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

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Running headline: Antibiofilm action of microencapsulated BAC

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Abstract

Aims

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

Methods and Results

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

Conclusions

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

Significance and Impact of the Study

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

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

Introduction

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

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

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

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

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

Materials and methods

Materials: bacteria and chemicals

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

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

Stock solution preparation

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

Complexes formation and spray-drying

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

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

Zeta potential measurement

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

Particle size measurement

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

Moisture content

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

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

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

Scanning electron microscopy (SEM)

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

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

Epifluorescence microscopy analysis

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

Cytotoxicity assay

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

Statistical analysis

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

Results

BAC-LM pectin interactions and properties of the formed complexes

Figure 1

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

Figure 2

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

Figure 3*Physicochemical properties of spray-dried microcapsules* **Figure 4**

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

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

Assessment of bacterial susceptibility to free and microencapsulated BAC

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

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

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

Antibiofilm assays

Table 2. The reduction (in log CFU cm⁻²) and viability percentage of *E. coli* and *L. 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

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

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

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

Impact of free and encapsulated BAC treatment on biofilm cells morphology

Figure 5

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

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

Cell Viability and biofilm architecture

Figure 6

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

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

Cytotoxicity of free and microencapsulated BAC

Figure 7

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

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

Discussion

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

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

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

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

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

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

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

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

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

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Conflict of Interest

There is no conflict of interest

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Figures Captions

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

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

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

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

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

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

Figure 7. *HeLa* cells viability percentage after 24 h exposure to concentrations (between 12.5 and 400 µg ml⁻¹) of free BAC ▣ ; pectin-complexed BAC-microcapsules (CBM) ▤ ; Spray 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) ■ .