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1 **Microencapsulation of benzalkonium chloride enhanced its antibacterial**
2 **and antibiofilm activity against *Listeria monocytogenes* and *Escherichia coli***

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18 Running headline: Antibiofilm action of microencapsulated BAC

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24

25 Abstract**26 Aims**

27 In this study, benzalkonium chloride (BAC) microcapsules were developed for surface
28 disinfection purpose and were evaluated against *Listeria monocytogenes* and *Escherichia coli*
29 biofilms.

30 Methods and Results

31 Microcapsules were prepared with two different strategies: uncomplexed BAC-microcapsules
32 (UBM), containing BAC and maltodextrins and complexed BAC-microcapsules (CBM)
33 containing BAC complexed by pectin and maltodextrins. The minimum inhibitory
34 concentrations (MICs) of free and microencapsulated BAC was investigated against two food
35 pathogens: *L. monocytogenes* and *E. coli*. The antibiofilm activities of UBM and CBM against
36 *L. monocytogenes* and *E. coli* biofilms formed on stainless steel at 37°C were evaluated and
37 compared to BAC used under its free form. MICs of encapsulated BAC were up to 4-fold lower
38 than those of free BAC. The UBM and CBM showed higher antibiofilm effect when compared
39 to the free BAC.

40 Conclusions

41 Overall, results demonstrated that microencapsulation enhanced the antibacterial activity of
42 BAC against *L. monocytogenes* and *E. coli* biofilms.

43 Significance and Impact of the Study

44 The application of such BAC microcapsule-based delivery systems can improve surface
45 disinfection procedures and reduce the required BAC concentrations and the related
46 cytotoxicity of this antimicrobial compound.

47 **Keywords:** Benzalkonium chloride, *Listeria monocytogenes*, *Escherichia coli*,
48 microencapsulation, antibiofilm activity

49 **Introduction**

50 Spray-drying microencapsulation is the most common method for turning a fluid solution into
51 dry particles due to its economical and easy operation (Abrahão *et al.* 2019). Spray-drying is
52 commonly used for microencapsulation in food and pharmaceutical industries. Applying spray-
53 drying for the encapsulation may impose stresses on the encapsulated active molecules through
54 exposure to high temperature, shearing, and dehydration. However, good control of the
55 operating parameters during the drying process makes it possible to reduce the residence time
56 of the capsules in the drying chamber and to protect the biological activity. Our previous
57 researches have proved the effectiveness of spray-drying microencapsulation of different active
58 compounds with pectin (Ben Amara *et al.* 2016, 2017).

59 Quaternary ammonium compounds (QACs) are the major class of cationic surfactants used as
60 disinfectants in domestic, industrial, and medical applications (Zhang *et al.* 2015). Their
61 structure consists of a positively charged nitrogen atom and four covalently bonded alkyl
62 groups. There is at least one hydrophobic hydrocarbon chain in these four alkyl groups, and
63 other alkyl groups are mostly short-chain moieties such as methyl or benzyl groups. The annual
64 worldwide consumption of QACs was reported as 500,000 tons in 2004 (Chen *et al.* 2018), the
65 production of QACs per year exceeds 344,000 tons in US (Phan 2019), and it is expected to
66 continuously increase in the future. QACs are generally considered to be biodegradable in an
67 activated sludge system. However, this degradation varies depending on the QAC
68 concentration, chemical structure, complexation with anionic surfactants, and microbial
69 acclimation (Zhang *et al.* 2015). Benzalkonium chloride (BAC) is a broad-spectrum
70 antimicrobial widely used to disinfect environmental surfaces in clinical and food industrial
71 settings (Kim *et al.* 2018). BAC has a benzyl group, two methyl groups and an alkyl chain
72 (C12-C18). BAC of various carbo chain lengths have diverse applications ranging from surface
73 disinfection in hospitals, food production, agriculture or dairy industry, to their use in eye drops

74 and hair shampoos (Kampf 2018; Mulder *et al.* 2018). On the other hand, BAC can also cause
75 genotoxic effects in mammalian and plant cells at environmentally relevant concentrations
76 (Ferk *et al.* 2007). The extensive use of QACs in domestic and industrial applications has
77 outcompeted its biodegradation in the environment, which results in its enrichment in
78 wastewaters and aquatic environment. When the concentration of some QACs exceeds certain
79 level in the environment, they become ecotoxic (Elersek *et al.* 2018). Therefore, there is a
80 growing concern on the presence of QACs in the environment, and the reduction of usage of
81 QACs should be a good study direction.

82 Pectin is a water soluble polysaccharide widely used in food manufacturing for its thickening
83 and gelling properties. Native pectins are high methoxyl pectin in which the majority of
84 carboxylic acid group are esterified by methanol and consequently non ionizable: therefore, in
85 the present work, LM pectin having a higher proportion of free ionized carboxylic acid groups
86 was considered in order to favor electrostatic interactions with positively charged BAC.

87 In this study, we use an electrostatic anchoring strategy to fix BAC inside microcapsules
88 containing pectin. The purpose is then to evaluate the effects of pectin complexation and spray-
89 drying microencapsulation on the antibiofilm activity of BAC. The cytotoxicity of both free
90 and microencapsulated BAC was evaluated. The overall goal was to improve the effectiveness
91 of this disinfectant through its microencapsulation while reducing the used amounts and
92 resulting cytotoxicity.

93 **Materials and methods**

94 *Materials: bacteria and chemicals*

95 The target strains used in this study were: *Listeria monocytogenes* ATCC 35152 and
96 *Escherichia coli* CIP 54127. Strains were maintained at -80°C in Tryptone Soy Broth (TSB,
97 Biokar Diagnostics, France) supplemented with 20% (v:v) glycerol. Pectin was from (Cargill,

98 Bauppte, France). The degree of esterification was from 22% to 28% and degree of acetylation
99 was from 20% to 23%. Maltodextrins DE 19 (dextrose equivalent value of 19) were obtained
100 from (Roquette-freres SA, Lestrem, France). Benzalkonium chloride (BAC), analytical grade
101 imidazole ($C_3H_4N_2$), acetic acid, sodium hydroxide (NaOH), hydrochloric acid (HCl), and
102 ethanol were purchased from (Sigma-Aldrich, St Quentin Fallavier, France). Distilled water
103 was used for the preparation of all solutions.

