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# Impact of growth temperature and surface type on the resistance of *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms to disinfectants

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ABSTRACT

Biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus aureus* on food-contact-surfaces represents a significant risk for the public health. In this context, the present study investigates the relationship between the environmental conditions of biofilm formation and the resistance to disinfectants. Therefore, a static biofilm reactor, called NEC-Biofilm System, was established in order to study the effect of growth temperature (20, 30 and 37 °C), and of the surface type (stainless steel and polycarbonate), on biofilm resistance to disinfectants. These conditions were selected to mimic the biofilm formation on abiotic surfaces of food processing industries. The antibiofilm assays were performed on biofilms grown during 24 h. The results showed that the growth temperature influenced significantly the biofilm resistance to disinfectants. These data also revealed that the growth temperature has a significant effect on the biofilm structure of both bacteria. Furthermore, the increase of the biofilm growth temperature increased significantly the *algD* transcript level in sessile *P. aeruginosa* cells, whereas the *icaA* one was not affected in *S. aureus* cells. Overall, our findings show that the biofilm structure and matrix cannot fully explain the biofilm resistance to disinfectant agents. Nevertheless, it underlines the intimate link between environmental conditions, commonly met in food sectors, and the biofilm resistance to disinfectants.



## 1. Introduction

*Pseudomonas aeruginosa* and *Staphylococcus aureus* are important opportunistic human pathogens, causing major problems in food sectors. In fact, *S. aureus* is among the common known cause of foodborne infections worldwide and the involvement of *P. aeruginosa* in such infections and food spoilage is also reported (Kim and Wei, 2007; Newell et al., 2010). Furthermore, most bacteria, in their natural and man-made ecosystems, are attached to surfaces and form a complex three-dimensional structure, called biofilm (Donlan and Costerton, 2002). The biofilm formation on food-contact-surfaces, in turn, leads to contamination of food products, which reduces their shelf-life or results in human foodborne diseases, and causes significant economic losses (Sharma and Anand, 2002; von Holy, 2006). Moreover, the environmental conditions encountered in food sectors have also been found to promote the biofilm formation and influence the biofilm resistance to disinfecting agents (Abdallah et al., 2014b, 2014c; Belessi et al., 2011; da Silva Meira et al., 2012; Nguyen and Yuk, 2013).

The biofilm populations have several advantages over their free-living counterparts, including the resistance to antimicrobial agents (Donlan and Costerton, 2002). The biofilm resistance is often linked to the biofilm matrix. In fact, the biofilm resistance mechanism involves antibacterial sequestration by matrix and prevents its penetration in biofilm deeper layers (Davison et al., 2010; Jang et al., 2006). The biofilm matrix is mainly composed of exopolysaccharides, proteins, nucleic acids and lipids (Flemming and Wingender, 2010). Different studies have shown that *P. aeruginosa* cells produce at least three exopolysaccharides: Alginate, Psl and Pel. The Alginate is a linear polyanionic exopolysaccharide composed of uronic acids and involves at least 24 genes for its biosynthesis (*algA*, *B*, *D*, *8*, *44*…) (Rehm, 2009). The Psl polysaccharide, which is synthesized by the polysaccharide synthesis locus (*psl*), consists of a repeating pentasaccharide, containing D-mannose, D-glucose and L-rhamnose (Byrd et al., 2009). Eleven

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genes, *pslACDEFGHIJKL*, are believed to be required for the Psl synthesis. The Pel polysaccharide, a glucose-rich polysaccharide is synthesized by the *pel* locus containing seven genes (*pelA* to *pelG*) (Ghafoor et al., 2011). Many *S. aureus* strains produce a poly-N-acetylglucosamine (PNAG) as a main exopolysaccharide and involve the intercellular adhesion (*icaADBC*) locus for the PNAG production (Arciola et al., 2012). Furthermore, different studies underlined that *P. aeruginosa* and *S. aureus* exopolysaccharides provide the structural scaffold of the biofilm and increase the resistance to antimicrobials such as antibiotics and disinfectants (Arciola et al., 2012; Yang et al., 2011). Subsequent studies showed that the biofilm resistance is related to the physiological state of sessile cells (Abdallah et al., 2014a, 2015; Campanac et al., 2002; Simoes et al., 2011). Therefore, a more thorough understanding of biofilm resistance mechanisms, as a function of environmental conditions, is necessary in order to improve the antibiofilm treatments in food processing industries.

In this regard, a static biofilm reactor was developed in order to study the biofilm resistance of *P. aeruginosa* and *S. aureus* to disinfecting agents This investigation is also intended to elucidate the effect of growth temperature (20, 30 and 37 °C), and surface type (stainless steel and polycarbonate), on the biofilm resistance to disinfectants formulated by Scientis laboratory (Scientis, France). The selected experimental conditions aimed to mimic the biofilm formation, under static conditions, on food processing equipment. The effect of these growth conditions was studied on the biofilm three-dimensional structure, using the confocal laser scanning microscopy. The expression of genes involved in the biosynthesis of exopolysaccharides was also quantified using the real time PCR in sessile cells grown under the different growth conditions. The present work thus endeavors to understand the relationship between the environmental conditions of biofilm formation and the resistance to disinfectant products in order to reduce the issues associated with the biofilm persistence.

## 2. Material and methods

### 2.1. Bacterial strain and culture conditions

The bacterial strains used for this study were *P. aeruginosa* CIP 103467 and *S. aureus* CIP 4.83. The strains were stored at −80 °C in Tryptic Soy broth containing 40% (v/v) of glycerol (TSB; Biokar Diagnostics, France). To prepare precultures, 100 mL from frozen stock cultures was inoculated into 5 mL of TSB and then incubated at the culture temperature (i.e., 20, 30 or 37 °C). The 20 °C pre-culture was incubated for 48 h, whereas those at 30 and 37 °C were incubated for 24 h. 1 mL of these preculture, containing $5 \times 10^4$ CFU, was used to inoculate 50 mL of TSB medium in 500 mL sterile flasks for bacterial cultures. Cultures were then incubated at 20, 30 and 37 °C, under shaking conditions at 160 rpm, and bacterial cells were harvested in the late exponential phase.

### 2.2. Slide preparation

The circular slides of stainless steel (304 L, Equinox, France), and polycarbonate (Plexilux, France), 41 mm in diameter and 1 mm thick, were soaked overnight in ethanol 95% (Fluka, Sigma-Aldrich, France) and then rinsed with distilled water. Rinsed slides were then soaked in 500 mL of 5% TDF4 detergent (Franklab SA, France), for 20 min at 50 °C under agitation conditions. The slides were then thoroughly rinsed 5 times, for 1 min under agitation in 500 mL of distilled water at 20 °C to eliminate detergent residues, followed by three washes with ultrapure water (Milli-Q® Academic, Millipore, France). Stainless steel slides were air-dried and sterilized by autoclaving at 121 °C for 15 min. The polycarbonate slides were sterilized in 95% ethanol for 10 min.

