role of chitosan in microbial anti-adhesion was, previously,  
299 investigated and results reveal that adsorption of chitosan to solid surfaces can be an effective  
300 strategy to reduce microbial adhesion, besides, combating pathogenic micro-organisms  
301 colonization [16]. Thus, to have a better understanding of chitosan anti-adhesive mechanism, it

302 seems to be compelling to study the effects of Cs AD and Mw on its anti-adhesive and disruptive  
303 activities.

### 304 3.3.1. Anti-adhesive activities of Cs derivatives

305 The anti-adhesive activity was investigated, through the pretreatment of polystyrene  
306 surfaces with Cs derivatives solutions, against *E. coli*, as microbe model. As it can be seen in  
307 **Fig. 3A**, the pretreatment of polystyrene surfaces with Cs and its derivatives revealed clearly  
308 an anti-adhesive effect against *E. coli* biofilm formation in a Cs concentration-dependent  
309 manner, *i.e.* higher inhibition values were reached at higher Cs concentrations. For example,  
310 subsequently to the surface treatment with the undigested CsIII, the bacterial adhesion  
311 inhibition decreased from more than 62% to about 16%, when Cs concentration decreased from  
312 12.5 mg ml<sup>-1</sup> to 0.01 mg ml<sup>-1</sup>. The same tendency was noted with the other tested Cs derivatives.  
313 Therefore, even at low concentration, Cs derivatives were very active and a diminution in  
314 bacterial adhesion was detected. The inhibition of biofilm formation could be ascribed to Cs  
315 and its derivatives to modify surface (coated with Cs molecules) hydrophobicity, interfering in  
316 the microbial adhesion and desorption process [33]. Additionally, previous reports have shown  
317 that chitosan has bactericidal effects [34]. Our data are in agreement with previous results  
318 suggesting that, at higher concentrations, the polystyrene surface became covered by Cs  
319 molecules that were adsorbed on bacterial cells to a higher degree due to the increase in the  
320 positive charge [35].

321 At the studied concentrations, the adhesion of *E. coli* was still limited by Cs and its  
322 derivatives, to variable extend, depending on Cs AD and Mw (**Fig. 3A**). The highest anti-  
323 adhesive effect was observed with undigested CsIII (AD of 8%), with an inhibition percentage  
324 of about 63%, at a concentration of 1.25% (w/v), which decreased concomitantly with the  
325 increase of the AD, reaching 60% and 57% for undigested CsII (AD of 13%) and undigested  
326 CsI (AD of 17%). The same tendency was noted with the prepared Cs derivatives,

327 independently of their Mw, where their anti-adhesive effects were negatively correlated with  
328 their AD and higher anti-adhesive effects were perceived with lower AD Cs derivatives. These  
329 results depict that the anti-adhesion activity of the surfaces coated with high AD (17%) Cs  
330 derivatives was significantly lower compared to the low AD (8%) Cs derivatives-coated  
331 surfaces by reason of the effective antibacterial activity of Cs derivatives. Higher amine groups,  
332 and thereby lower AD, allowed higher positive charge density, which confers stronger  
333 antibacterial activity against *E. coli* [34,36].

334 Moreover, the inhibitory effect was found to be dependent on the Cs Mw (Fig. 3A). Based  
335 on the obtained data, for the same AD, the inhibition of *E. coli* adhesion, subsequently to  
336 polystyrene surfaces treatment with Cs, decreased with the decrease of the Mw. For example,  
337 for an AD of 8% (CsIII), the inhibition of adhesion diminished from 63% for the undigested  
338 CsIII to 57% and 54% for CsIII-1 and CsIII-3, respectively, at a concentration of 1.25% (w/v).  
339 Inhibition values of 57% and 49% were noted with undigested CsI and CsI-1, respectively, vs.  
340 36% for CsI-3. This finding could be mostly attributed to the drop of Cs solutions' viscosity,  
341 after digestion with cellulase, generating Cs derivatives with low Mw, and further explained by  
342 the decrease in positive charge density of Cs. Hence, our results suggest a positive correlation  
343 between the adsorbed Cs and the efficiency to reduce the adhesion stage of *E. coli*, therefore,  
344 the further development of biofilms can be blocked. Otherwise, bacterial adhesion was found  
345 to be depended not only on bacterial charge and surface type, but also, on Cs charge interfering  
346 in its effects [34,35,37]. This phenomenon confirmed our findings that during the process of  
347 bacterial growth, low Mw Cs derivatives exhibited weak bactericidal effect.