104 *Stock solution preparation*

105 Imidazole-acetate buffer solutions (5 m mol l^{-1}) were prepared by dispersing weighed amounts
106 of imidazole and acetic acid into distilled water. The stock solutions were prepared by
107 dispersing BAC, pectin or maltodextrins powders into imidazole-acetate buffer stirring at room
108 temperature until total hydration, and then the pH was adjusted to 7 by using HCl (0.1 or 1.0
109 mol l^{-1}) or NaOH (0.1 or 1.0 mol l^{-1}). The concentration of each stock solution (w:w) was: BAC
110 1%, pectin 3%, and maltodextrins 50%. The pH of each solution was adjusted again to 7 before
111 analysis.

112 *Complexes formation and spray-drying*

113 The suspensions of complexes were prepared by mixing the stock solutions and imidazole-
114 acetate buffer to the desired concentration. Stock maltodextrins solutions were added to the
115 prepared suspensions, to obtain a final concentration (w:w) of 20% maltodextrins DE 19. Four
116 complex pectin/BAC ratios of 1, 2, 2.5 and 3 were prepared. For complexed BAC-
117 microcapsules study, only the ratio 3 was selected (20% maltodextrins DE 19, 0.4% BAC, 1.2%
118 pectin). The mixtures were stirred for 30 min and then spray-dried using a laboratory scale
119 device equipped with a 0.5 mm nozzle atomizer (Mini Spray-Dryer Büchi B-290, Switzerland).
120 The operational conditions of the drying process were as follow: feed flow rate: 0.5 l h^{-1} , inlet
121 air temperature: $180 \pm 2^\circ\text{C}$, and outlet air temperature: $80 \pm 5^\circ\text{C}$. After spray-drying, the
122 powders were collected in sealed containers and stored at 4°C until analysis. Reconstituted

123 suspensions were prepared by dispersing weighted amounts (the same dry matter as before
124 spray-drying) of spray-dried powders in imidazole-acetate buffer solutions (5 m mol l⁻¹, pH 7)
125 and stirring for 1 h.

126 *Zeta potential measurement*

127 The electric charge of free molecules or complexes was determined using a Zetasizer Nano
128 ZS90 (Malvern Instruments, Malvern, UK). The zeta-potential (ZP) of BAC and pectin was
129 measured in imidazole-acetate buffer at different pH values. If necessary, the samples were
130 diluted with corresponded pH imidazole-acetate buffer. Each test was repeated at least three
131 times. The mean ZP values (\pm SD: standard deviation) were obtained from the instrument.

132 *Particle size measurement*

133 Particle size distribution of complexes were assessed by a laser diffraction instrument
134 (Mastersizer 3000, Malvern Instruments, Malvern, UK) with a value of 1.33 for water phase.
135 To measure the particle size distribution of spray-dried microcapsules, the powder was slowly
136 added into the measurement chamber containing ethanol (refractive index: 1.36) to avoid the
137 dissolution of maltodextrins during the measurement. The spray-dried powders were
138 continuously stirred throughout the measurement to ensure that the samples were
139 homogeneous. The volume mean particle diameter (D₄₃) was calculated by the software from
140 the three injections of three separate samples with five readings per sample.

141 *Moisture content*

142 The moisture content of spray-dried microcapsules was measured by drying in an oven at 160°C
143 under air circulation for 24 h. Spray-dried microcapsules (~1 g) were placed in caps that were
144 weighed before and after oven drying. Moisture content values were calculated as a fraction of
145 the initial microcapsules weight lost during drying and were reported on a wet basis.

146 *Bacterial susceptibility testing*

147 *L. monocytogenes* and *E. coli*, were cultured overnight in TSB at 37°C. Cells were pelleted by
148 centrifugation (5000 g, 5 min, 20°C) then washed with Potassium Phosphate Buffer (PPB; 100
149 m mol l⁻¹, pH 7). Bacterial suspensions were diluted in TS broth to 10⁶ CFU ml⁻¹. The Minimum
150 inhibitory concentrations (MICs) of free-BAC, uncomplexed BAC-microcapsules (UBM) and
151 complexed BAC-microcapsules (CBM) were determined by measuring kinetically, the
152 development of turbidity by vertical photometry using a Bioscreen C (Labsystems, Helsinki,
153 Finland), (Khelissa *et al.* 2018). Briefly, 100 µl of bacterial suspension were added to the plate
154 wells containing serial 2-fold dilutions from microcapsules or free BAC stock solution prepared
155 in TSB. A growth control (bacteria with microcapsule-free TSB), and a sterility control (with
156 only microcapsule-free TSB) were added to each plate. Plates were incubated at 37°C under
157 continuous shaking in the Bioscreen C which measured the optic density (OD) at 600 nm every
158 2 h during 24 h. The MIC was defined as the lowest concentration preventing the bacterial
159 growth, as measured by optical density. The experiment was repeated three times and mean OD
160 600 nm values were plotted versus time.

161 *Biofilm installation and disinfection assay*

162 All bacterial strains were precultured in TSB (5 ml) for 24 h at 37°C. Cultures were prepared
163 by inoculating 50 ml of TSB with 10⁴ CFU ml⁻¹ from the preculture and incubated at 37°C
164 overnight. Cells were and harvested by centrifugation (5000 g, 5 min, 20°C) and washed twice
165 with PPB. Bacterial suspensions were adjusted to 10⁸ CFU ml⁻¹ in PPB (OD 600 nm 0.110 ±
166 0.005). Stainless steel slides (50 x 25 mm) were covered with 3 ml of the cell suspension
167 adjusted to 10⁷ CFU ml⁻¹ and allowed to stand at 20 °C for 1 h. Afterwards, slides were rinsed
168 in PPB, to remove loosely attached cells. Each slide was covered with 3 ml of TSB and
169 incubated statically at 37°C for 24 h. After 24 h of incubation, slides covered with biofilm were
170 rinsed with PPB then placed horizontally in 20 ml of the tested solution (at the MIC) and
171 incubated for 10 min. Afterwards, slides were withdrawn from the tested solution and immersed

172 in 20 ml of neutralizing solution (Toté *et al.* 2010) containing 1 g of 1 mm diameter glass beads
173 in 100 ml sterile pot to stop the antibacterial action. In order to detach the biofilm cells were
174 from the stainless steel surface, pots were vortexed for 30 s followed by a sonication for 5 min
175 (37 kHz, 20 °C) (Elmasonic S60H, Elma, Germany). Thereafter, pots were vortexed again for
176 30 s and serial dilutions were realized in TS. Samples of 100 µl were spread onto TSA plates
177 and incubated at 37°C for 24 h. The number of viable and culturable cells was counted on plates
178 and results were expressed in log CFU cm⁻². For the control assays, the disinfectant solution
179 was replaced by the TS. The cell viability percentage in treated biofilms was determined after
180 staining with the LIVE/DEAD® BacLight Bacterial Viability kit (Invitrogen Molecular Probes,
181 USA). Briefly, 10⁷ CFU ml⁻¹ of detached cells were filtered through a 0.2 µm-pore-size black
182 polycarbonate filters (Millipore, France) and stained for 10 min in the dark. Stained cells were
183 washed once with 1 ml of distilled water, and filters were air dried in the dark then observed
184 under epifluorescence microscopic (Olympus BX43, Germany) for enumeration. The green
185 cells (viable) and red ones (dead) were counted over 50 microscopic fields. The percentage of
186 viable cells was calculated as follows: (Number of green cells x 100) / total number of both red
187 and cells. Results represent the average of three independent experiments and in each case, two
188 slides were used.