### 2.3. Description of NEC Biofilm System

The system consists of several assembled pieces of stainless steel and a rubber O-ring (Fig. 1A). The lower part (1) is made of stainless steel and constitutes the circular basis of the system. On the upper flat face, the O-ring (2) can be used to fit perfectly one circular test slide (3). Then a stainless steel cylinder (4) can be placed in order to form the well of the biofilm formation. This cylinder has two orifices on its lateral wall in order to ensure oxygen supply for the bacterial growth. A collar clamp (5) was used to provide tightness and a metal cover (6) was used to ensure the sterility of the closed system (7). This system is called NEC-Biofilm System (Fig. 1B). All system parts are autoclavable at 121 °C for 20 min.

### 2.4. Cell suspension preparation

Cells of 20, 30 and 37 °C cultures were harvested by centrifugation for 10 min at 3500 *g* (20 °C). Bacteria were washed twice with 20 mL of potassium phosphate buffer (PB; 100 mM, pH 7) and finally resuspended in 20 mL of PB. To disperse cells, a sonication at 37 kHz was carried out for 5 min at 25 °C (Elmasonic S60H, Elma, Germany). Subsequently, bacteria were resuspended in the PB to a cell concentration of $1 \times 10^8$ CFU/mL by adjusting the optical density to $OD_{620\ nm} = 0.110 \pm 0.005$ (Ultrospec 1100 pro, GE Healthcare, formerly Amersham Biosciences, United Kingdom). Standardized cell suspensions were diluted 10 fold in order to make a cell concentration of $10^7$ CFU/mL for bacterial adhesion experiments.

### 2.5. Biofilm formation assay

The biofilm formation was initiated by the deposition of 3 mL of bacterial suspension ($10^7$ CFU/mL) in the sterile well of each reactor and then incubated at 20 °C for 60 min. After the bacterial adhesion, the 3 mL were removed and the slides were gently washed twice using 5 mL of PB in order to remove loosely adherent cells. Then 5 mL of TSB

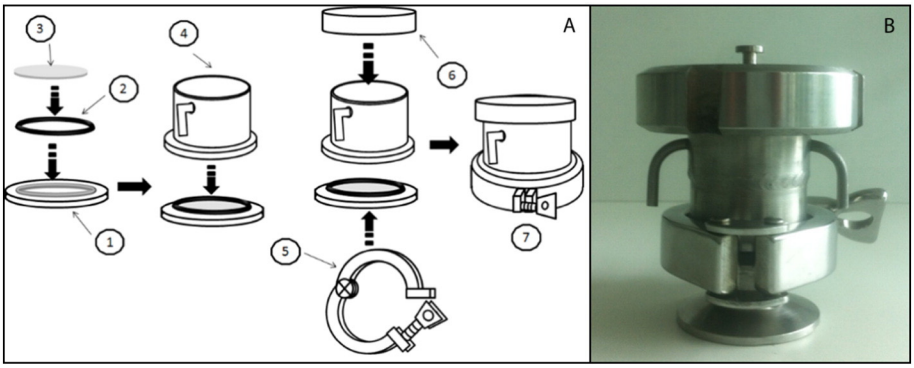


**Fig. 1.** Description of the static biofilm system. A presents the different pieces of the assembled system. B presents NEC Biofilm System.

were deposited in each well and the closed systems were incubated for 24 h at the same temperature of pre-cultures and cultures. After an incubation time of 24 h, the old culture medium was removed and biofilms were washed twice with 5 mL of PB in order to remove planktonic cells. Thereafter, slides were used for the quantification of biofilm biomass, the confocal microscopy analysis and the antibiofilm assay. In order to quantify the biofilm biomass, sessile cells were detached in 20 mL of Tryptone Salt broth (TS; Biokar Diagnostics, France) containing 1 g of 1 mm glass beads in 100 mL sterile pot. Pots were vortexed for 30 s followed by a sonication for 5 min (37 kHz, 5 min, 25 °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 Tryptic Soy Agar broth plates (TSA; Biokar Diagnostics, France) and incubated at 37 °C for 24 h. The number of attached cells was counted on plates and the results are expressed in log CFU/cm$^2$. The results represent the mean of three independent experiments and in each experiment two slides were used.

### 2.6. Antibiofilm assay

For the antibiofilm treatments, rinsed slides were placed horizontally in 30 mL of the disinfectant solutions and incubated for the recommended incubation time (Scientis, France). The composition and the characteristics of the disinfectants used here are shown in Table 1. Afterwards, slides were withdrawn from the disinfectant solution and immersed in 10 mL of neutralizing solution to stop the antibacterial action. The neutralizer contains a combination of Tween 80 (30 g/L), Saponin (30 g/L), Lecithin (30 g/L), Sodium Thiosulphate (5 g/L), L-Histidin (1 g/L) and Tryptone Salt broth (9.5 g/L) (Toté et al., 2010). For the confocal microscopy analysis, slides were placed in Petri dishes and the action of biocide was stopped by the deposition of 3 mL of neutralizing solution on the upper face. Attached cells were detached and counted as described above. For the control assays, the disinfectant solution was replaced by the Tryptone Salt broth. The results represent the average of three independent experiments and in each case, two slides were used.

### 2.7. Confocal and epifluorescence microscopy analysis

Treated biofilms were stained with LIVE/DEAD BacLight kit (Invitrogen Molecular Probes, USA), according to the manufacturer instruction for 15 min in the dark. After sessile cells staining, confocal microscopic observations were performed using a Nikon A1R laser confocal microscope (Nikon, Japan), equipped with a 20× Plan Fluor water immersion objective. The three-dimensional biofilm structure, after treatment with the Tryptone Salt broth (TS) and the P2 product, were explored by a series of xy images with a z-step of 0.5 μm. Signals were recorded using the green (excitation 488 nm, emission 515/30 nm) and red (excitation 568 nm, emission 600/50 nm) channels. Images were reconstructed for 3-dimensional visualization using the NIS-Elements AR software (Nikon, Japan). The percentage of viable cells in the initial population of biofilms was determined after staining with the LIVE/DEAD® BacLight Bacterial Viability kit. Briefly, 10$^7$ CFU of detached cells were filtered through a 0.2 μm pore-size polycarbonate membrane filters (Millipore, France) and stained for 15 min in the dark. Stained cells were washed once with 1 mL of saline solution, 0.85% of NaCl, and filters were placed on microscopic slides for the epifluorescence microscopic enumeration. The viable cells (green ones) and the dead cells (red ones) were counted in 50 microscopic fields. Each microscopic field contained between 70 and 100 cells. The results are expressed as mean of the three independent experiments and two slides were used for each experiment.

**Table 1**
Composition and characteristic of disinfectant products[a].

| Disinfectants | Antimicrobial | Final concentration (ppm) | Action time (min) |
|---|---|---|---|
| P1 | DDAC | 137.5 | 15 |
| P2 | DDAC | 490 | 15 |
| | BDA | 180 | |
| P3 | DDAC | 125 | 15 |
| | ADBAC | 475 | |

*ADBAC*: Alkyldimethylbenzylammonium chloride.
*BDA*: Bis (3-aminopropyl) dodecylamine.
*DDAC*: Didecyldimethylammonium chloride.
[a] The concentration and the action time were recommended by the manufacturer (Scientis).