### 348 3.3.2. Pre-formed biofilm disruption effects (post-treatment)

349 Besides the inhibition of biofilms adhesion (initial stage of biofilm formation), the  
350 disruption of pre-formed biofilms, which consists in removing the attached microorganisms

351 from the biotic or abiotic surface after biofilm formation, is another interesting phenomenon to  
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2 352 be studied.  
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5 353 As illustrated in **Fig. 3B**, the efficacy of biofilm disruption decreased with decreasing Cs  
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7 354 derivatives concentration from 12.5 to 0.01 mg ml<sup>-1</sup>, and interestingly the percentages of  
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9 355 disruption remained nearly constant above a concentration of 0.2 mg ml<sup>-1</sup> Cs, independently of  
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11 356 Cs AD and Mw. Indeed, Cs was probably efficient in penetration and adsorption at the interface  
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13 357 between the solid surface and the pre-formed biofilm, interfering with surface adhesion, to  
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15 358 promote its detachment [15].  
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19 359 In line with the pre-treatment test (adhesion inhibition), disruption of pre-formed biofilm  
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21 360 values increased with the decrease of Cs AD, the maximum disruption was observed with  
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23 361 undigested CsIII (91%), followed by undigested CsII (85%) and undigested CsI (76%). This  
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25 362 finding could be related to high surface activity of Cs with lower AD, promoting the reduction  
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27 363 of interfacial tension [38]. Otherwise, Cs with lower AD and high Mw could mold, around the  
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29 364 cells, an impermeable layer, delaying afterward the channels and preventing essential nutrients  
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31 365 transport. As a result, the cell wall would be destabilized, ultimately cell death [35].  
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36 366 Conversely, for the same AD, the effectiveness of Cs in disrupting pre-formed biofilm  
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38 367 (post-treatment) increased with decreasing its Mw. For example, disruption values of 91%, 94%  
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40 368 and 97% were reached after treatment with undigested CsIII, CsIII-1 and CsIII-3, respectively.  
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42 369 Likewise, undigested CsII, CsII-1 and CsII-3 allowed the elimination of 84%, 88% and 91% of  
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44 370 the pre-formed biofilms, respectively (**Fig. 3B**). This effect may be explained by the fact that  
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46 371 at lower Mw, Cs infiltration and adsorption at the interface between the solid surface and the  
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48 372 adherent bacteria forming a biofilm are easier and more pronounced, allowing derivatives to  
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50 373 exert their disturbing effect, and thereby, the death of bacteria [15,39].  
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56 374 The present study is opportune to have a better and extended understanding of the  
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58 375 mechanisms of inhibition of biofilm formation, whether by adhesion inhibition or by disruption  
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376 of pre-formed biofilms, testifying the effectiveness of Cs and Cs derivatives as attractive  
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2 377 alternatives for bacterial pathogens control in food and hospital fields. AD and Mw strongly  
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4 378 govern antimicrobial and anti-adhesive activities of chitosan, and AD further modulates  
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7 379 chitosan solubility and viscosity [15]. Accordingly, Mw and AD are structural key traits that  
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10 380 should be taken into consideration based on the perceived application. In literature, a growing  
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12 381 number of reports on the antimicrobial and anti-adhesive activities of chitosans, but a  
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14 382 comparison between works is difficult due to differences between the test organisms, assay  
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17 383 methods used and characteristics of the tested chitosan preparations.  
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#### 20 384 **3.4. Study of blue crab chitosan and its derivatives anti-coagulant activity**

23 385 Coagulation is the haemostasis second step that involves a cascade of enzymatic reactions  
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25 386 including clotting factors. coagulation intervenes in platelet aggregate, obtained at the end of  
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28 387 primary haemostasis, promoting bleeding breaking. Coagulation of human blood cascade  
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30 388 involves two intrinsic and extrinsic pathways, that joins at the stage of factor Xa (FXa)  
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32 389 formation by factors IXa (FIXa) and FVIIa, respectively. The thrombin conversion of soluble  
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35 390 fibrinogen into fibrin filaments consists of the coagulation ultimate step. In the case of an  
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38 391 inequity in this equilibrium, either bleeding or thrombus formation occur. Several of critical  
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40 392 cardiovascular diseases result from fibrin accumulation in blood vessels, interfering with blood  
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43 393 flow [40]. Multitude of drugs have been developed for the treatment of blood clots and thereby,  
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45 394 preventing the damage they cause, behaving as blood clotting power thinner and clot growth  
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47 395 preventor. Unfractionated heparin, low molecular weight heparin, and warfarin are the most  
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50 396 common anti-coagulants used. Currently, researchers are prompted to strive new and better  
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52 397 anti-coagulants with reduced side risks [41-43].  
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54 398 Chitosan and chitosan derivatives are reported as anti-coagulants in literature, owing to  
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57 399 their structural similarity with heparin (N-acetylglucosamine and iduronic acids are constituent  
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59 400 units of heparin) [44].  
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401 As a second promising application of blue crab Cs, the anti-coagulant activity of the  
402 prepared Cs derivatives, with different AD and Mw, was evaluated, by the classical coagulation  
403 assays of APTT, QT and TT. APTT is the measurement of the activity of all coagulation factors  
404 in the intrinsic pathway, while QT allow the screening of the activity of the extrinsic pathway.  
405 TT measurement reflects the investigation of fibrin polymerization process determining the  
406 fibrin formation time from fibrinogen after known amounts of thrombin addition to the plasma  
407 sample.