189 *Scanning electron microscopy (SEM)*

190 The morphology of biofilm bacterial cells treated for 10 min with free BAC or CBM at MICs
191 was assessed by SEM and compared to negative control where cells were treated with Tryptone
192 Salt (TS). Biofilm treatment cells were recovered according to Abdallah *et al.*, (2014) and
193 diluted (10-fold) Tryptone Salt (TS). Samples of 1 mL volume from the diluted biofilm cell
194 suspensions were filtered through a 0.2-µm-pore-size polycarbonate membrane filter
195 (Schleicher & Schuell, Dassel, Germany) then fixed overnight with 2% glutaraldehyde,
196 cacodylate buffer 0.1 mol l⁻¹, pH 7, at 4°C. Fixed cell samples were dehydrated in an ascending

197 ethanol series (50%, 70%, 95%, and 100% (×2) (v:v) ethanol), for 10 min at each concentration
198 and dried. Fixed bacterial samples were carbon coated and examined at an accelerating voltage
199 of 3 kV.

200 *Epifluorescence microscopy analysis*

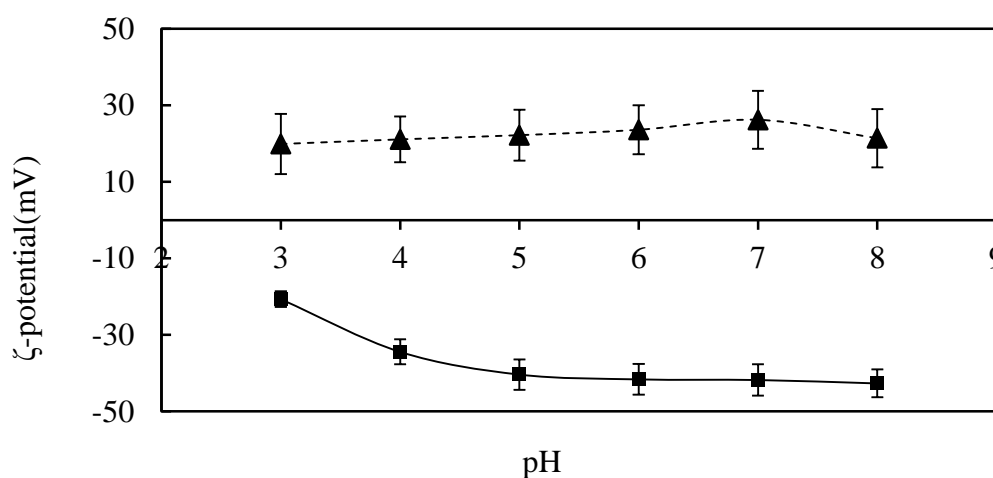
201 Biofilms were treated with free and encapsulated BAC and for the control biofilm were treated
202 with TS. Biofilms were stained with LIVE/DEAD BacLight kit (Invitrogen Molecular Probes,
203 USA), according to the manufacturer instruction for 15 min. Biofilms were washed by gently
204 dipping the stainless steel coupons in distilled water, air dried in the dark then observed under
205 epifluorescence microscopic (Olympus BX43, Germany). The green cells were considered
206 viable and red ones were defined as non-viable.

207 *Cytotoxicity assay*

208 The *HeLa* cell line derived from cervical carcinoma from a 31 year old female (ATCC® CCL-
209 2™, ECACC, Sigma Aldrich, Saint-Quentin Fallavier, France) were cultured and maintained
210 in Dulbecco's Modified Eagle's medium (DMEM, Gibco®) supplemented with 10% fetal
211 bovine serum (FBS, Gibco®) and 1% penicillin-streptomycin (Gibco®) in a humidified
212 incubator at 37 °C and 5% CO₂. Cells were seeded at a density of 10⁴ cells per well in a 96-well
213 plate and grown for 24 h before assay. The culture medium was replaced with a fresh medium
214 that contains the compounds from 12.5 to 400 µg ml⁻¹. After 24 h, the old medium was removed
215 and cells were washed with PBS. The cell viability was evaluated using resazurin cell viability
216 method. Briefly, 100 µl of the resazurin solution (11 µg ml⁻¹) in complete medium were added
217 to each well and the plate was incubated for 4 h in the humidified incubator. The fluorescence
218 emission of each well was measured at 593 nm (20-nm bandwidth) with an excitation at 554
219 nm (18-nm bandwidth) using a Cytation™ 5 Cell Imaging Multi-Mode Reader (BioTek
220 Instruments SAS, France). Each condition was replicated three times and the mean fluorescence
221 value of non-exposed cells (treated with TSB) was taken as 100% cellular viability.

222 *Statistical analysis*

223 All experiments were carried out at least three times. Statistical study was performed with IBM
224 SPSS 19 statistics software following the one-way ANOVA. Results are considered
225 significantly different when $P < 0.05$.