### 2.8. Isolation of total RNA

After scrapping sessile cells, bacteria were harvested by centrifugation at 4500 *g* for 10 min (20 °C) and pellets, containing about 5 × 10$^9$ CFU, were resuspended in 5 mL of RNA later for 10 min at 20 °C (Ambion, Carlsbad, CA, USA). Cells were then harvested, washed twice with PB and stored at −80 °C until the RNA extraction. The frozen cell pellets were resuspended in 250 μL of lysis buffer (Tris–EDTA: 10 mM Tris–HCl, 1 mM EDTA, pH 8.0), supplemented with lysozyme (2.9 mg/mL; Sigma-Aldrich, France) for *P. aeruginosa* and lysostaphin (0.4 mg/mL; Sigma-Aldrich, France) for *S. aureus*. The total RNA isolation was performed in an RNase-free environment using the GeneJET RNA Purification Kit (Fisher Scientific, France) per the manufacturer's instructions. Using the absorbance ratio A260/A280, the purity of the RNA was determined and samples were stored at −80 °C until use.

### 2.9. Reverse transcriptase and real-time PCR analysis

A sample of 1 μg of RNA was reverse transcribed with random hexamers using the RevertAid H Minus First Strand complementary cDNA synthesis kit (Fermentas, France) in accordance with the manufacturer's protocol. After the enzyme inactivation at 70 °C for 5 min, cDNA samples were diluted 1:10 in DNase-free water (Invitrogen, France) and stored at −20 °C until use. The primer design, for genes involved in the biosynthesis of *P. aeruginosa* and *S. aureus* exopolysaccharides, was carried out using Primer3 software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). The gene specificity of all primers was confirmed using BLAST searches in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). Primer sequences are shown in Table S1. Gene expression levels were measured using the StepOnePlus Real time PCR System (Applied Biosystems, USA). The reactions were carried out in a final volume of 20 μL, containing 1 μL of diluted cDNA, 2.5 ng of each primer, 10 μL of IQ SYBR® Green Supermix (100 mM KCl, 40 mM Tris–HCl at pH 8.4, 0.4 mM) of each dNTP (dATP, dCTP, and dTTP), iTaq DNA polymerase (50 U/mL), 6 mM of $MgCl_2$, 20 nM fluorescein and stabilizers. The PCR conditions were: 1 cycle at 95 °C for 5 min, followed by 40 cycles consisting of 95 °C for 20 s, 57 °C for 20 s. Finally, a melt-curve analysis immediately followed the amplification at 95 °C for 15 s, cooling to 60 °C for 20 s, and a slow rise in temperature to 95 °C at a rate of 0.5 °C/10 s with continuous acquisition of fluorescence decline. Final PCR products were analyzed by melting curves and electrophoresis in order to ensure the specificity of amplification. Quantification of target genes and housekeeping genes was performed in triplicate reactions for three independent experiments. The ribosomal gene 16S rRNA was used as a housekeeping gene. In all plates, a negative control was performed (without cDNA). The transcript level of each gene was relatively quantified by the calculation of ΔCT. The ΔCT denotes the difference in threshold cycle between the target and the control gene. The transcript level was expressed as the n-fold of relative difference to a growth condition according to the ΔΔ-CT method (Livak and Schmittgen, 2001). The fold increase is expressed as $2^{\Delta\Delta\text{-CT}}$. For *P. aeruginosa*, the transcript level of *pslA*, *pelA* and *algD* in the 30 and 37 °C sessile cells were calculated with respect to the 20 °C ones. For *S. aureus*, the transcript

level of *icaA* in 30 and 37 °C sessile cells was calculated with respect to its expression in 20 °C sessile cells.

### 2.10. Quantification of total protein and carbohydrate of the biofilm matrix

The protein and carbohydrate concentrations in the matrix of biofilms, grown on stainless steel and polycarbonate, were quantified after an incubation time of 24 h. After biofilms rinsing with saline solution (0.85% NaCl), biofilms were recovered by scraping surface, aspirating and expelling at least 10 times with 6 mL of ultrapure water. The suspensions were homogenized for 30 s, followed by a sonication (5 min, 37 kHz). The cells were removed by centrifugation at 5000 × g for 15 min. The supernatants were filtered through 0.2 μm Millipore filters and then used for biochemical assays. The protein concentrations were quantified using the Bradford method with bovine serum albumin as the standard (Bradford, 1976). The total carbohydrate content was measured using the Phenol–Sulfuric method with glucose as the standard (Dubois et al., 1956). The results were presented in μg/cm$^2$ as the mean of three independent experiments and in each experiment, two slides were used.

### 2.11. Data analysis

The results are presented as mean values and the standard error to the mean (SEM). Data analysis was performed using Sigma Plot 11.0 (Systat Software, USA), using one-way ANOVA (Tukey's method) to determine the significance of differences.

## 3. Results

### 3.1. Effect of growth temperature and surface type on the biofilm formation of P. aeruginosa and S. aureus

The 24 h biofilms were observed on stainless steel and polycarbonate slides after cell staining with the LIVE/DEAD® Kit. The results showed that *P. aeruginosa* and *S. aureus* biofilms colonized the surface of slides whatever the growth temperature and the surface type (data not shown). The enumeration of viable cells, using the epifluorescence microscope, underlined that *P. aeruginosa* and *S. aureus* biofilms harbored predominantly viable cells. The percentage of viable cells in *P. aeruginosa* and *S. aureus* biofilms was respectively of *ca* 86.7 and 92.7% irrespective of the growth temperature and the surface type (Table 2). Furthermore, the results showed that neither the growth temperature nor the surface type influenced the biofilm biomass. The biofilms of *P. aeruginosa* and *S. aureus* presented a bacterial biomass of *ca* 7.8 and 8.1 log CFU/cm$^2$, respectively (Table 2).

### 3.2. Effect of growth temperature and surface type on the biofilm resistance to disinfectants

The antibiofilm efficacy of disinfectants was investigated on *P. aeruginosa* and *S. aureus* biofilms grown at 20, 30 and 37 °C during 24 h, on stainless steel and polycarbonate. This investigation aimed to evaluate the impact of environmental conditions of biofilm formation on the resistance to disinfectants. The results showed that treatment TS (Tryptone Salt broth), used as a negative control, slightly reduced the initial population of *P. aeruginosa* and *S. aureus* biofilms. However, this reduction did not exceed the 0.7 log CFU/cm$^2$ whatever the growth temperature, the bacterium and the surface type (Table 3). The observed reduction, after the treatment TS, is probably due to the elimination of remaining loosely adherent cells.

The results underlined that the biofilm resistance of both bacteria depended on the growth temperature, the surface type and the disinfectant product. Table 3 showed that the increase of growth temperature resulted in a significant increase of *P. aeruginosa* biofilm resistance to the treatment P1. The treatment P1 of *P. aeruginosa* biofilms, grown at 20, 30 and 37 °C, reduced the initial population respectively to 2.8, 3.0 and 6.3 log CFU/cm$^2$ on the stainless steel and respectively to 3.7, 4.2 and 6.3 log CFU/cm$^2$ on the polycarbonate (Table 3). The treatment P2 of *P. aeruginosa* biofilms reduced completely the initial population of biofilms grown on stainless steel whatever the growth temperature (Table 3). Similar results were observed when biofilms were grown at 20 °C on the polycarbonate. However, the biofilms grown on the polycarbonate at 30 and 37 °C presented respectively a culturable count of 1.4 and 2.3 log CFU/cm$^2$ after the treatment P2 (Table 3). The results presented in Table 3 also suggest that the treatment P3 reduced the initial population of 20, 30 and 37 °C biofilms respectively to 6.2, 5.0 and 4.7 log CFU/cm$^2$ on the stainless steel and respectively to 6.9, 6.1 and 5.5 log CFU/cm$^2$ on the polycarbonate.

