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ORIGINAL ARTICLE

Comparative study of growth temperature impact on the susceptibility of biofilm-detached and planktonic *Staphylococcus aureus* cells to benzalkonium chloride

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Abstract
The present study investigated and compared the effect of growth temperature on the susceptibility of biofilm-detached and planktonic *Staphylococcus aureus* cells, to benzalkonium chloride (BAC). This study also highlights the impact of BAC on the bacterial physiology and the role of membrane fluidity regulation as a bacterial resistance mechanism. The minimum inhibitory concentration of BAC was characterized with micro-dilution growth inhibition assay. The BAC treatment was performed on *S. aureus* cultured at 20 °C and 37 °C, for 24 h. The morphology of *S. aureus* cells was examined using scanning electron microscopy. The loss of bacterial membrane integrity after BAC treatment was studied by monitoring the intracellular potassium ion leakage using the atomic absorption spectroscopy. The bacterial membrane total fatty acid composition, controlling the membrane fluidity, was analyzed by GC/MS. The results showed that the resistance of *S. aureus* cells to BAC increased with the increase of growth temperature. The planktonic cells were more susceptible to BAC than biofilm-detached ones. The rise of growth temperature resulted in an increase of *S. aureus* membrane rigidity. Furthermore, a higher membrane fluidity was observed in planktonic cells when compared to that in the biofilm-detached ones. The resistance of *S. aureus* seems to depend on the growth temperature. Compared to planktonic cells, biofilm-detached cells showed a greater resistance to BAC. The BAC targets and disturbs the bacterial membrane. Membrane fluidity modulation is likely a one of resistance mechanisms for *S. aureus* to BAC at the cellular scale. Therefore, disinfection procedures, in food sector, should be adapted for bacteria detached from biofilm.

Keywords *Staphylococcus aureus* · Biofilm-detached cells · Planktonic cells · Growth temperature · Membrane fluidity · Susceptibility to BAC

Introduction

The emergence of *Staphylococcus aureus* in food sector represents a significant risk for public health. In fact, this bacterium is commonly involved in foodborne diseases (FBDs) (Denayer et al. 2017). In order to fight against FBDs, disinfectants are constantly applied to maintain a high level of surface hygiene in food fields (Khelissa et al. 2017a). Such procedure is thought to be effective to decrease the microbiological risk in the highly affected areas. Bacteria are often attached to surfaces and form a complex structure, called biofilm (Tuson and Weibel 2013). The biofilm-associated cells are physiologically different from the planktonic ones (Chua et al. 2014). Furthermore, biofilm-structured cells have a better resistance to disinfectants than their planktonic counterparts (Bridier et al. 2011). In the food sector, bacteria are usually exposed to different environmental constraints such as temperature changes, shear forces, and pH (Simões et al. 2010; Berne et al. 2018). The exposure of biofilms to high shear forces may result in cell dispersal (Berne et al. 2018). Thus, the biofilm represents a bacterial reservoir which, once detached, serves as a continuous source of contamination resulting in severe FBDs.

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The biofilm resistance mechanisms to antimicrobial agents may be explained in several ways (Bridier et al. 2011). The biofilm resistance mechanisms should be observed at the macroscopic and the microscopic levels. At the macroscopic scale, it seems to be related to the production of an extracellular matrix, composed of extracellular polymeric substances (EPS), that hinder the disinfectant diffusion (Bridier et al. 2011). However, several studies highlighted the fact that the biofilm matrix cannot always explain the biofilm resistance to disinfectants (Abdallah et al. 2014). Therefore, the biofilm resistance at the microscopic or cellular scale, which is thought to be linked to the modification of bacterial physiology, should be explored. In addition, bacterial cells may adapt to unfavorable growth conditions by modifying their membrane lipid composition (Chihib et al. 2005). The bacterial membrane, composed of phospholipids and proteins, constitutes the first line of bacterial defense against antimicrobial. The fatty acid composition controls the fluidity of bacterial membrane and may hinder the penetration of antimicrobial into cells (Dubois-Brissonnet et al. 2016; Malanovic and Lohner 2016).

In this regard, the purpose of this work was to study the effect of growth temperature (20 °C and 37 °C) on the resistance of *S. aureus* cells, issued from planktonic culture or detached from biofilm formed on stainless steel, to BAC. This study also aimed to study the membrane fluidity, of planktonic and biofilm-detached *S. aureus*, in order to characterize its involvement as a resistance mechanism to BAC treatment at the cellular level.