226 **Results**227 *BAC-LM pectin interactions and properties of the formed complexes*

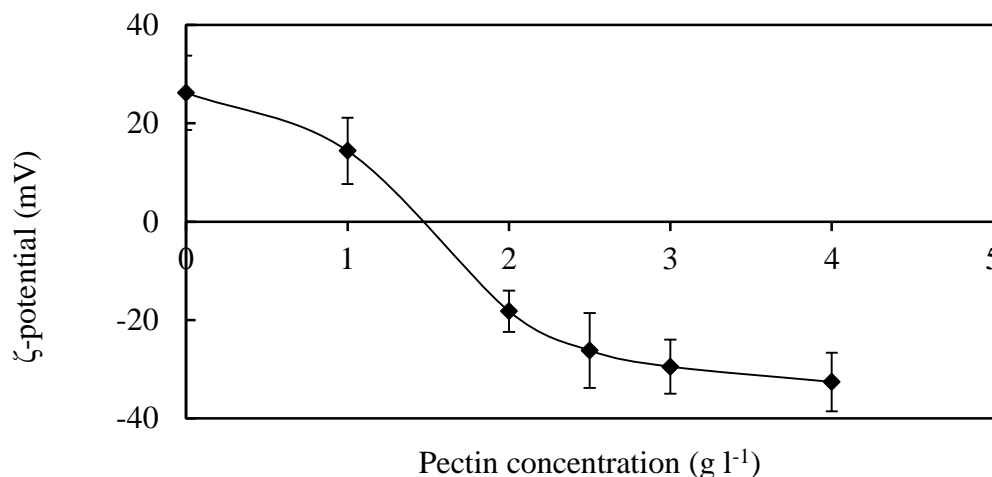
228

229 **Figure 1**

230

231 To choose optimum pH for the complexation, the zeta-potential (ZP) of BAC and pectin as
232 function of pH was measured as shown in Figure 1. At the pH range from 3 to 8, pectin was
233 negatively charged. In detail, the absolute value of ZP for pectin obviously increased with
234 increasing the pH from 3 to 5, which was due to the deprotonation of carboxyl groups. With
235 continual increase of pH over 5, the changes in ZP value became unobvious, which indicated
236 the end of deprotonation of carboxyl groups. The zeta-potential of BAC was overall stable at
237 around +20 mV whatever the pH value. At pectin/BAC mass ratio 1.5, the complexes of BAC
238 and pectin became electrical neutral at pH 7 (Figure 2).

239



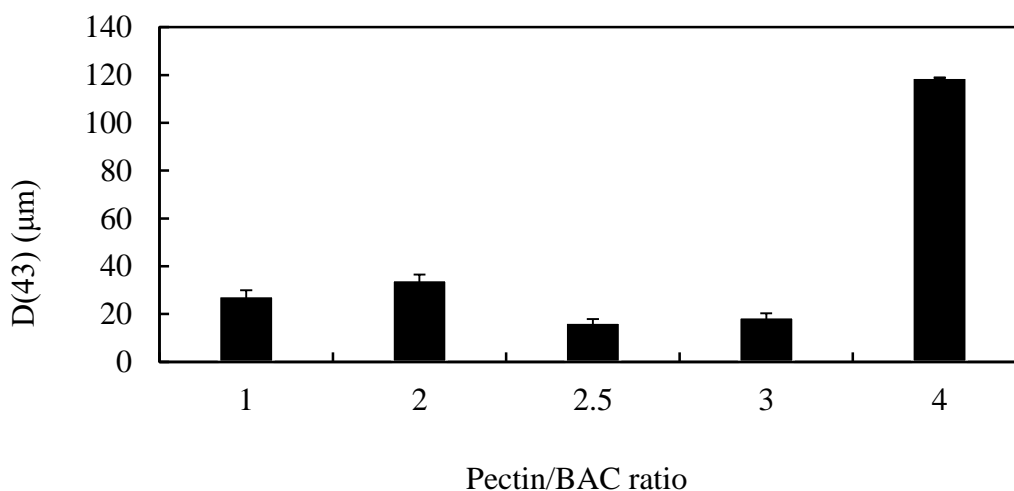
240

241

Figure 2

242

243 The ZP of the complexes reached a plateau after the pectin/BAC mass ratio reached 3, which
 244 could be indicated that positively charged BAC groups were fully occupied by pectin (Figure
 245 2). Therefore, this ratio was chosen for the following experiments as it allowed to have small
 246 complexes having a compact structure (Figure 3). Nevertheless, at ratio 4, the high pectin
 247 amount resulted in the dissociation of pectin/BAC complexes. This could be due to the bridging
 248 of BAC molecules which significantly ($P < 0.05$) increased the size of complexes (Figure 3).
 249 For high concentrations of pectin, the complexes can dissociate and a single molecule of BAC
 250 could bound to several chains of pectin which increases the apparent size of the complexes.

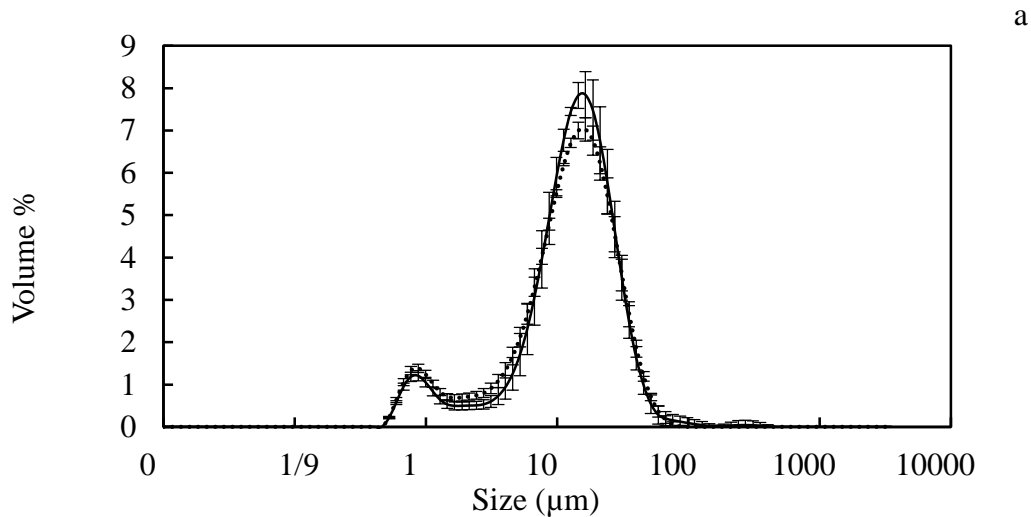


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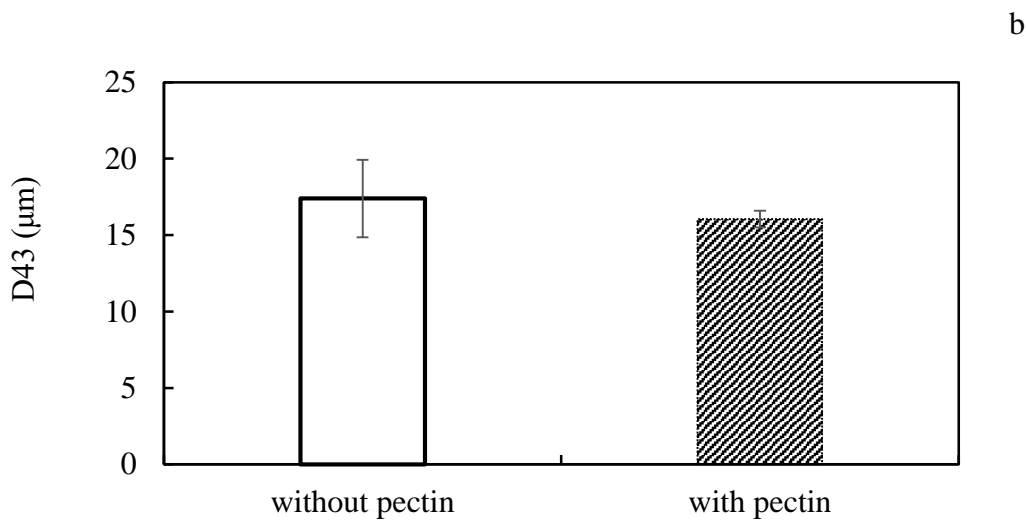
252