The increase of growth temperature significantly increased the *S. aureus* biofilm resistance to all tested disinfectants. The treatment P1 of *S. aureus* biofilms, grown at 20, 30 and 37 °C, reduced the culturable count respectively to 1.6, 3.6 and 4.6 log CFU/cm$^2$ on the stainless steel and respectively to 2.0, 3.3 and 5.6 log CFU/cm$^2$ on the polycarbonate (Table 3). The results also underlined that the treatment P2 reduced the initial population of *S. aureus* biofilms, grown at 20 °C, to 0 log CFU/cm$^2$ whatever the surface type used (Table 3). However, the treatment P2 of *S. aureus* biofilms, grown at 30 °C and 37 °C, reduced the initial population respectively to 1.2 and 2.8 log CFU/cm$^2$ on the stainless steel and respectively to 1.7 and 2.3 log CFU/cm$^2$ on the polycarbonate (Table 3). When the growth temperature of *S. aureus* biofilms increased from 20 to 37 °C, the recovered biomass, after the treatment P3, increased from 3.8 to 6.9 log CFU/cm$^2$ on the stainless steel and from 2.8 to 7.1 log CFU/cm$^2$ on the polycarbonate (Table 3).

**Table 2**
Effect of growth temperature on *P. aeruginosa* and *S. aureus* biofilm formation on the stainless steel (SS) and the polycarbonate (PC)[a].

| Log CFU/cm$^2$ | | | | | | |
|---|---|---|---|---|---|---|
| | 20 °C | | 30 °C | | 37 °C | |
| | SS | PC | SS | PC | SS | PC |
| *P. aeruginosa* | | | | | | |
| Culturable biomass | $7.7 \pm 0.2^{ax}$ | $7.8 \pm 0.2^{ax}$ | $7.6 \pm 0.1^{ax}$ | $7.7 \pm 0.3^{ax}$ | $7.9 \pm 0.4^{ax}$ | $7.9 \pm 0.1^{ax}$ |
| % of viability | $82 \pm 3^{ax}$ | $83 \pm 2^{ax}$ | $82 \pm 5^{ax}$ | $80 \pm 1^{ax}$ | $86 \pm 7^{ax}$ | $87 \pm 5^{ax}$ |
| *S. aureus* | | | | | | |
| Culturable biomass | $8.2 \pm 0.1^{a}$ | $7.7 \pm 0.2^{a}$ | $7.9 \pm 0.4^{ax}$ | $8.3 \pm 0.2^{ax}$ | $8.3 \pm 0.1^{ax}$ | $8.2 \pm 0.1^{ax}$ |
| % of viability | $93 \pm 2^{ax}$ | $93 \pm 2^{ax}$ | $91 \pm 3^{ax}$ | $89 \pm 1^{ax}$ | $94 \pm 2^{ax}$ | $93 \pm 3^{ax}$ |

[a] The data represent the mean of recovered viable and culturable cells count (log CFU/cm$^2$), and the percentage of viable cells (%) ± SEM. Between growth temperatures (a) and attachment surfaces (x) under the same condition, the mean values with the same letters are not significantly different ($P > 0.05$).

**Table 3**
Effect of growth temperature on the resistance of *P. aeruginosa* and *S. aureus* biofilms to disinfectant products[a].

| Log CFU/cm$^2$ | | | | | | |
|---|---|---|---|---|---|---|
| | 20 °C | | 30 °C | | 37 °C | |
| | SS | PC | SS | PC | SS | PC |
| *P. aeruginosa* | | | | | | |
| TS | $7.5 \pm 0.1^{ax}$ | $7.5 \pm 0.3^{ax}$ | $7.4 \pm 0.1^{ax}$ | $7.3 \pm 0.2^{ax}$ | $7.6 \pm 0.3^{ax}$ | $7.3 \pm 0.1^{ax}$ |
| P1 | $2.8 \pm 0.7^{a}$ | $3.7 \pm 0.2$ | $3.0 \pm 0.3^{a}$ | $4.2 \pm 0.2$ | $5.1 \pm 0.5$ | $6.3 \pm 0.2$ |
| P2 | $0 \pm 0^{ax}$ | $0 \pm 0^{x}$ | $0 \pm 0^{a}$ | $1.4 \pm 0.3$ | $0 \pm 0^{a}$ | $2.3 \pm 0.3$ |
| P3 | $6.2 \pm 0.2$ | $6.9 \pm 0.1$ | $5.0 \pm 0.5^{a}$ | $6.1 \pm 0.2$ | $4.7 \pm 0.3^{a}$ | $5.5 \pm 0.2$ |
| *S. aureus* | | | | | | |
| TS | $7.5 \pm 0.4^{abx}$ | $7.7 \pm 0.3^{ax}$ | $7.4 \pm 0.1^{a}$ | $7.8 \pm 0.2^{a}$ | $7.8 \pm 0.2^{bx}$ | $7.7 \pm 0.4^{ax}$ |
| P1 | $1.6 \pm 0.6^{ax}$ | $2.0 \pm 0.1^{x}$ | $3.6 \pm 1.7^{abx}$ | $3.3 \pm 0.4^{x}$ | $4.6 \pm 0.2^{b}$ | $5.6 \pm 0.5$ |
| P2 | $0 \pm 0^{ax}$ | $0 \pm 0^{x}$ | $1.2 \pm 1.3^{abx}$ | $1.7 \pm 0.3^{x}$ | $2.8 \pm 0.6^{bx}$ | $2.3 \pm 0.7^{x}$ |
| P3 | $3.8 \pm 0.3$ | $2.8 \pm 0.1$ | $6.8 \pm 0.6^{ax}$ | $7.3 \pm 0.3^{ax}$ | $6.9 \pm 0.2^{ax}$ | $7.1 \pm 0.4^{ax}$ |

[a] The data represent the mean of recovered viable and culturable cells count (log CFU/cm$^2$) ± SEM. Between growth temperatures (a, b) and attachment surfaces (x) under the same condition, the mean values with the same letters are not significantly different ($P > 0.05$).

### 3.3. Effect growth temperature, and surface type, on the biofilm structure and biofilm removal with disinfectant

The structure of biofilm was studied as a function of biofilm growth conditions and antibiofilm treatments. This investigation aimed to understand the relationship between the biofilm architecture and the resistance to disinfectants. Figs. 2–5 show the reconstruction of the three-dimensional structure of biofilms grown at 20, 30 and 37 °C during 24 h, after the treatment TS and P2. The treatment P2 was selected due to its high antibacterial efficacy against *P. aeruginosa* and *S. aureus* biofilms.