408 The results of the anti-coagulant activity of the different types of Cs are listed in **Table**  
409 **3**. Regarding the control (distilled water), APTT, QT and TT of 31,2 s, 14,8 s and 14,4 s,  
410 respectively, were reached. Above a concentration of 12.5 µg/ml plasma, all Cs derivatives  
411 were endowed with an anti-coagulant potential, since APTT, QT and TT times were effectively  
412 prolonged compared to distilled water in a Cs dose-dependent manner. **For example, the APTT**  
413 **and QT, prolonged from 74.7 s to >120 s and from 15.7 s to 19.1 s, respectively, when the**  
414 **concentration of undigested CsIII in the reaction mixture increased from 12.5 µg/ml to 25**  
415 **µg/ml. The TT increased from 15.9 s to 16.7 in the presence of 12.5 µg/ml and 25 µg/ml of**  
416 **undigested CsIII, respectively.** Results of APTT and TT of heparin sodium were reported to  
417 reach 79.7 s and 51.2 s at 14 µg/ml plasma, respectively [45]. APTT and QT assay of heparin  
418 sulphate showed 61.2 s and 31.4 s at the concentration of 50 µg/ml plasma, respectively [46].  
419 However, considering their AD and average Mw, the different types of Cs derivatives displayed  
420 diverse anti-coagulant activity patterns.

421 Considering the AD of the different types of tested CCB derivatives (**Table 3**), APTT and  
422 QT values increased with the decrease of the AD ( $p < 0.05$ ). Indeed, undigested CsIII allowed  
423 the most substantial prolongation of APTT by more than 2-folds (74.7 s), compared with that  
424 obtained with distilled water (31.2 s), when added at a concentration of 12.5 µg/ml plasma in  
425 the reaction medium. QT screening displayed the same trend. Indeed, values were, similarly,

426 improved with the decrease of derivatives AD, reaching 17 s, 18.1 s and 19.1 s, with addition  
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2 427 of undigested CsIII, undigested CsII and undigested CsI, respectively, at a concentration of 25  
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5 428  $\mu\text{g/ml}$  plasma, vs. QT of 14.8 s with distilled water.

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7 429 As shown in **Table 3**, results of the present study reveal that the anti-coagulant potential,  
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10 430 in terms of APTT and QT, was interestingly more marked with low Mw Cs derivatives. In fact,  
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12 431 at a concentration of 12.5  $\mu\text{g/ml}$  plasma, APTT of undigested CsIII (74.7 s) was lower than  
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14 432 those obtained with CsIII-1 and CsIII-3, with values of 93.2 s and  $>120$  s, respectively. The  
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16 433 same tendency was noted with the other derivatives (undigested and Cellulase-treated CsII and  
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18 434 CsI), with APTT that increased significantly with the decrease in the Mw of Cs derivatives  
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21 435 ( $p<0.05$ ). These findings were further confirmed by the study of Cs derivatives activity in the  
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24 436 extrinsic coagulation pathway (QT), where the lower the Mw of the derivatives was, the better  
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27 437 was their anti-coagulant activity ( $p<0.05$ ). The highest times were, therefore, reached with CsI-  
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29 438 3 (19.7 s), CsII-3 (20.5 s) and CsIII-3 (21.2 s), at a concentration of 25  $\mu\text{g/ml}$  of plasma,  
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31 439 probably correlated with the higher water solubility of the CCB derivatives prepared, having  
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34 440 low DA and Mw [47]. Additionally, Cs derivatives with the lowest AD could have the greatest  
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36 441 degree of similarity with regards to heparin structure. The positively charged groups  $-\text{NH}_3^+$   
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39 442 increase the adsorption of proteins through the electrostatic effect, which increases the  
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41 443 activation of intrinsic cascade [48].

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44 444 Conversely, the screening of the effects of Cs AD and Mw on its anti-coagulant behavior,  
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46 445 considering the TT that provides information about the evaluation of the common pathway of  
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49 446 coagulation, reveal that clotting time decreased with the decrease of AD and Mw (**Table 3**).  
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51 447 Subsequently to the addition of 25  $\mu\text{g/ml}$  plasma, in the reaction mixture, the highest TT values  
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54 448 were attained with the undigested CsI (21.3 s), undigested CsII (19 s) and undigested CsIII  
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56 449 (16.7 s), followed by CsI-1 (19.3 s), CsII-1 (17.9 s) and CsIII-1 (16.5 s). The lowest TT  
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58 450 prolongation values were reached in the presence of CsI-3 (17.3 s), CsII-3 (16.8 s) and CsIII-3  
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2 452 (16.3 s). This finding could be ascribed to lower complex-forming ability of Cs derivatives  
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5 453 (lower Mw) with the blood components, such as factor Xa [44]. A previous study showed that  
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7 454 the higher molecular weight fractions of low molecular weight chitosan polysulfate exhibited  
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9 the greatest anticoagulant activity as assessed using the TT assay [49].