Figure 3

253

254 *Physicochemical properties of spray-dried microcapsules*

255



256

Figure 4

257

258

259 The spray-dried BAC microcapsules were white color with low moisture content of 3.70% and
260 5.15% for UBM and CBM, respectively. Pectin is a polyanionic biopolymer which has a high
261 affinity with water molecules, which explains the highest water content for CBM. On the other
262 hand, for the 2 types of capsules, these water contents should make it possible to have good
263 storage capacities. The particle size of spray-dried microcapsules with and without pectin
264 showed no significant difference ($P > 0.05$) in their particle size distribution (Figure 4 a). For

265 details, the mean diameters (D_{43}) were ranged between 17.3 μm for spray-dried BAC without
 266 pectin and 16 μm for spray-dried BAC with pectin microcapsules (Figure 4 b). These particle
 267 distribution properties should make it possible to have fluid powders with good properties of
 268 use whatever the type of microcapsules.

269

270 *Assessment of bacterial susceptibility to free and microencapsulated BAC*

271

272 **Table 1.** Minimal inhibitory concentration (in mg l^{-1}) of free and encapsulated BAC against *E.*
 273 *coli* and *L. monocytogenes*.

Bacterial strains	free BAC	CBM	UBM
<i>E. coli</i>	12.5	3.12	6.25
<i>L. monocytogenes</i>	6.25	1.56	3.12

274 Abbreviations: BAC, benzalkonium chloride; CBM: complexed BAC-microcapsules; UBM:
 275 uncomplexed BAC-microcapsules.

276

277 The MIC of CBM and UBM microcapsules were determined and compared to those of free
 278 BAC against *L. monocytogenes* ATCC 35152 and *E. coli* CIP 54127. The obtained results are
 279 shown in Table 1. The MICs of the encapsulated BAC were significantly ($P < 0.05$) lower than
 280 those of free BAC against all studied strains. In addition, the MICs of CBM were 2-fold ($P <$
 281 0.05) lower than those of UBM whatever the studied strain (Table 1). *L. monocytogenes* was 2-
 282 fold more susceptible to all studied BAC forms than *E. coli* (Table 1).

283 *Antibiofilm assays*

284

285 **Table 2.** The reduction (in log CFU cm⁻²) and viability percentage of *E. coli* and *L.*
 286 *monocytogenes* biofilm biomass after treatments with free or microencapsulated BAC.

Bacteria	Viable cells %			Biomass log reduction		
	BAC	CBM	UBM	BAC	CBM	UBM
<i>E. coli</i>	69 ± 2	57 ± 3	65 ± 2	1.8 ± 0.2	3.1 ± 0.1	2.1 ± 0.1
<i>L. monocytogenes</i>	52 ± 3	37 ± 2	48 ± 3	2.5 ± 0.3	3.6 ± 0.2	2.3 ± 0.1

287

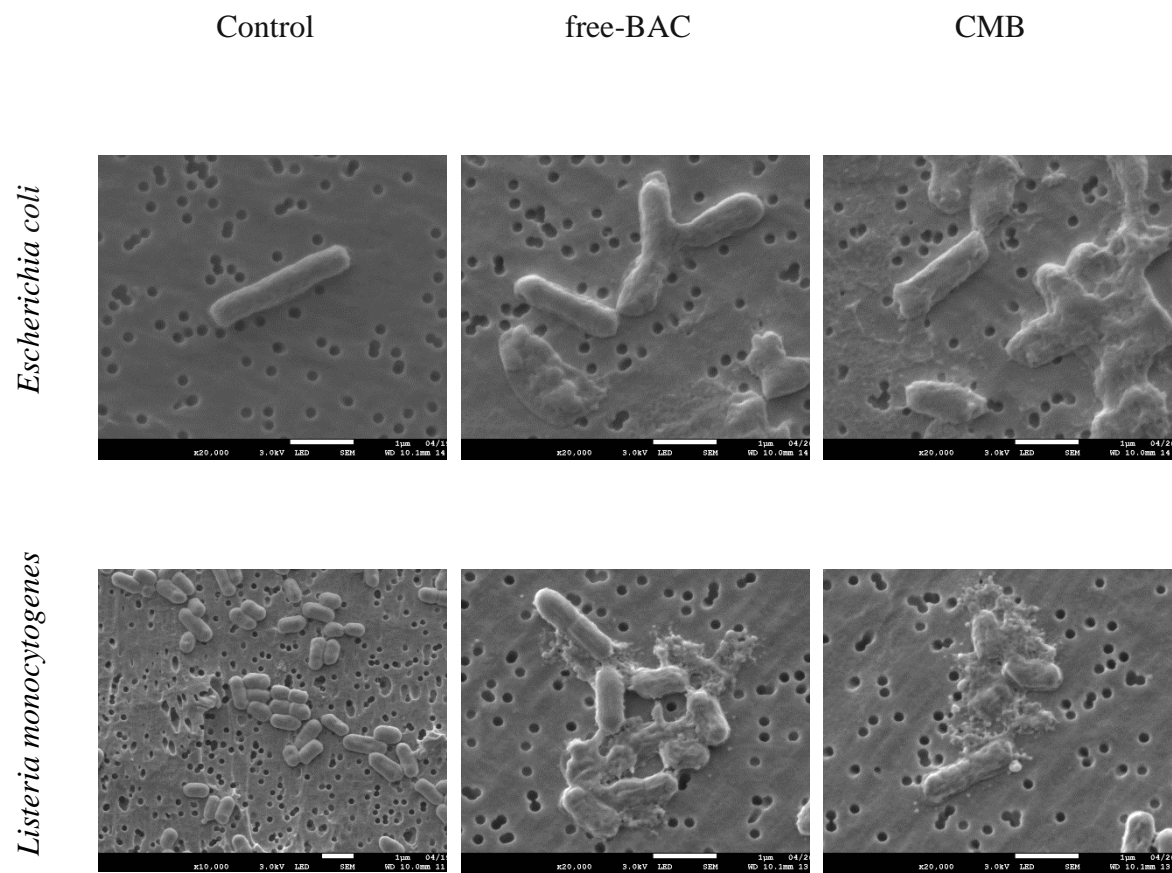
288 **Abbreviations:** BAC: benzalkonium chloride; CBM: complexed BAC-microcapsules; UBM:
 289 uncomplexed BAC-microcapsules. Data presented as means (±SD) of three independent
 290 repeats.