The CLSM analysis revealed that *P. aeruginosa* biofilms covered the entire surface of stainless steel, and polycarbonate, slides whatever the growth temperature (Figs. 2 and 3). Moreover, the change of growth temperature promoted significant changes in the three-dimensional (3D) structure of *P. aeruginosa* biofilms. Figs. 2A and 3A showed that *P. aeruginosa* formed thick biofilms when growing at 20 °C on both surfaces. Under this condition, *P. aeruginosa* biofilms formed mushroom-shaped structures which are connected with thin cell layers on the stainless steel and thick ones on the polycarbonate (Figs. 2A and 3A). When growing at 30 and 37 °C, this bacterium formed flat and compact biofilms on both surfaces (Figs. 2C, E, 3C and E). Furthermore, the

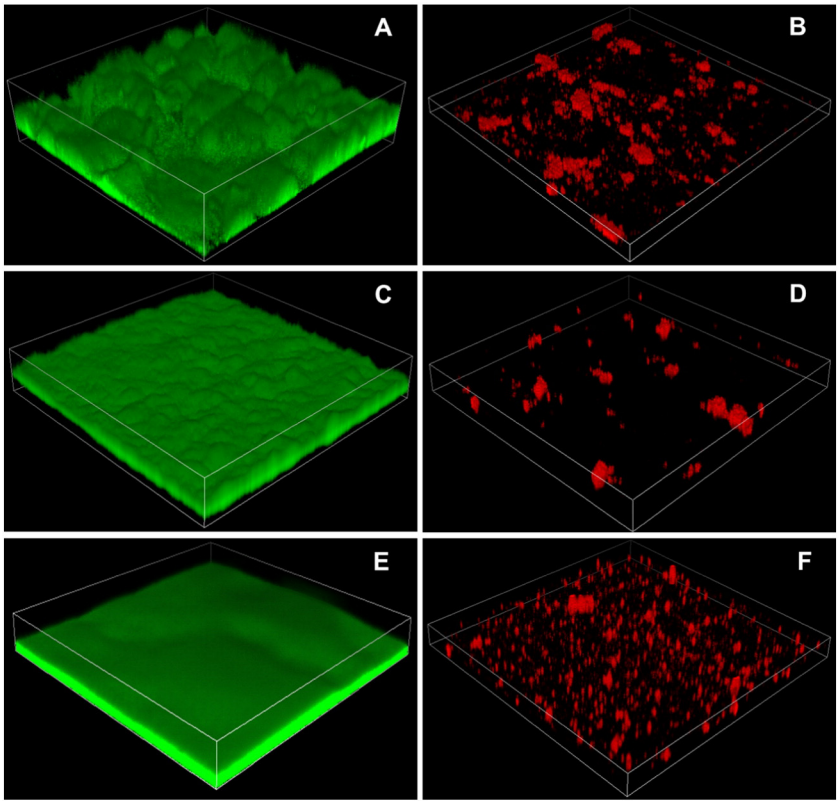


**Fig. 2.** Effect of growth temperature on the *P. aeruginosa* biofilm structure and removal on stainless steel. A, C and E represent respectively biofilms grown at 20, 30 and 37 °C after exposition to Tryptone Salt broth. B, D and F represent respectively the biofilms grown at 20, 30 and 37 °C after exposition to P2 disinfectant.

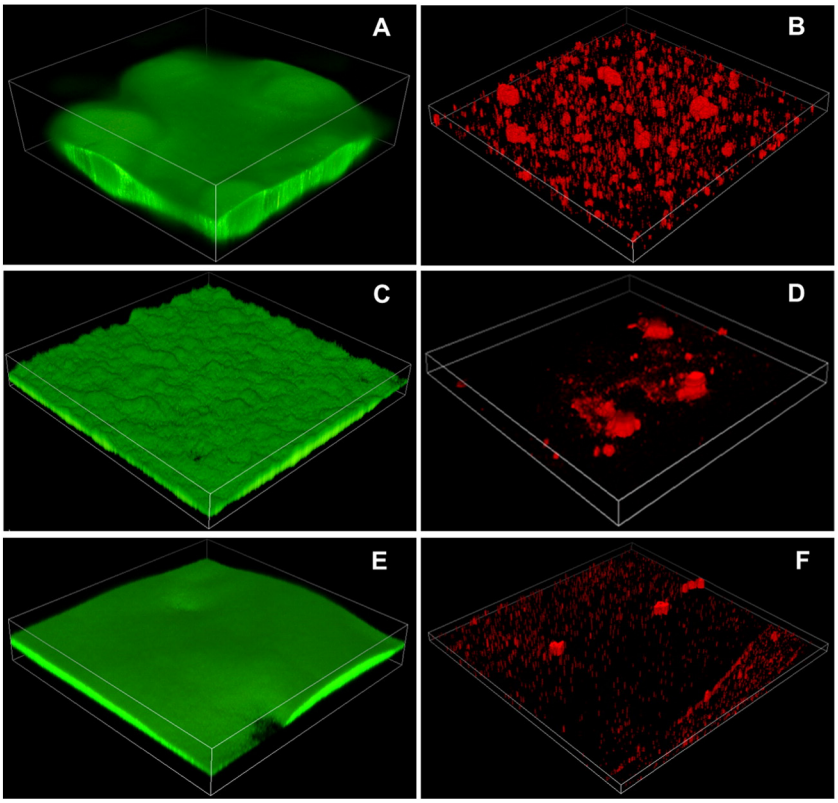


**Fig. 3.** Effect of growth temperature on the *P. aeruginosa* biofilm structure and removal on polycarbonate. A, C and E represent respectively biofilms grown at 20, 30 and 37 °C after exposition to Tryptone Salt broth. B, D and F represent respectively the biofilms grown at 20, 30 and 37 °C after exposition to P2 disinfectant.