Materials and methods

Bacterial culture conditions and suspension preparation

Staphylococcus aureus CIP 4.83 strain was stored (− 80 °C) in tryptic soy broth (TSB) (Biokar Diagnostics, France) with 40% (*v*/*v*) of glycerol. Precultures were prepared by adding 100 μL from stock tube to 5 mL of TSB and incubated at 20 °C (for 48 h) or 37 °C (for 24 h). The cultures were started by inoculating 10^4 CFU/mL from the preculture in 50 mL of TSB and incubation under continuous shaking (160 rpm) at 20 °C or 37 °C. Cultures were stopped at the late exponential phase and cells were pelleted (5000*g*, 5 min, 20 °C). Bacteria were washed twice with potassium phosphate buffer (PPB; 100 mM, pH 7). Finally, the bacterial suspensions were adjusted to 10^8 CFU/mL in PPB, by fixing the optical density at 600 nm to 0.110 ± 0.005, using a Jenway 6320D UV/visible light spectrophotometer.

Stainless steel slide preparation

The stainless steel (SS) (304 L, Equinox, France) slides were first immersed in acetone (Fluka, Sigma-Aldrich, France) for 1 h, then rinsed under distilled water. The slides were soaked in DDM ECO detergent (1%) for 10 min at 20 °C (ANIOS, France) and rinsed under distilled water then under ultrapure water (Milli-Q® Academic, Millipore, France). The SS slides were dried and autoclaved (20 min, 120 °C).

Development of biofilm

The SS slides (90 × 90 mm) were covered with 12 mL of the corresponding cell suspension (20 °C or 37 °C) adjusted to 10^7 CFU/mL and incubated at 20 °C for 1 h to allow the bacterial adhesion. Afterwards, slides were rinsed twice, by immersion in PPB, to remove loosely attached cells. Then, each slide was covered with 12 mL of TSB and incubated statically, at 20 °C or 37 °C for 24 h. Following the 24-h incubation, slides covered with biofilm were rinsed with PPB. The strongly attached bacteria were recovered into 10 mL of PPB by surface scraping, harvested by centrifugation (5000*g*, 5 min, 20 °C), and then washed once with 20 mL of PPB. The bacterial suspension concentrations were adjusted in PPB to 10^8 CFU/mL as above cited.

BAC minimum inhibitory concentration determination

The minimum inhibitory concentration (MIC) of BAC was determined by micro-dilution growth inhibition assays, using a Bioscreen C (Labsystems, Helsinki, Finland). *Staphylococcus aureus* cells were cultured, as previously, in Mueller-Hinton Broth (MHB) (Bio-Rad, France). One hundred microliters of *S. aureus* suspension (10^6 CFU/mL) were added to the plate wells containing decreasing BAC concentrations ranging from 25 to 0 mg/L (in MHB). Two BAC-free controls were included, one with only MHB (sterility control) and the other with MHB + bacteria (growth control). The micro-dilution plates were incubated in the Bioscreen C at 37 °C under continuous shaking. The $OD_{600\ nm}$ was measured every 2 h for 48 h. The MIC was the lowest concentration of the BAC that prevented growth, as measured by optical density. Log $OD_{600\ nm}$ values were plotted versus time (h). The lag time, of each growth curve, was estimated by extrapolating the linear portion of Log $OD_{600\ nm}$ versus time plot back to the initial $OD_{600\ nm}$. The growth rate (μ) was calculated during the exponential growth phase using the formula: $dN/dt = kN$, where N is the Log $OD_{600\ nm}$ of cells, t the time, and k is the growth rate constant.

Disinfection of *S. aureus* cells

For the disinfection assay, 1 mL of bacterial suspension, adjusted to 10^7 CFU/mL, was introduced to 1 mL of 6 mg/L BAC solution (prepared in PPB). After 5-min contact time at 20 °C, 1 mL of this mixture was transferred into 9 mL of neutralizing solution (Abdallah et al. 2014) to stop the antibacterial action. Thereafter, tenfold serial dilutions were done in PPB. Samples of 100 μL were spread onto tryptic soy agar broth plates (TSA; Biokar Diagnostics, France) and incubated at 37 °C for 24 h. After the incubation time, the number of viable and culturable cells was counted on the plates and the results were expressed in Log CFU/mL. For the control assays, the disinfectant solution was replaced by PPB.