291 The antibiofilm efficacy of CBM and UBM used at their MICs was investigated on *L.*
 292 *monocytogenes* and *E. coli* biofilms and compared to that of free BAC, respectively. The results
 293 obtained were expressed as log CFU cm⁻² reductions in treated biofilms, compared to untreated
 294 controls (Table 2). The treatment with free BAC resulted in log reduction of the initial
 295 biomasses of *E. coli* and *L. monocytogenes* biofilms by 1.8 and 2.5 log CFU cm⁻² ($P < 0.05$),
 296 respectively (Table 2). Similar results were observed after treatment with UBM (Table 2). The
 297 treatment with CBM resulted in a significant log reduction of *ca* 3.1 and 3.6 log CFU cm⁻² (P
 298 < 0.05), for *E. coli* and *L. monocytogenes*, respectively. CBM showed higher antibiofilm effect
 299 as compared to UBM ($P < 0.05$). Furthermore, the percentage of viable cells after staining with
 300 the SYTO9 and propidium iodide (PI), showed that untreated *E. coli* and *L. monocytogenes*
 301 biofilms were composed mainly of viable cells (respectively of *ca* 93 and 96%, data not shown).
 302 The results presented in Table 2 also suggest that the treatment with free BAC reduced the
 303 viability of *E. coli* and *L. monocytogenes* biofilms to 69 and 52% respectively ($P < 0.05$). After
 304 treatment with CBM and UBM the remaining *E. coli* viable cells percentage was of *ca* 57 and
 305 65 % respectively ($P < 0.05$). The viability percentages of *L. monocytogenes* after treatment

306 with CBM and UBM were of 37 and 48% respectively (Table 2). Upon antibacterial treatment
307 the viability percentage results were in agreement with the cellular biomass reduction (Table
308 2). In the free and microencapsulated BAC-treated biofilms the decrease of viable cells
309 indicates the increase of percentage of membrane-compromised stained with PI (Table 2).

310 *Impact of free and encapsulated BAC treatment on biofilm cells morphology*

311



312

313

Figure 5

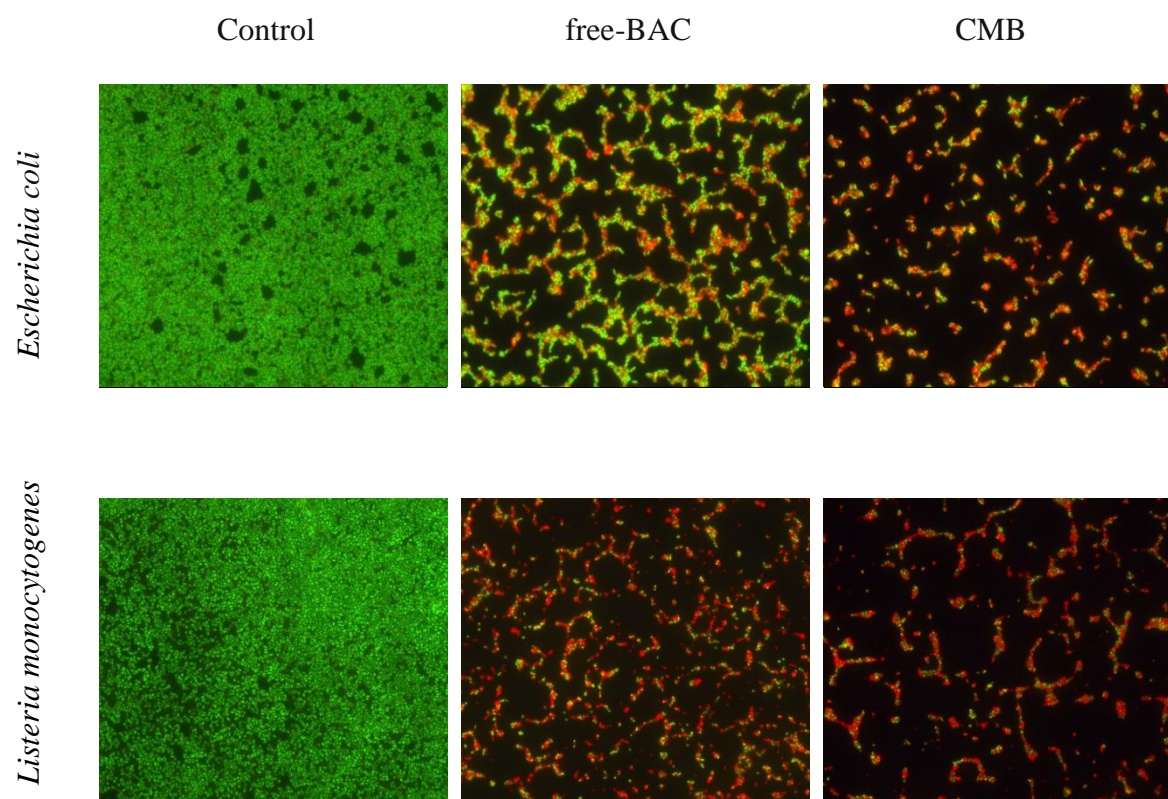
314

315 To investigate the morphological modifications of *E. coli* and *L. monocytogenes* biofilm cells
316 after the treatment with either free-BAC or BAC microcapsules. Bacterial samples were
317 analyzed by SEM. As the effect of both free BAC and UBM on treated cells morphology was
318 similar, UBM-treated cells micrographs were not shown (Figure 5). The negative control (cells

319 treated with TS) showed intact cells which exhibited a normal and smooth lining (Figure 5).
320 However, whatever the studied bacteria strain, the exposure to both free-BAC and CBM
321 solutions (concentration adjusted to the MIC) for 15 min led to significant structural collapse
322 (Figure 5). Cells seemed irreversibly lysed, deformed and showed holes in their cell walls
323 (Figure 5).