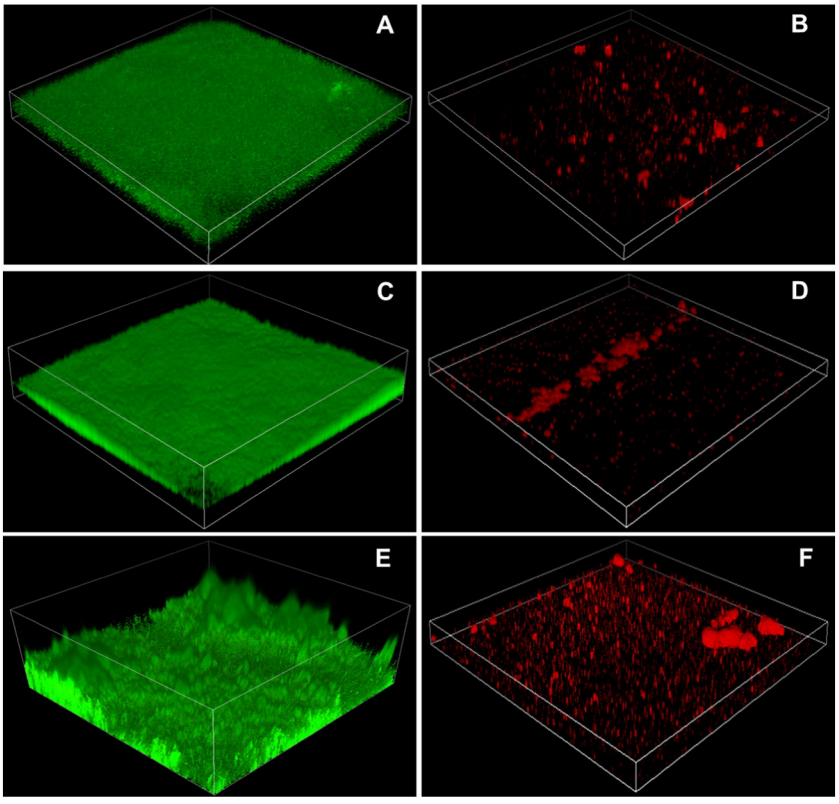


**Fig. 4.** Effect of growth temperature on the *S. aureus* biofilm structure and removal on stainless steel. A, C and E represent respectively biofilms grown at 20, 30 and 37 °C after exposition to Tryptone Salt broth. B, D and F represent respectively the biofilms grown at 20, 30 and 37 °C after exposition to P2 disinfectant.

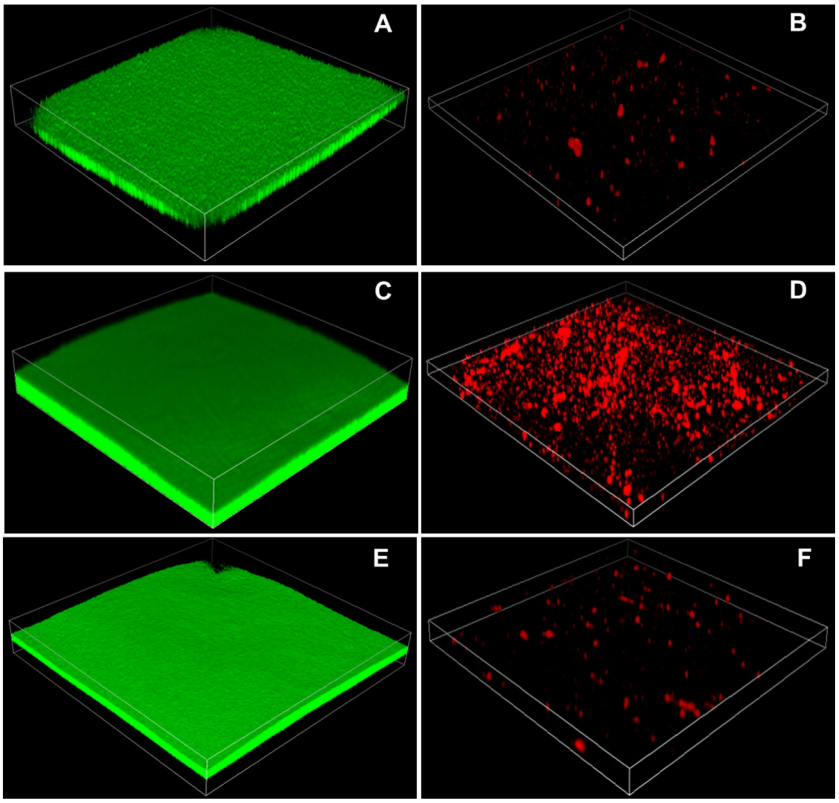


**Fig. 5.** Effect of growth temperature on the *S. aureus* biofilm structure and removal on polycarbonate. A, C and E represent respectively biofilms grown at 20, 30 and 37 °C after exposition to Tryptone Salt broth. B, D and F represent respectively the biofilms grown at 20, 30 and 37 °C after exposition to P2 disinfectant.

treatment P2 showed a significant effect on *P. aeruginosa* biofilm removal whatever the growth temperature and the surface type used.

The analysis of *S. aureus* biofilms showed that this bacterium formed a flat and regular structure at 20 and 30 °C on both stainless steel and polycarbonate surfaces (Figs. 4A, C, 5A and C). However, *S. aureus* formed an irregular biofilm at 37 °C with valley and spire structures on the stainless steel (Fig. 4E), and a flat and regular one on the polycarbonate (Fig. 5E). Furthermore, Figs. 4 and 5 showed that *S. aureus* formed a more compact biofilm on the polycarbonate than that on the stainless steel surface whatever the growth temperature. The results also showed that the highest sanitizing effect of the treatment P2 was recorded when *S. aureus* biofilms were grown at 20 °C on both surfaces (Figs. 4B and 5B) and at 37 °C on the polycarbonate (Fig. 5F). However, the biofilms grown at 30 °C on both surfaces (Figs. 4D and 5D), and at 37 °C on stainless steel (Fig. 4F), were the most resistant to the treatment P2 sanitizing effect.

### *3.4. Effect of growth temperature, and surface type, on the transcript level of genes involved in the biosynthesis of exopolysaccharides*

After the investigation of the biofilm structure, the effect of growth temperature and surface type was performed on the transcript level of *pslA*, *pelA* and *algD* in the sessile *P. aeruginosa* cells and of *icaA* in *S. aureus* ones.

For *P. aeruginosa*, the results showed that the increase of growth temperature increased significantly the *algD* transcript level. The increase of growth temperature from 20 to 37 °C increased significantly the transcript level of *algD* by 2 and 4 fold respectively in stainless steel and polycarbonate sessile cells ($P < 0.05$) (Fig. 6). The increase of growth temperature from 20 to 37 °C also increased the transcript level of *pslA* by 2.3 fold in sessile stainless steel cells ($P < 0.05$). The *pslA* transcript level was not influenced by the change of growth temperature in sessile polycarbonate cells ($P > 0.05$) (Fig. 6). Fig. 6 also

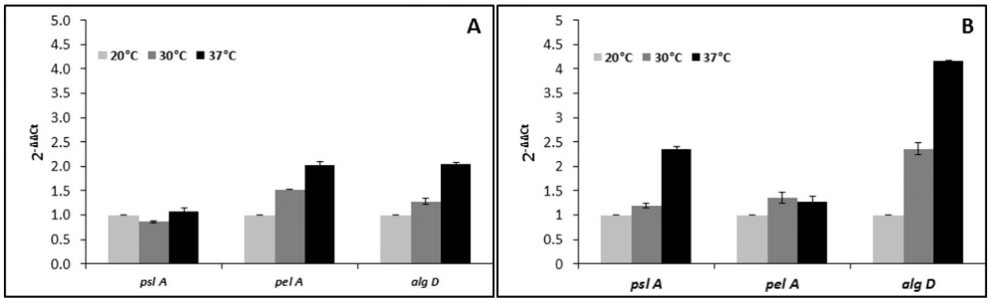


**Fig. 6.** The effect of growth temperature and surface type on the expression of *pslA*, *pelA* and *algD* in the sessile *P. aeruginosa*. Cells were recovered from 24 h biofilms grown at 20, 30 and 37 °C on polycarbonate (A) and stainless steel (B). The transcript level of each gene in 30 and 37 °C sessile cells was normalized to its expression in the 20 °C ones.