BAC-induced cell membrane permeabilization

Biofilm-detached and planktonic *S. aureus* cells grown at 20 and 37 °C were concentrated to 10^{10} CFU/mL. Five milliliters of the concentrated bacterial suspensions was introduced into silicone cap glass reaction vessels (100-mL wide-necked flasks) containing 45 mL of BAC solution prepared in HEPES buffer (final concentration of 3 mg/L in 50 mL final volume) or HEPES buffer (negative control). The K^+ concentration at time zero was measured in a tenfold dilution of the bacterial suspension filtrate (0.2 μm, Sartorius™ Minisart™ NML Syringe Filters, France) before contact with BAC solution. After the introduction of bacterial suspension to the reaction vessel containing the BAC solution, samples (4 mL) were filter sterilized at 5, 10, 15, 20, 30, 60, and 90 min. Each sample was removed using a sterile plastic syringe attached to a sterile needle to enable easy access to the reaction mixture suspension through the silicon cap. The K^+ concentration in filtrate samples was determined using a Varian SpectrAA 55/B atomic absorption spectrometer in flame emission mode (wavelength 766.5 nm; slit 0.7 nm high; air-acetylene flame).

Cellular fatty acid extraction and analysis

Biofilm-detached and planktonic *S. aureus* cells were harvested as cited above, either by scrapping cells embedded in biofilm from the rinsed coupons or by centrifuging planktonic culture, then resuspended in 10 mL of PPB. Cells were sonicated (37 kHz, 5 min) and vortexed for 30 s. Cells were pelleted by centrifugation (10,000*g*, 10 min, 4 °C), and pellets, containing 10^9 CFU/mL, were washed twice with cold distilled water. Cells were subjected to the saponification and methylation (Chihib et al. 2005). Fatty acid methyl ester extraction was realized as described previously by Chihib et al. (2005). Data analysis was performed with GC-2014 gas chromatograph (Shimadzu, Japan) with a capillary column (Zebron ZB-FFAP, Phenomenex, Australia) and connected to mass spectrometer (Thermo-Finnigan Trace DSQ, Thermo Fisher Scientific, USA).

Scanning electron microscope observation

The morphology of *S. aureus* cells upon BAC treatment was assessed by scanning electron microscope (SEM). One milliliter volume of the BAC- or PPB-treated then neutralized planktonic or biofilm-detached cell suspensions was filtered through a 0.2-μm-pore-size polycarbonate membrane filter (Schleicher & Schuell, Dassel, Germany) then fixed for 4 h with 2% glutaraldehyde, in cacodylate buffer 0.1 M pH 7, at 20 °C. Fixed samples were then dehydrated in an ascending ethanol series (50, 70, 95, and 2 × 100% (*v*/*v*) ethanol), for 15 min at each concentration. Samples were critical point dried and coated with thin carbon film before examination in the SEM. Microscopy was performed with a Hitachi S4700 microscope at 3 kV.

Statistical analysis

The results are presented as mean values and their standard error of the mean. Data analysis was performed using Sigma Plot 11.0 (Systat Software Inc.), using one-way ANOVA (Tukey's method). Results were considered significant at a P value of < 0.05.

Results

Determination of the BAC minimum inhibitory concentration

The results of Fig. 1 showed that at a concentration of 0.2 mg/L, cells have the same growth behavior of the control (cells without BAC). At a concentration of 0.4, 0.8, and 1.5 mg/L of BAC, the growth profile was different from the control ($P < 0.05$). The results showed that the BAC minimum inhibitory concentration was of 3 mg/L. Figure 1 showed also that the growth rate (μ) and lag time (λ) were dependent on the BAC concentration. In fact, our results showed that the μ value of *S. aureus* cultures was of 0.056 h^{-1} when the BAC concentrations were ranging from 0 to 1.5 mg/L (Fig. 1). Furthermore, the lag time consistently extended with increased BAC concentrations (Fig. 1). When the BAC concentrations increased from 0 to 1.5 mg/L, the λ increased from 8 to 20 h.

Susceptibility of *S. aureus* cells to BAC treatment

The susceptibility of biofilm-detached and planktonic *S. aureus* cells, grown at 20 °C and 37 °C, to BAC treatment