10 455 Data of the present study proposed that blue crab chitosan and its derivatives affected  
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12 456 mainly the intrinsic pathway and have a potential anti-coagulant capacity. Consequently, further  
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14 457 work is in progress to establish clear relationship between Mw and AD and the anti-coagulant  
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16 458 activity and to understand their exact mechanism of action.  
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#### 20 459 **4. Conclusion**

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23 460 In the present work, the effects of AD and average Mw of Cs derivatives on their bio-  
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25 461 functionalities were investigated. Data reveal that Cs crystallinity decreased with the increase  
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27 462 of its AD and the decrease of its Mw, displaying more disordered structure. Thermal stability  
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29 463 and degradation behavior, in terms of degradation and transition temperatures, were improved  
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31 464 with lower AD. Higher thermal stability was, moreover, noted with higher Mw Cs derivatives.  
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33 465 Advantageously, Cs derivatives would be potential alternative for bacterial pathogens control  
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35 466 in food industry, since they were found to display anti-adhesive and pre-formed biofilms  
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37 467 disruptive effects. The highest anti-adhesive effect was observed with lower AD and higher  
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39 468 Mw Cs derivatives. Cs derivatives, and particularly those with lower Mw, were found more  
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41 469 efficient in disrupting pre-formed biofilms. Scanning electron microscopy, confocal laser  
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43 470 scanning microscopy, analysis of the exopolysaccharide and biofilms metabolic activity  
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45 471 screening could be very useful tools to have a better understanding of the relationship between  
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47 472 Mw and AD and the anti-adhesive activity and to establish their exact mechanism of action.  
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49 473 Additionally, Cs derivatives were found to be effectively endowed with anti-coagulant activity,  
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51 474 mainly for the intrinsic coagulation pathway, to be applied in biomedical and pharmaceutical  
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2 475 industries. However, no clear relationship, between AD and Mw and Cs derivatives anti-  
3 476 coagulant effect, could be yet founded and further studies are required.  
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9  
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**Table 1:** Acetylation degrees and molecular weight of prepared blue crab chitosan and derivatives.

Cellulase hydrolysis (h)	CsI		CsII		CsIII	
	AD (%)	Mw (g mol <sup>-1</sup> )	AD (%)	Mw (g mol <sup>-1</sup> )	AD (%)	Mw (g mol <sup>-1</sup> )
0	17	125 600	13	118 900	8	115 000
1	Nd	17 800	Nd	59 270	Nd	78 430
3	17	10 440	14	18 540	9	16 040

**Nd:** not determined.

Acetylation degree was determined based on the <sup>13</sup>C NMR spectra.

Average molecular weight was determined based on the SEC chromatograms.

**Table 2:** Blue crab chitosan derivatives thermal stability/degradation behavior as function of their AD and Mw.

Parameters	AD of 17%			AD of 13%			AD of 8%			
	UN_CsI	CsI-1	CsI-3	UN_CsII	CsII-1	CsII-3	UN_CsIII	CsIII-1	CsIII-3	
Phase 1	$\Delta W$ (%)	8	8	8	7	8	8	6	7	7
	Td (°C)	53	49	41	55	52	48	58	53	51
	Tmax (°C)	203	111	99	218	127	99	224	143	123
	Tf (°C)	253	174	142	260	180	170	267	215	192
Phase 2	$\Delta W$ (%)	58	62	64	57	61	63	56	60	62
	Td (°C)	276	240	207	281	256	222	289	259	238
	Tmax (°C)	460	306	295	466	319	304	474	402	355
	Tf (°C)	495	427	395	496	442	409	497	454	446
Residue (R; %)	34	30	28	36	31	29	38	33	31	
Tg (°C)	188	166	142	191	172	157	202	188	173	

Cs: chitosan; UN\_Cs: undigested chitosan; Cs-1: Cs treated with Cellulase for 1 h; Cs-3: Cs treated with Cellulase for 3 h;  $\Delta w$ : weight loss (%); Td: initial degradation temperature; Tmax: maximal degradation temperature; Tf: final degradation temperature; Tg: glass transition temperature.



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**Table 3:** Blue crab chitosan derivatives anti-coagulant activity as function of their AD and Mw, based on the activated partial thromboplastin time (APTT), Quick time (QT) and Thrombin time (TT). Values (seconds) presented are the mean of triplicate analyses.