324 *Cell Viability and biofilm architecture*

325



326

327

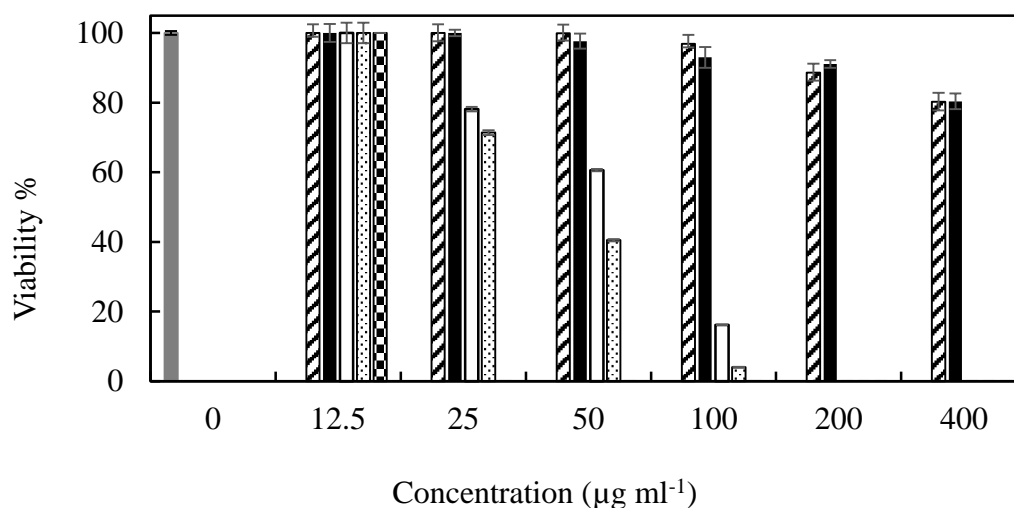
Figure 6

328

329 Figure 6 showed epifluorescence microscopy images of 24 h *E. coli* and *L. monocytogenes*
330 biofilms stained with SYTO9 and PI after treatment with free and encapsulated BAC. The
331 control (biofilm treated with TS) showed a thick and covering viable biomass mainly stained
332 with SYTO9. The treatment with BAC and CBM resulted in a significant removal of biofilm

333 biomass from the SS coupon. Furthermore, the density of remaining biofilm cells stained with
334 PI after antibacterial treatment increased significantly. The results demonstrated a higher level
335 of cell dispersion after treatment with CBM as a very thin cell layer mostly stained with PI was
336 observed (Figure 6). According to our results, there was a correlation between the cellular
337 membrane integrity and the remaining biofilm cell biomass after treatment with both free BAC
338 and CBM which highlights the possible modes of action of these antimicrobials.

339 *Cytotoxicity of free and microencapsulated BAC*



340

341

Figure 7

342

343 The viability of *HeLa* cells was assessed after 24 h contact with free and micro-encapsulated
344 BAC concentrations ranging from 12.5 to 400 $\mu\text{g ml}^{-1}$ (Figure 7). The TSB was used as a
345 negative control. Two additional controls consisting of microcapsules formulated without BAC
346 (empty UBM and CBM). Figure 7 showed that TSB had no negative effect on *HeLa* cells
347 viability. When used at concentrations between 12.5 and 100 $\mu\text{g ml}^{-1}$, both empty UBM and
348 CBM had no cytotoxic effect on *HeLa* cells which showed a viability percentage of *ca* 100 %
349 after being in contact with empty microcapsules for 24 h. However, at concentrations of 200
350 and 400 $\mu\text{g ml}^{-1}$ the viability of *HeLa* cells was reduced by 10 and 20 % respectively, whatever

351 the studied empty microcapsules (Figure 7). Contact with BAC at $12.5 \mu\text{g ml}^{-1}$ was not toxic to
352 *HeLa* cells which remained 100 % viable (Figure 7). Our findings, underlined that exposure to
353 BAC concentrations greater than, or equal to $25 \mu\text{g ml}^{-1}$ was significantly cytotoxic ($P < 0.05$)
354 and led to 100 % *HeLa* cells viability reduction (Figure 7). Furthermore, CBM and UBM
355 demonstrated reduced cytotoxicity towards *HeLa* cell, with a minimum needed concentration
356 of $200 \mu\text{l ml}^{-1}$ to completely kill the *HeLa* cells. Nevertheless, CBM were significantly more
357 cytotoxic than UBM ($P < 0.05$) (Figure 7). These results confirm the ability of
358 microencapsulation to enhance the antibacterial effect of BAC while reducing its health hazard
359 even at doses that would otherwise be completely toxic.

360 **Discussion**

361 BAC is different from biomacromolecules like pectin having a complex molecular structure.
362 The antimicrobial used in this study has a simpler molecular structure which consists of
363 quaternary cationic nitrogen atom linked with a benzyl group, two methyl groups and an alkyl
364 chain (C16). The molecular structure of BAC could induce, in aqueous phase, the formation of
365 micelles by inside hydrophobic long alkyl chains that can be surrounded by positively charged
366 nitrogen atoms. The structure of BAC micelles with positively charged nitrogen atoms on the
367 surface would not change with the change of pH (Ilari *et al.*, 2014). Considering that pectin and
368 BAC carried opposite charges at the tested pH range, the most common pH for several
369 applications, pH 7, was chosen for the formation of electrostatic complexes in this study. Pectin
370 is net negatively charged at pH 7, and can combine sufficiently with positively charged BAC
371 via electrostatic attractive interactions. Therefore, the ZP of pectin/BAC complexes gradually
372 decreased with increasing the amount of added pectin (Figure 2).

373 Furthermore, our results showed that CBM and UBM microcapsules had low moisture content
374 which was slightly higher for those containing pectin. This could be due to the water holding
375 capacity of pectin. In this context, Botrel *et al.* (2017) reported that the differences in the

376 moisture content presented between particles were due to the affinities of the materials to water
377 and moisture diffusivities through the wall matrix. This moisture content is fitted in the ideal
378 moisture content range as 3-5% (Crouter and Briens 2014).