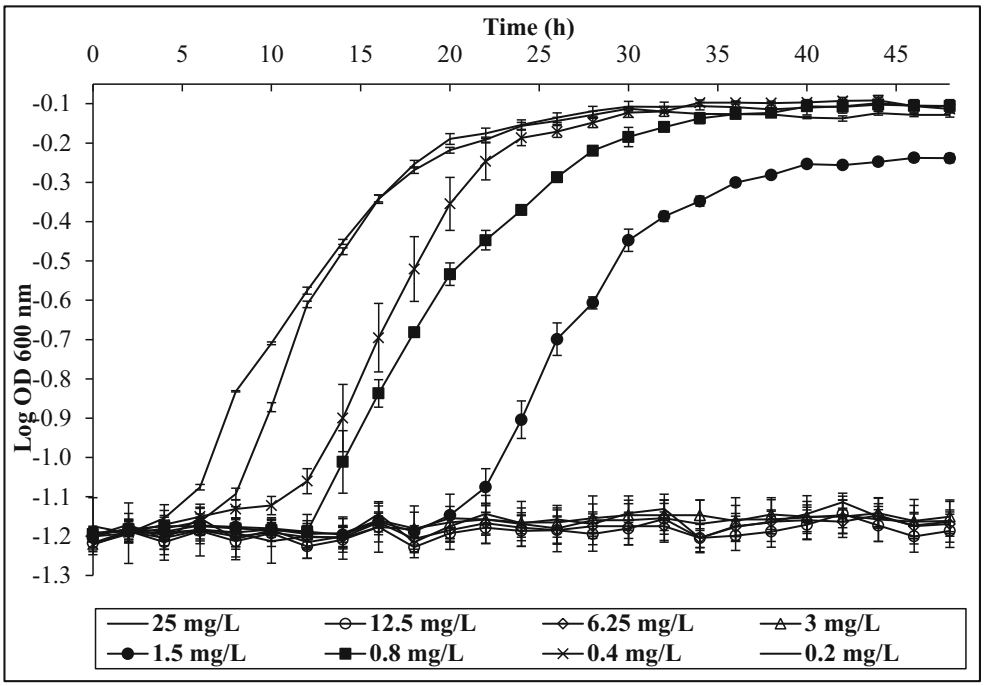

Fig. 1 Growth curves of *Staphylococcus aureus* grown at 37 °C for 48 h, measured at the optic density (OD) of 600 nm under different benzalkonium chloride concentrations. The concentration at which there was a linear growth inhibition was considered the MIC. The OD at 600 nm in Mueller-Hinton Broth without bacterial inoculum was measured to ensure the sterility of the growth medium (Only MHB)

was studied. The results showed that the PPB treatment, used as the negative control, had no significant effect on the initial population of *S. aureus*, whatever the studied conditions ($P < 0.05$) (Fig. 2). The average of viable and culturable counts of cells treated with PPB was of *ca* 7.3 Log CFU/mL (Fig. 2).

The treatment of biofilm-detached cells grown at 20 °C and 37 °C, for 5 min at a BAC concentration of 3 mg/L, resulted in a significant reduction of the initial viable and culturable count of 3.2 and 1.8 Log CFU/mL, respectively ($P < 0.05$) (Fig. 2). However, the treatment of planktonic cells grown at 20 °C and 37 °C, for 5 min at a BAC concentration of 3 mg/L, significantly reduced the initial viable and culturable count by 4.3 and 3.1 Log CFU/mL, respectively ($P < 0.05$) (Fig. 2). Furthermore, the results also showed that the remained viable and culturable count of biofilm-detached cells after BAC treatment was of *ca*. 1.3 Log CFU/mL higher than that of planktonic cells whatever the cell growth temperature ($P < 0.05$) (Fig. 2).

Effect of BAC on *S. aureus* cell membrane permeability

Measurements of K^+ efflux from the bacterial cells were realized to assess the cell resistance to BAC treatment. This was carried out by monitoring the extracellular K^+ concentration in biofilm-detached and planktonic *S. aureus* cells grown at 20 °C and 37 °C for 24 h (Fig. 3). Our results also showed that the HEPES buffer addition (control) had no effect on K^+ efflux which remained stable whatever the studied condition. The addition of BAC at a final concentration of 3 mg/L resulted in an immediate increase of extracellular K^+. Figure 3 showed that K^+ efflux was higher in planktonic than in biofilm-detached cell suspensions whatever the growth temperature condition ($P < 0.05$). Five minutes after the BAC addition, the extracellular K^+ concentration increased to 4.8 and 1.7 mg/L in the planktonic cell suspensions grown at 20 °C and 37 °C, respectively ($P < 0.05$) (Fig. 3).