Tests	Concentration µg/ml	CsI			CsII			CsIII			Distilled water
		UN	I-1	I-3	UN	II-1	II-3	UN	III-1	III-3	
APTT	6.25	27.4	29	30.6	28.6	30.1	32.5	29.8	31.1	34.3	
	12.5	33.7	36.3	38.8	54.2	64.8	72.4	74.7	93.2	>120	31.2
	25.0	68.5	73.7	78.8	73.9	89.9	96.3	>120	>120	>120	
QT	6.25	14	14.3	14.5	14.4	14.4	14.8	14.7	15.5	15.8	
	12.5	14.5	15.3	15.5	14.9	15.8	16.3	15.7	16.2	17.7	14.8
	25.0	17	18.5	19.7	18.1	19.3	20.5	19.1	20.3	15.8	
TT	6.25	16.2	15.8	14.9	15.4	15	14.3	14.5	14.1	14	
	12.5	18.5	17.7	16	17.2	16.6	15.2	15.9	15.5	14.4	14.4
	25.0	21.3	19.3	17.3	19	17.9	16.8	16.7	16.5	16.3	

Cs: chitosan; UN: undigested chitosan; Cs-1: Cs treated with Cellulase for 1 h; Cs-3: Cs treated with Cellulase for 3 h.

## Figures captions

**Figure 1:**  $^{13}\text{C}$  NMR spectra of Cs derivatives: Cs with AD of 17% (A), Cs with AD of 13% (B) and Cs with AD of 8% (C). Cs: chitosan; UN\_Cs: undigested chitosan; Cs-3: Cs treated with Cellulase for 3 h.

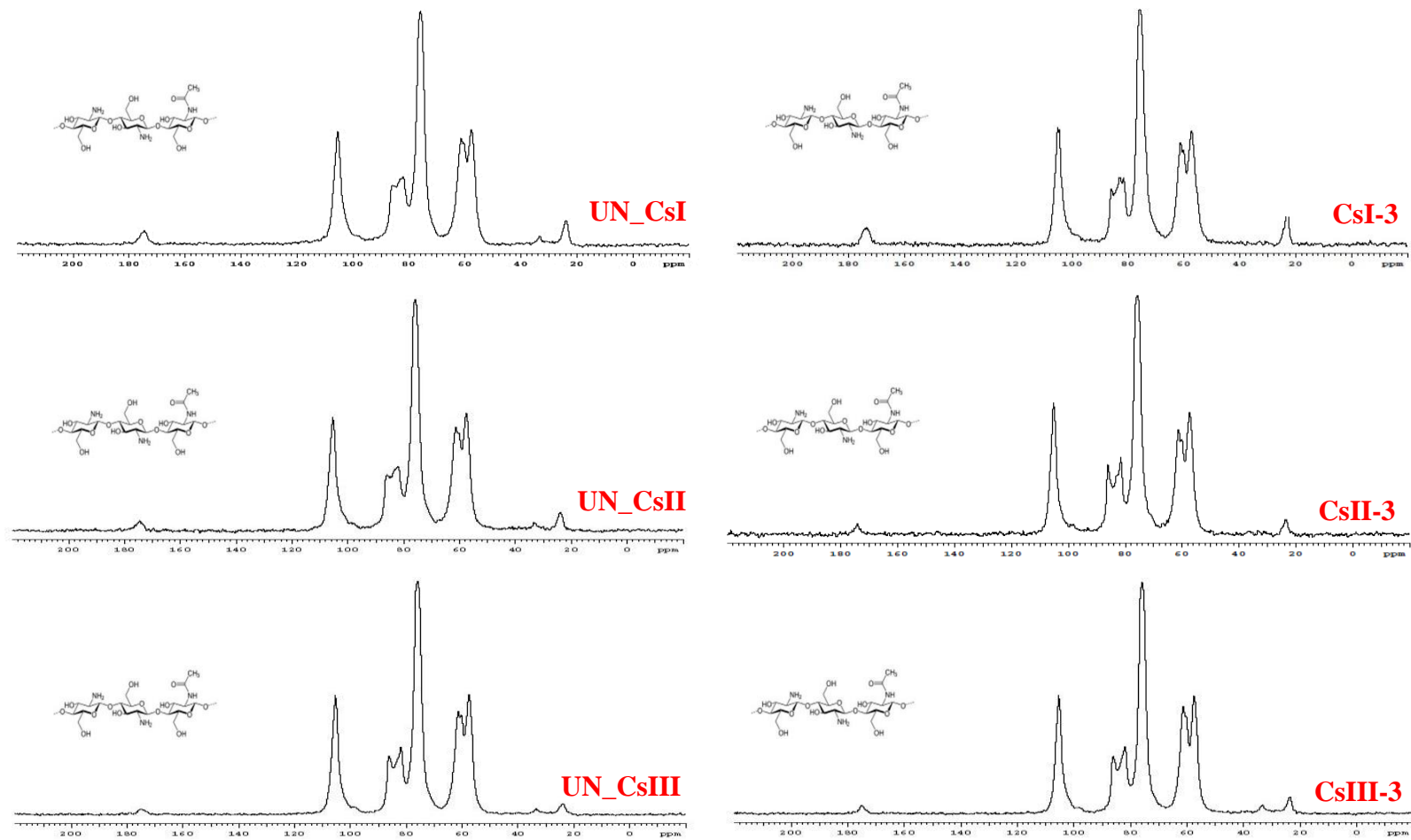
**Figure 2:** XRD spectra of Cs and Cs derivatives: Cs with AD of 17% (A), Cs with AD of 13% (B) and Cs with AD of 8% (C). Cs: chitosan; UN\_Cs: undigested chitosan; Cs-1: Cs treated with Cellulase for 1 h; Cs-3: Cs treated with Cellulase for 3 h.