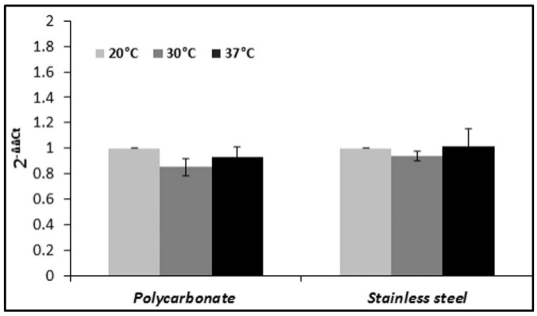


**Fig. 7.** The effect of growth temperature and surface type on the expression of *icaA* in the sessile *S. aureus*. Cells were recovered from 24 h biofilms grown at 20, 30 and 37 °C on stainless steel and polycarbonate. The transcript level of each gene in 30 and 37 °C sessile cells was normalized to its expression in the 20 °C ones.

showed that the increase of growth temperature from 20 to 37 °C increased significantly the transcript level of *pelA* by 1.3 and 2 fold respectively in sessile stainless steel and polycarbonate cells ($P < 0.05$) (Fig. 6).

For *S. aureus*, the results showed that the *icaA* transcript level was not influenced by the change of the growth temperature on either surfaces ($P > 0.05$) (Fig. 7). In fact, the transcript level of *icaA* in the sessile cells grown at 30 and 37 °C on stainless steel and polycarbonate was of *ca* 1 after its normalization to the 20 °C transcript level (Fig. 7).

### 3.5. Effect of growth temperature, and surface type, on the production of biofilm matrix

The effect of the growth temperature and the surface type were studied simultaneously on the major component of biofilm matrix (Table 4). This study aimed to understand the involvement of the biofilm matrix in the biofilm resistance to disinfectants. For *P. aeruginosa*, Table 4 indicated that the increase of growth temperature, from 20 to 37 °C, increased the concentration of total protein and carbohydrate of the biofilm matrix by 1.5 and 1.9 fold, respectively. For *S. aureus*, the rise of growth temperature, from 20 to 37 °C, induced 1.5 and 2 fold increases in the protein concentration on stainless steel and polycarbonate, respectively (Table 4). However, the increase of growth temperature for *S. aureus* biofilms did not influence the carbohydrate concentration of biofilm matrix regardless the surface type (Table 4).

## 4. Discussion

The persistence of biofilms in food sectors represents a significant threat for the development of foodborne illness and can cause a rapid material deterioration and failure (Donlan and Costerton, 2002). To fight against biofilms, several static and continuous-flow biofilm systems have been used to study the biofilm formation and to screen the antibiofilm efficacy of disinfectant products (Coenye and Nelis, 2010). The static biofilm systems, such as (MTP)-based systems, have several advantages over continuous flow systems (Ceri et al., 1999; Theraud et al., 2004). In fact, these systems are characterized by the simplicity of the experimental procedures, the ease of experiments and the high screening capacity. However, the (MTP)-based systems have some disadvantages concerning the limitation in the surface test choice and the low reproducibility (Chavant et al., 2007; Pitts et al., 2003). In this context, the present work has led to the setup of a static biofilm system, NEC Biofilm System, which presents a facility in the experimental procedure (i.e. biofilm formation and antibiofilm assays) and permits to study the antibiofilm efficiency of disinfectant products. The system also presents another advantage concerning its ability to receive all solid substrata, thereby enabling study of different solid surfaces. In addition, this system presents an easy accessibility to sessile cells, which allowed the characterization of the biofilm formation and disinfectant efficacy either by the cell counting or by the microscopic observation.

The results showed that NEC Biofilm System has ensured reproducibility in *P. aeruginosa* and *S. aureus* biofilm formation on the stainless steel and the polycarbonate. Moreover, our data underlined that the final biomass of the 24 h biofilms were not significantly affected by the change of growth temperature and the surface type. These results are in agreement with the results of Abdallah et al. (2014c), da Silva Meira et al. (2012) and Smith and Hunter (2008), who showed that the growth temperature and the surface type do not significantly influence the biofilm formation under static conditions. However, other studies showed that the increase of the growth temperature may increase the biofilm biomass (Choi et al., 2013; Hostacka et al., 2010; Vazquez-Sanchez et al., 2013). It is worthwhile to note here that the difference in the experimental procedure (i.e. the culture medium the surface and strain) may explain the discrepancy between studies. Moreover, Buckingham-Meyer et al. (2007) confirmed that biofilm growth conditions significantly influence the biofilm formation and resistance to disinfectants. Furthermore, our results demonstrate that the resistance of *P. aeruginosa* and *S. aureus* biofilms depended on the environmental conditions of the biofilm formation. These results seem to be consistent with other studies (Abdallah et al., 2014c; Belessi et al., 2011; Nguyen and Yuk, 2013), that showed the influence of growth temperature on the biofilm resistance to disinfecting agents. In accordance with the finding of Grobe et al. (2002), our results also indicated that the efficacy of disinfectant product is dose dependent. In fact, the treatment P2 (DDAC: 490 ppm) showed a higher antibiofilm efficacy than the treatment P1 (DDAC: 137.5 ppm). Furthermore, the increase of growth temperature increased the resistance of *S. aureus* biofilms to the treatment P3 (ADBAC: 475 ppm; DDAC: 125 ppm), and decreased that of *P. aeruginosa* to the same treatment. Such results may suggest that the biofilm resistance to disinfectants seems to be dependent on the bacterial genus. The ADBAC and DDAC belong to quaternary ammoniums and have different structures. Moreover, Ioannou et al. (2007) reported that the ADBAC formed a single monolayer at the end of primary uptake, while the DDAC formed a double monolayer. Thus, the efficacy of disinfectant products also seems to be related to the structure of active agent, which may change its cellular uptake and interactions with biofilm matrix components.

**Table 4**
Total proteins and carbohydrates in the matrix of biofilms grown at 20, 30 and 37 °C[a].

| μg/cm² | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | *P. aeruginosa* | | | *S. aureus* | | |
| | | 20 °C | 30 °C | 37 °C | 20 °C | 30 °C | 37 °C |
| PC | Total protein | 14.8 ± 1.7 | 21.1 ± 0.5 | 24.3 ± 0.4 | 16.0 ± 4.4 | 15.4 ± 2.1 | 24.5 ± 1.4 |
| | Total carbohydrate | 6.1 ± 1.1 | 13.8 ± 1.7 | 11.3 ± 0.9 | 3.8 ± 1.1 | 4.5 ± 0.6 | 6.6 ± 2.1 |
| SS | Total protein | 14.7 ± 1.4 | 15.3 ± 3.1 | 20.4 ± 2.1 | 14.9 ± 4.9 | 17.8 ± 1.2 | 29.8 ± 1.7 |
| | Total carbohydrate | 7.4 ± 1.4 | 13.2 ± 1.1 | 13.7 ± 1.9 | 5.2 ± 1.3 | 6.2 ± 1.4 | 7.4 ± 0.9 |

[a] The biofilms were grown during 24 h on stainless steel (SS) and polycarbonate (PC). The concentrations of proteins and carbohydrates are presented in μg/cm² ± SEM.