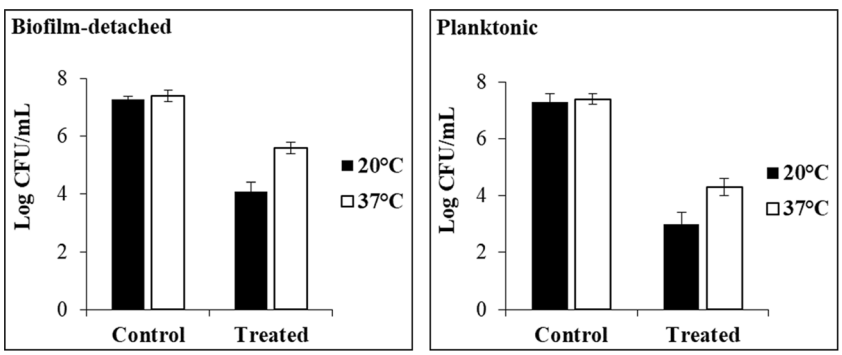

Fig. 2 Susceptibility of biofilm-detached and planktonic *Staphylococcus aureus* cells, grown at 20 °C and 37 °C for 24 h, to benzalkonium chloride treatment (BAC). The bacterial count is presented in Log CFU/mL after 100 mM potassium phosphate buffer (control) or BAC treatment (3 mg/L for 5 min)

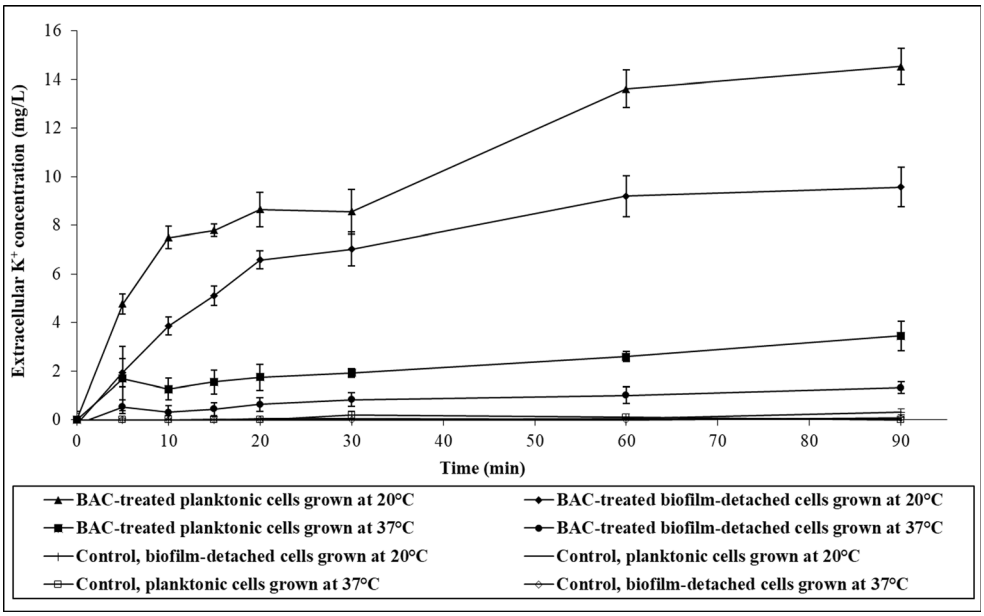

Fig. 3 Kinetics of intracellular potassium leakage from biofilm-detached and planktonic *Staphylococcus aureus* cells, grown at 20 °C and 37 °C for 24 h. The K^+ concentration is presented in milligrams per liter. The treatment with HEPES buffer represents the negative control

Under the same conditions, the extracellular K^+ increased to a concentration of 1.9 mg/L and 0.5 mg/L in the biofilm-detached cell suspensions grown at 20 °C and 37 °C, respectively (Fig. 3).

After 90 min of BAC addition into bacterial suspensions grown at 20 °C, the extracellular K^+ concentration reached concentrations of 10 and 15 mg/L in suspension of biofilm-detached and planktonic cells ($P < 0.05$) (Fig. 3). However, when cells were issued from cultures grown at 37 °C, the K^+ extracellular concentration in planktonic and biofilm-detached suspensions increased to a concentration of 5 and 2.5 mg/L, respectively ($P < 0.05$) (Fig. 3).

Impact of BAC treatment on *S. aureus* morphology changes

To investigate structural modifications of *S. aureus* biofilm-detached and planktonic cells after the exposition to BAC treatment, bacterial samples were analyzed by SEM. The untreated biofilm-detached and planktonic *S. aureus* cells cultivated at 20 °C and 37 °C looked round and exhibited an undamaged normal smooth lining (Fig. 4). However, when biofilm-detached and planktonic *S. aureus* cells were exposed for 5 min to a BAC concentration of 3 mg/L, significant morphological changes were observed (Fig. 4). *Staphylococcus aureus* planktonic cells grown at 20 °C showed holes in their cell walls (Fig. 4). *Staphylococcus aureus* biofilm-detached cells, cultivated at 20 °C, showed multiple dents on their surfaces (Fig. 4). However, the morphological changes of *S. aureus* biofilm-detached and planktonic cells, grown at 37 °C, seemed to be less pronounced than those of their 20 °C counterparts (Fig. 4). Hence, the BAC-treated planktonic cells, grown at 37 °C, were less round and their membrane seemed to be rougher, wrinkled, and deformed compared with untreated cells (Fig. 4). The treated *S. aureus* biofilm-detached cells, grown at 37 °C, became generally distorted in shape and had few holes in their cell walls (Fig. 4).