**Figure 3:** TGA (A) and DTG (B) thermograms of Cs and Cs derivatives. Cs: chitosan; UN\_Cs: undigested chitosan; Cs-1: Cs treated with Cellulase for 1 h; Cs-3: Cs treated with Cellulase for 3 h.

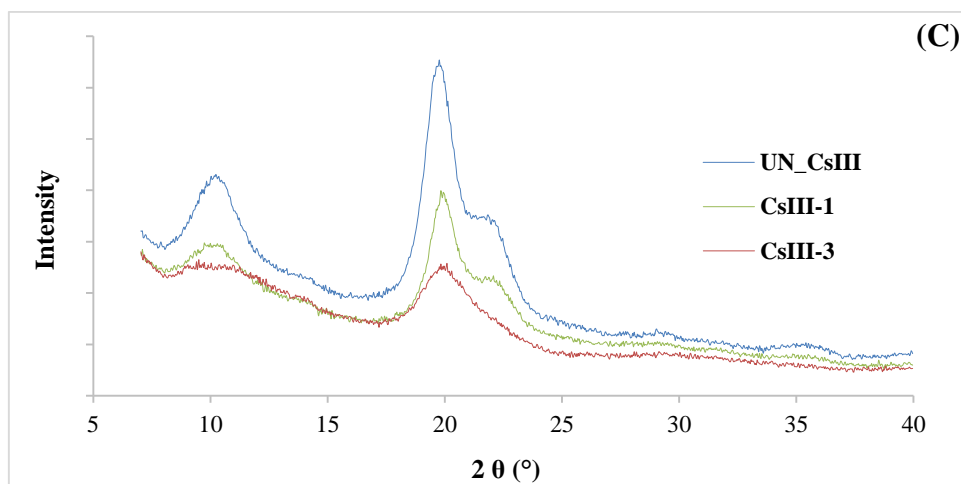
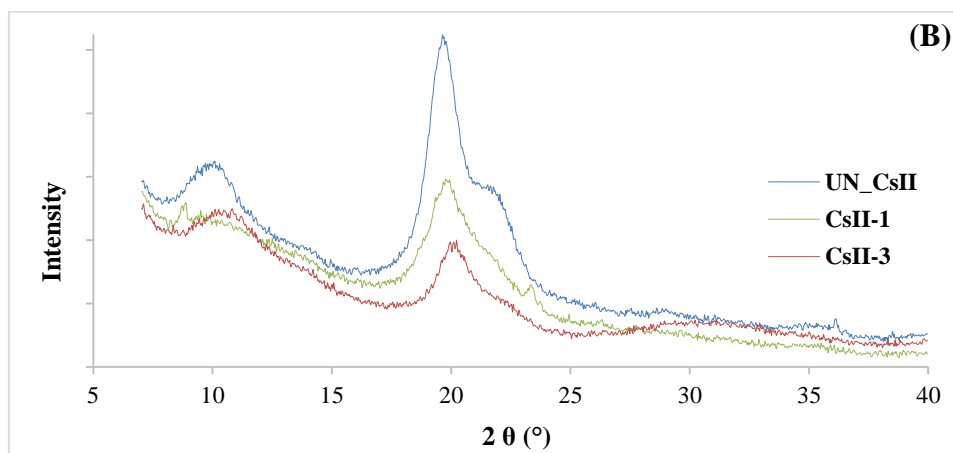
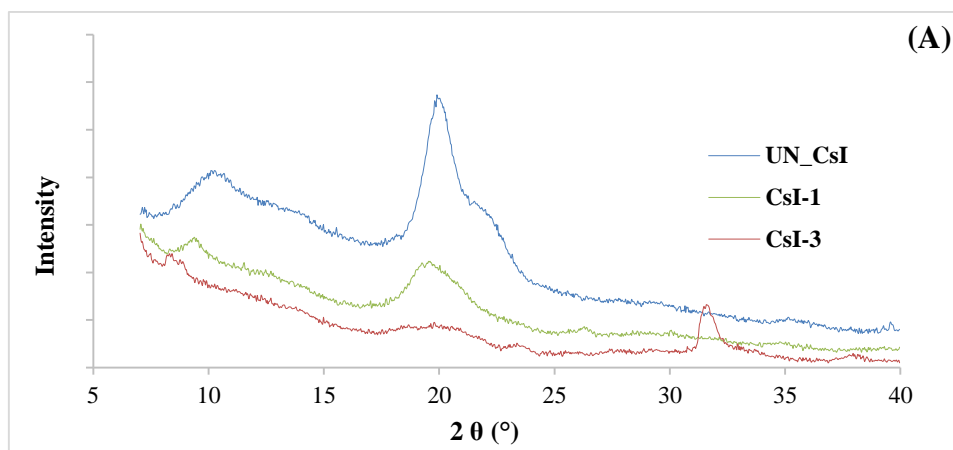
**Figure 4:** Microbial adhesion inhibition (A) and pre-formed biofilm disruption effect (B) of Cs and Cs derivatives. Cs: chitosan; UN\_Cs: undigested chitosan; CsI: Cs with AD of 17%; CsII: Cs with AD of 13%; CsIII: Cs with AD of 8%; Cs-1: Cs treated with Cellulase for 1 h; Cs-3: Cs treated with Cellulase for 3 h.