379 The size distribution of spray-dried microcapsules with and without pectin showed no
380 significant difference ($P > 0.05$). This result was expected since the same drying conditions
381 were used for all the feed solutions (temperature, pulverization pressure). However, Sansone *et*
382 *al.* (2011) have shown that the presence of pectin affects the maltodextrin/pectin particle size
383 significantly. To assess the susceptibility of *L. monocytogenes* and *E. coli* to the different form
384 of BAC, the MIC of CBM and UBM microcapsules were determined and compared to those of
385 free BAC. Our results showed that the encapsulation of BAC reduced the its MIC significantly
386 ($P < 0.05$). In addition, the MICs of CBM were 2-fold ($P < 0.05$) lower than those of UBM
387 whatever the studied strain. We demonstrated that treatment with CBM used at the MIC reduced
388 significantly the viability and cultivability of *L. monocytogenes* and *E. coli* biofilm cells. The
389 antibiofilm effect of CMB was significantly greater than those of free BAC and UBM whatever
390 the studied strain. These results are consistent with those obtained by Wang *et al.* (2019) for
391 citral microcapsules which showed an increase of the antibacterial effect of this essential oil
392 against several food pathogens when pectin was added to emulsified citral. This could be due
393 to the presence of a proper concentration of BAC in close of bacterial cells that could result
394 from a controlled release of BAC encapsulated in presence of pectin.

395 It has been reported that the antibacterial activity of many antimicrobial agents including BAC
396 results in the disruption of the cell membrane integrity through the insertion of the antimicrobial
397 agent in the cell membrane, leading to the collapse of the membrane and to the leakage of
398 cytoplasmic pool or penetration of the compound into the cell, affecting internal homeostasis
399 (McLandsborough *et al.* 2006; Khelissa *et al.* 2018, 2019). Thus, we observed the morphology
400 of bacterial cells after antibacterial treatment with SEM. SEM micrographs showed the

401 structural collapse and the irreversible lysis of cell walls. These observations are consistent with
402 our previously reported studies regarding the damage made by BAC to bacterial cells membrane
403 integrity (Khelissa *et al.* 2018, 2019). Like any other QAC, BAC is toxic for mammalian cells.
404 Our findings, underlined that exposure to BAC concentrations greater than, or equal to 25 μg
405 ml^{-1} killed HeLa cells up to 100 %. Our results, are consistent with those reported by Deutschle
406 *et al.* (2006), who reported that BAC used at low concentration (0.01%) nearly kill all human
407 respiratory epithelial BEAS-2B cell line after 2 h of exposure. These results highlighted the
408 health and environmental hazards associated to the BAC even at very low concentrations. In
409 addition, cytotoxicity assays showed apoptotic effects for lower concentrations and necrotic
410 effects for higher concentrations of BAC (Deutschle *et al.* 2006).

411 Exposure of *HeLa* cells to high concentrations of empty microcapsules (formulated without
412 BAC) resulted in up to 20% reduction of initial viability. This could be explained by the high-
413 dose-toxicity effect over 200 $\mu\text{g ml}^{-1}$. It has been demonstrated that pectin-derived acidic
414 oligosaccharides in mammalian assays were classified as cytotoxic only at highly
415 concentrations (Garthoff *et al.* 2010; Fiume *et al.* 2016). Our results showed that the
416 microencapsulation of BAC significantly reduced its cytotoxicity toward *HeLa* cells. The
417 significant reduction in BAC toxicity is an important step in advancing the developed UBM
418 and CBM towards industrial application.

419 In summary, BAC was successfully encapsulated by electrostatic complexation with pectin
420 using spray-drying technique. We studied the effect of complexation and spray-drying on the
421 antimicrobial action of BAC. The results showed that the complexation of BAC with pectin
422 would control its release kinetics from microcapsules, and therefore improve its effectiveness
423 against bacteria contaminating food. The increase of the pectin amount influenced the particle
424 size of BAC/pectin complexes before spray-drying. Microbiological tests demonstrated that
425 complexed BAC microcapsules (CBM) had the highest antimicrobial activity than

426 uncomplexed ones (UBM). Exposure of *E. coli* and *L. monocytogenes* biofilms MICs of free
427 and microencapsulated BAC resulted in the modification of overall biofilm cell viability, cell
428 morphology, and cell biomass dispersal. In addition, both CBM and UBM were more efficient
429 than free BAC when they are applied against several food pathogen biofilms. The application
430 of such a BAC microcapsule-based delivery system can improve the surface disinfection
431 procedures and reduce the generated chemical wastes that seems to be a good strategy to
432 maintain hygiene in concerned sectors.

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437 **Conflict of Interest**

438 There is no conflict of interest

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516 **Figures Captions**

517 Figure 1. Zeta-potential of benzalkonium chloride (BAC) --▲-- and pectin --■-- in imidazole
518 acetate buffer at different pH.

519 Figure 2. Zeta-potential of BAC/pectin complexes as a function of pectin concentration at pH
520 7 (the concentration of BAC is constant at 1 g l⁻¹).

521 Figure 3. Particle size distribution of BAC/pectin complexes as a function of pectin/BAC ratio.

522 Figure 4. Particle size distribution and corresponding average size of spray-dried
523 microcapsules; (a) Particle size distribution of BAC spray-dried microcapsules formulated with
524 pectin or without pectin —; (b) corresponding average size of BAC spray-dried
525 microcapsules formulated with pectin ▨ or without pectin □.

526 Figure 5. Scanning electron micrographs of *Escherichia coli* and *Listeria monocytogenes*,
527 biofilm cells grown at 37 °C for 24 h, after treatment with free benzalkonium chloride (free-
528 BAC) or pectin-complexed BAC-microcapsules (CBM) at the minimum bactericidal
529 concentration for 10 min. The control represents cells treated with Tryptone Salt Buffer.

530 Figure 6. Fluorescence microscopy images of *Escherichia coli* and *Listeria monocytogenes*,
531 biofilm cells grown at 37 °C for 24 h on the stainless steel after treatment with free
532 benzalkonium chloride (free-BAC) or pectin-complexed BAC-microcapsules (CBM) at the
533 minimum inhibitory concentration. Cells were visualized after staining with SYTO-9 (green
534 fluorescence for living bacteria) and propidium iodide (red fluorescence for dead bacteria). The
535 control represents cells treated with Tryptone Salt Buffer.

536 Figure 7. *HeLa* cells viability percentage after 24 h exposure to concentrations (between 12.5
537 and 400 µg ml⁻¹) of free BAC ▣; pectin-complexed BAC-microcapsules (CBM)▤; Spray
538 dried pectin microcapsules without BAC (empty CBM) ■; uncomplexed BAC-microcapsules

539 (UBM) □; Uncomplexed microcapsules formulated without BAC (empty UBM) ▣. The control
540 represents cells treated with Tryptone Soy Broth (TSB) ■ .