Effect of growth temperature on *S. aureus* membrane fatty acid profiles

Membrane fatty acid (FA) profile of biofilm-detached and planktonic *S. aureus* cells, grown for 24 h at 20 °C and 37 °C, was analyzed (Fig. 5). This investigation was performed to study the effect of growth temperature on the membrane fatty acid composition which controls the membrane fluidity. Figure 5 showed that the amounts of anteiso C15 (aC15) were maintained at a stable level whatever the growth temperature ($P > 0.05$). The results also showed that the aC15 amount of biofilm-detached cell membranes was significantly lower than that of planktonic cells ($P < 0.05$). Moreover, the total long-chain FA amounts, the aC19, C18, and C20, of biofilm-detached cells were 1.3-fold higher than their planktonic counterparts whatever the growth temperature ($P < 0.05$) (Fig. 5). The increase of growth temperature from 20 to 37 °C promoted an increase in long-chain FA amounts including aC19, C18, and C20 ($P < 0.05$) (Fig. 5).

Discussion

Biofilm-detached cells constitute a major source of bacterial dissemination and contamination of food contact surfaces (Khelissa et al. 2017b). Thus, it is of importance to conduct research on biofilm-detached cells to further assess their associated microbiological risk and to optimize appropriate disinfection procedures. *Staphylococcus aureus* is a pathogenic