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**Fig. 1**



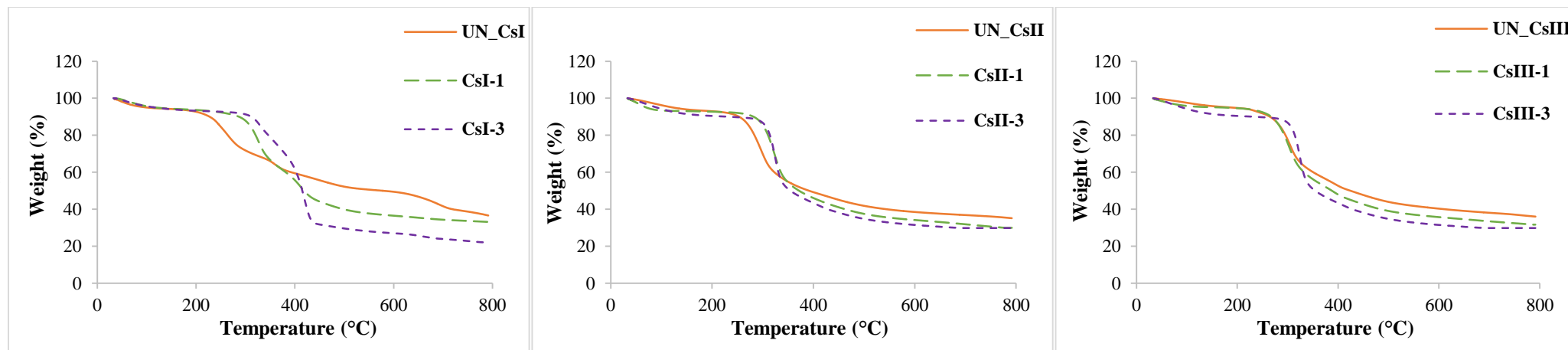
**Fig. 2**



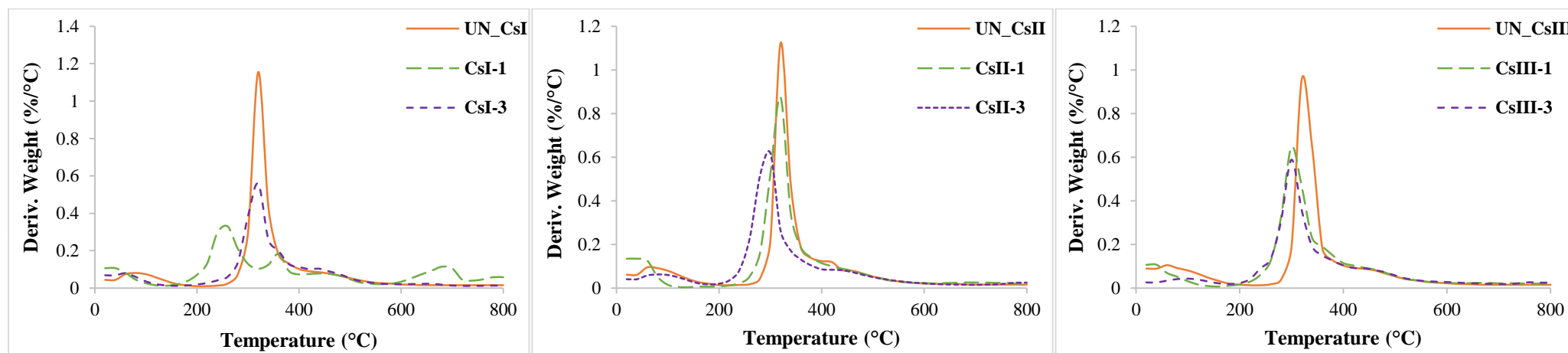
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**Fig. 3**

**(A)**

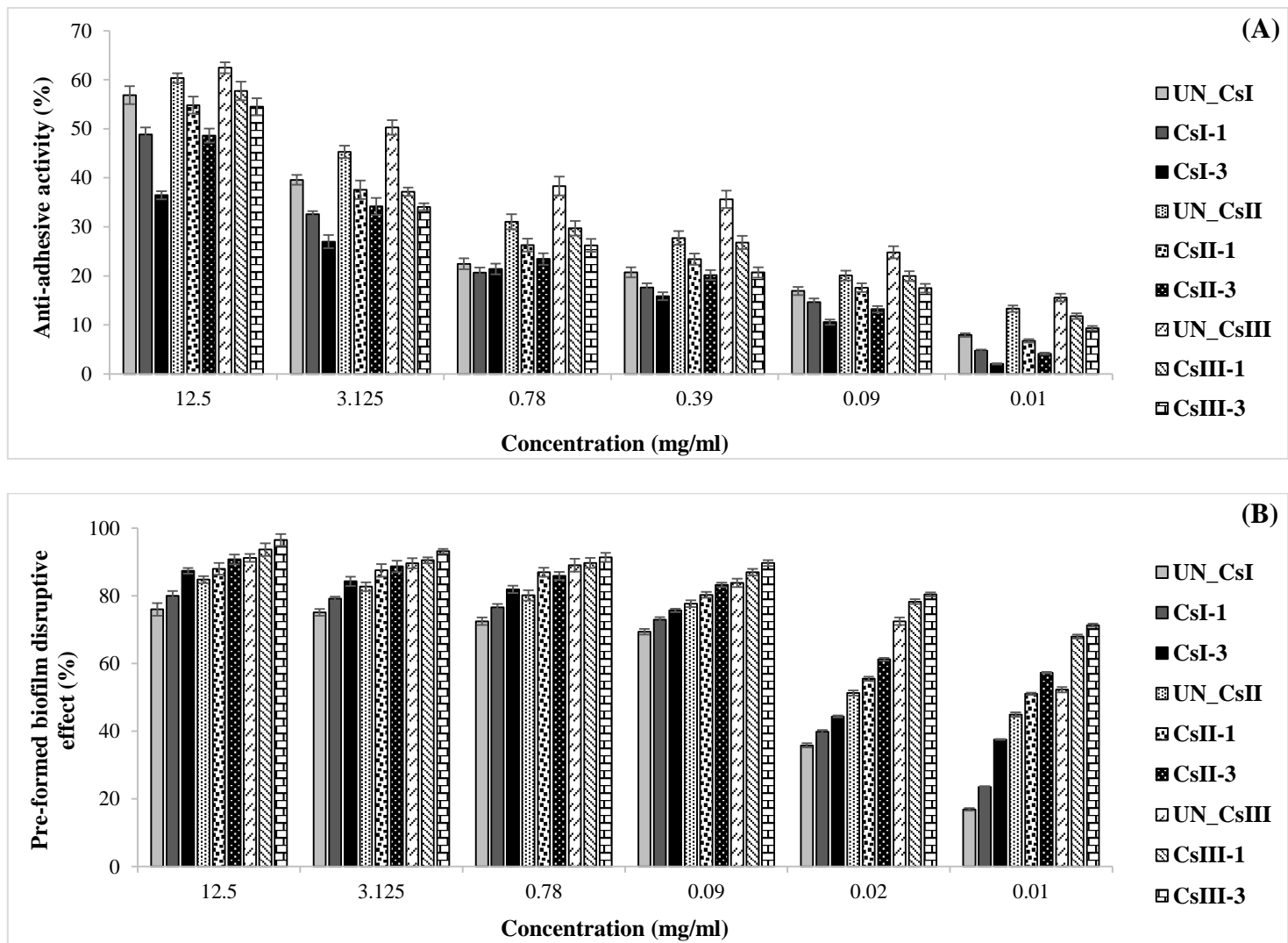


**(B)**



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**Fig. 4**



Sfax, 24<sup>th</sup> of April 2020

### **Credit author statement**

**Marwa HAMDI:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing-Editing, Visualization.

**Rim NASRI:** Resources, Supervision.

**Ikram BEN AMOR:** Investigation.

**Suming LI:** Resources, Supervision.

**Jalel GARGOURI:** Investigation.

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