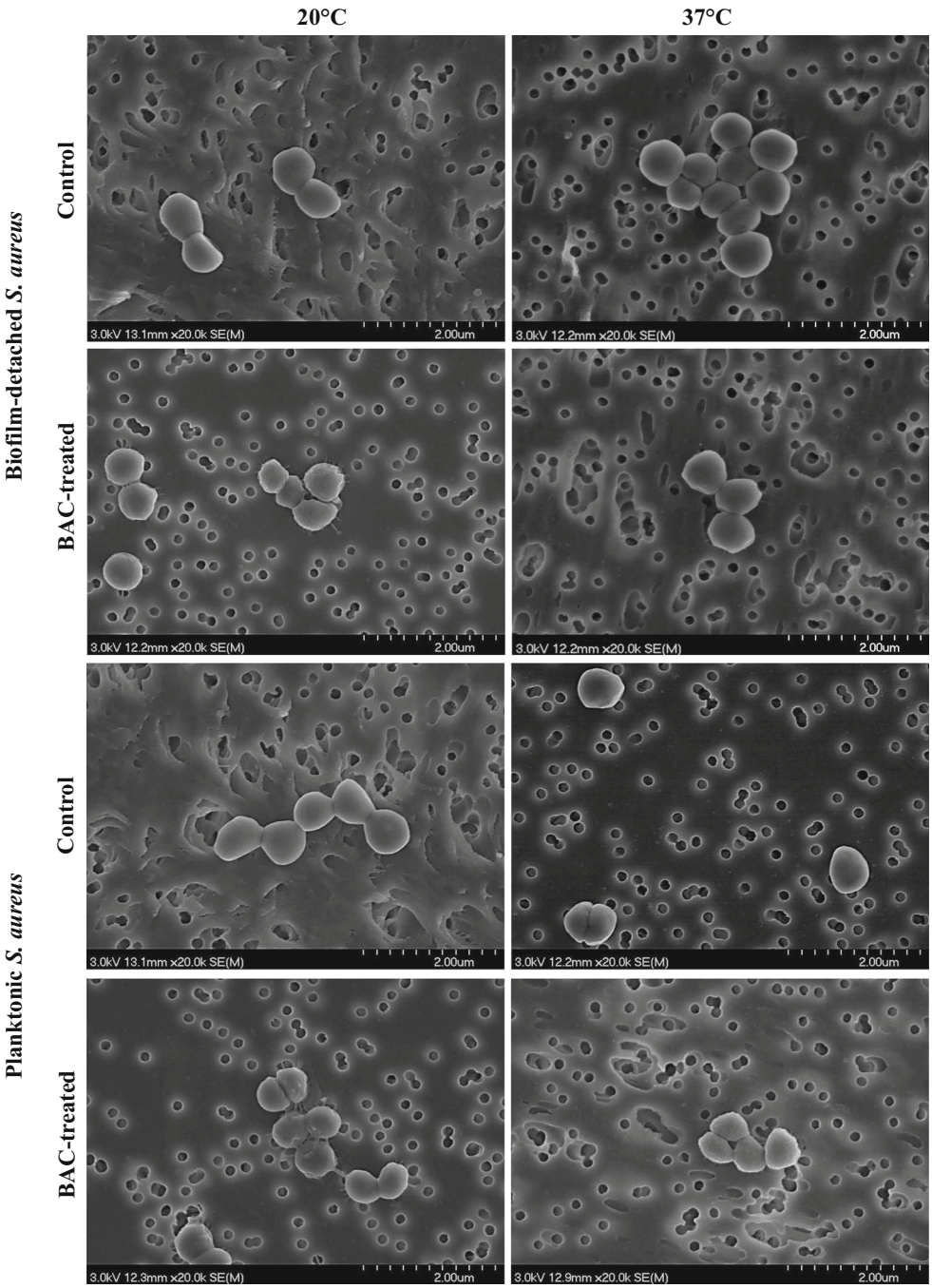

Fig. 4 Scanning electron micrographs of biofilm-detached and planktonic *Staphylococcus aureus* cells grown at 20 °C and 37 °C for 24 h, after treatment with benzalkonium chloride at MIC. The control represents cells treated with potassium phosphate buffer

bacterium, associated with serious FBDs, and able to adhere and form biofilms on food contact surfaces (Kadariya et al. 2014; Abdallah et al. 2015; Khelissa et al. 2017a). Our findings showed that biofilm-detached cell phenotype is highly different from that of the planktonic one. Cells grown under biofilm state are known to be more resistant to antimicrobial agents than those grown under floating state (Davies 2003; Batoni et al. 2016). This resistance is often associated with the extracellular matrix, a compact structure, which may prevent disinfectants from penetrating and reaching the bacterial cells (Abdallah et al. 2014, 2015). The goal in the present work is to investigate the resistance at the cellular level when bacteria are detached from biofilm.

Our results underlined that the rise of growth temperature from 20 to 37 °C increased the resistance of both biofilm-detached and planktonic *S. aureus* cells to BAC treatment. Moreover, the resistance of biofilm-detached cells to BAC was significantly higher than that of planktonic cells whatever the studied growth temperature. However, Rollet et al. (2009) reported that the sessile, biofilm-detached, and planktonic *Pseudomonas aeruginosa* showed the same antibiotic susceptibility profile. Our results highlight the fact

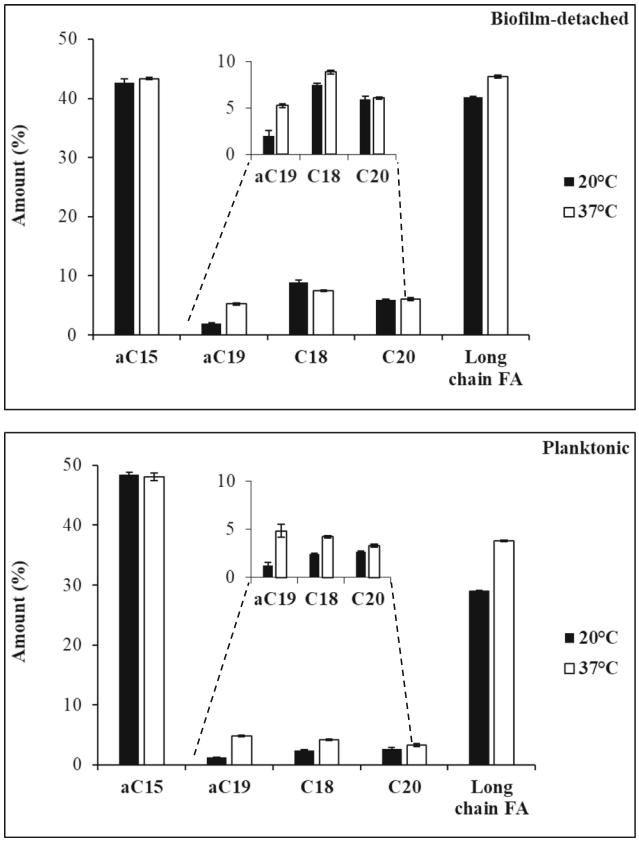

Fig. 5 Membrane fatty acid composition of biofilm-detached and planktonic *Staphylococcus aureus* cells, grown at 20 °C and 37 °C for 24 h. a: anteiso

that biofilm-detached cells in food processing industry represent a serious public health problem. In fact, after being released from biofilm, these cells represent a real threat as they acquired a high resistance profile and require an effective antimicrobial treatment. It has been reported that quaternary ammonium compounds have the bacterial membrane as a main target (Gilbert and Moore 2005; Abdallah et al. 2014). In this context, our results showed that when biofilm-detached and planktonic *S. aureus* cells were exposed to a BAC concentration of 3 mg/L, an immediate K^+ leakage was measured. The K^+ efflux rate decreased with the increase of the growth temperature from 20 to 37 °C. These results also showed that at a given growth temperature, the K^+ leakage was higher in planktonic than in biofilm-detached cells. Thus, the BAC bactericidal activity depends both on the physiological state and on the growth temperature. These findings were comforted by the morphological modifications of bacterial cells when exposed to BAC and analyzed by SEM. Cells treated with a BAC concentration of 3 mg/L were less bulky, and their membrane seemed to be rougher, wrinkled, and deformed compared with untreated cells. This could be a result of the high cell wall–BAC interaction that, in addition to disrupting cell membranes, promotes the release of intracellular material and thereby significantly changes cell homeostasis.

Regarding our data, and in order to explain the observed results, the modification of the cellular membrane fatty acid composition, which controls the membrane fluidity, was investigated. In the present study, *S. aureus* biofilm-detached cells displayed a significantly higher SFA amount compared to planktonic cells, and this is due to the high increase in the amount of long-chain SFA, including aC19, C18, and C20. Furthermore, the amounts of aC19, C18, and C20 of biofilm-detached and planktonic cells incubated at 37 °C were significantly higher than those incubated at 20 °C. Zhang and Rock (2008) have underlined that the straight-chain saturated fatty acids are linear and are also known to pack together to make a rigid membrane bilayer with a high phase transition. Nevertheless, the amounts of aC15 remained at a stable level in both in *S. aureus* studied cell populations. At the same time, aC15 amounts were lower in biofilm-detached cells than in planktonic ones whatever the studied growth temperature. It has been reported that aC15 has a low melting point which makes it a major determinant of membrane fluidity for many Gram-positive bacteria (Kaneda 1991). In addition, the fatty acid melting point decreases as their chain length shortens (Annous et al. 1997). The phase transition temperatures of the phosphatidylcholine containing aC19:0 (36.7 °C) and C18 (26 °C) are significantly higher than those of phosphatidylcholine containing aC15 (− 13.9 °C) (Schindler 1980; Suutari and Laakso 1994). Thus, the membrane fatty acid profiles of the studied *S. aureus* cells would have probably resulted in a lower fluidity of the biofilm-detached cell membranes when compared to those of planktonic cells. This could explain the greater resistance of the biofilm-detached cells to BAC treatment. Furthermore, the transition to a fatty acid profile with stable amounts of aC15, the increase of aC19:0, C18, and C20 amounts in biofilm-detached and planktonic cells incubated at 37 °C, suggests that the bacterial membranes may be less fluid at high growth temperatures. Wang et al. (2016) recently showed that the increase of growth temperature decreased the fluidity of *S. aureus* membrane in response to electroporation. Thus, the increase of growth temperature probably decreased the permeability of *S. aureus* membranes to BAC. Taken together, our findings may explain the increase of planktonic and biofilm-detached cells resistance to BAC treatment with the increase of growth temperature and as well as the greater resistance of biofilm-detached cells to BAC treatment compared to their planktonic counterparts. Overall, the results related to the membrane fluidity corroborate the membrane integrity monitored by K^+ efflux findings.

In conclusion, our work showed that the resistance of *S. aureus* to BAC is dependent on the growth temperature. In addition, the bacterial physiological state, whether biofilm-detached or planktonic, is a determinant parameter related to bacterial susceptibility to disinfectant. Our approach aimed to

identify the aspects of bacterial physiology that are affected by BAC activity, beginning with an initial focus on antibacterial activity followed by an assessment of cell membrane integrity and changes in membrane fluidity. *Staphylococcus aureus* is extremely susceptible to BAC which had high antimicrobial effect leading to membrane collapse and irreversible loss of membrane integrity with consequent leakage of intracellular K^+. The resistance behavior correlated with the membrane fluidity. Therefore, it is suggested that modification of membrane fatty acid composition seems to be a possible resistance mechanism, at the cellular scale, for *S. aureus* cells.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent N/A

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