In line with the findings of Chaturongkasumrit et al. (2011) and Schlisselberg and Yaron (2013), our findings underlined that the surface type has an effect on the biofilm resistance to disinfectants. Moreover, our results also showed that the surface type presents an effect on the biofilm structure which may influence the biofilm resistance to disinfectants. In fact, the change of biofilm structure may decrease the diffusion of antibacterial inside biofilms and decrease the antibiofilm efficacy of disinfectants (Xu et al., 1996; Yang et al., 2011). Therefore, the compactness of *P. aeruginosa* biofilms, grown at 20 °C on the polycarbonate, may explain the resistance of these biofilms to P1 and P3 treatments in comparison to biofilms grown on stainless steel. Furthermore, the limitation diffusion hypothesis may explain the results obtained on the treatment of *S. aureus* biofilms. In fact, the increase of growth temperature promoted simultaneously an increase in the *S. aureus* biofilm thickness/compactness and resistance to disinfectant products. However, the validity of such hypothesis appears to be dependent on the disinfectant product. Indeed, the *P. aeruginosa* biofilms grown at 20 °C showed the highest sensitivity to the treatment P1 despite the apparent biofilm compactness. Thus, our findings suggest that the biofilm structure cannot always explain the biofilm resistance to antimicrobial agents. Furthermore, our data showed that some disinfectants completely eliminated viable bacteria but they were not able to remove completely the biofilm from the surface.

In order to explain the biofilm resistance to disinfectants, we studied the effect of growth temperature and surface type on the expression of exopolysaccharide genes. For *P. aeruginosa*, the increase of *algD* transcript level, with the rise of growth temperature, probably induced an increase in the Alginate production. The increase of *algD* was also accompanied by a significant increase of the carbohydrate concentration. The increase of such negatively charged exopolysaccharide may increase the sequestration of positively charged antimicrobials (e.g. the quaternary ammonium) which may impede the diffusion these cationic antimicrobials inside biofilms. Our results also showed that the increase of growth temperature resulted only in an increase of *pslA* transcript level in *P. aeruginosa* cells on the stainless steel. Ma et al. (2009) reported that Psl promotes both cell–cell and cells–surfaces interactions and increases the stability of the mature biofilm. However, the involvement of this exopolysaccharide in the biofilm resistance to disinfectants in our conditions is unclear since the expression of *pslA* was not significantly influenced in sessile polycarbonate cells.

For *S. aureus*, our finding showed that neither the growth temperature nor the surface type did influence the *icaA* transcript level. These results are at variance with that of Cerca and Jefferson (2008), who showed that the growth temperature increased the PNAG expression in sessile *Escherichia coli* cells. Furthermore, the increase of *S. aureus* biofilm resistance to disinfectants with the increase of growth temperature suggests that the PNAG is not an essential element of *S. aureus* biofilm resistance to disinfectants. Moreover, Campanac et al. (2002) showed that the resistance of *S. aureus* biofilms to quaternary ammonium Chloride (QAC) seems to be linked to the sessile cell phenotype rather than the presence of the extracellular matrix.

In conclusion, NEC-Biofilm System used here allowed us to study the relationship between the environmental conditions of biofilm formation and the biofilm resistance to disinfectant products. The results underlined that the biofilm resistance is influenced by the environmental conditions commonly met in food sectors. It is therefore of interest to pay more attention to environmental conditions of biofilm formation, such as the growth temperature and the surface type, when testing the disinfectant efficacy against biofilms. In addition, our finding showed that the biofilm structure and matrix do not fully explain the biofilm resistance to disinfectants. Other factors related to the physiological state of the cells may be responsible for the biofilm resistance to disinfecting agents.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2015.07.022.

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## References

Abdallah, M., Benoliel, C., Drider, D., Dhulster, P., Chihib, N.E., 2014a. Biofilm formation and persistence on abiotic surfaces in the context of food and medical environments. Arch. Microbiol. 196, 453–472.
Abdallah, M., Benoliel, C., Jama, C., Drider, D., Dhulster, P., Chihib, N.E., 2014b. Thermodynamic prediction of growth temperature dependence in the adhesion of *Pseudomonas aeruginosa* and *Staphylococcus aureus* to stainless steel and polycarbonate. J. Food Prot. 77, 1116–1126.
Abdallah, M., Chataigne, G., Ferreira-Theret, P., Benoliel, C., Drider, D., Dhulster, P., Chihib, N.E., 2014c. Effect of growth temperature, surface type and incubation time on the resistance of *Staphylococcus aureus* biofilms to disinfectants. Appl. Microbiol. Biotechnol. 98, 2597–2607.
Abdallah, M., Benoliel, C., Ferreira-Theret, P., Drider, D., Dhulster, P., Chihib, N.-E., 2015. Effect of culture conditions on the resistance of *Pseudomonas aeruginosa* biofilms to disinfecting agents. Biofouling 31, 49–59.
Arciola, C.R., Campoccia, D., Speziale, P., Montanaro, L., Costerton, J.W., 2012. Biofilm formation in *Staphylococcus* implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials. Biomaterials 33, 5967–5982.
Belessi, C.E., Gounadaki, A.S., Psomas, A.N., Skandamis, P.N., 2011. Efficiency of different sanitation methods on *Listeria monocytogenes* biofilms formed under various environmental conditions. Int. J. Food Microbiol. 145 (Supplement 1), S46–S52.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Buckingham-Meyer, K., Goeres, D.M., Hamilton, M.A., 2007. Comparative evaluation of biofilm disinfectant efficacy tests. J. Microbiol. Methods 70, 236–244.
Byrd, M.S., Sadovskaya, I., Vinogradov, E., Lu, H., Sprinkle, A.B., Richardson, S.H., Ma, L., Ralston, B., Parsek, M.R., Anderson, E.M., Lam, J.S., Wozniak, D.J., 2009. Genetic and biochemical analyses of the *Pseudomonas aeruginosa* Psl exopolysaccharide reveal overlapping roles for polysaccharide synthesis enzymes in Psl and LPS production. Mol. Microbiol. 73, 622–638.
Campanac, C., Pineau, L., Payard, A., Baziard-Mouysset, G., Roques, C., 2002. Interactions between biocide cationic agents and bacterial biofilms. Antimicrob. Agents Chemother. 46, 1469–1474.
Cerca, N., Jefferson, K.K., 2008. Effect of growth conditions on poly-N-acetylglucosamine expression and biofilm formation in *Escherichia coli*. FEMS Microbiol. Lett. 283, 36–41.
Ceri, H., Olson, M.E., Stremick, C., Read, R.R., Morck, D., Buret, A., 1999. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J. Clin. Microbiol. 37, 1771–1776.
Chaturongkasumrit, Y., Takahashi, H., Keeratipibul, S., Kuda, T., Kimura, B., 2011. The effect of polyesterurethane belt surface roughness on *Listeria monocytogenes* biofilm formation and its cleaning efficiency. Food Control 22, 1893–1899.
Chavant, P., Gaillard-Martinie, B., Talon, R., Hebraud, M., Bernardi, T., 2007. A new device for rapid evaluation of biofilm formation potential by bacteria. J. Microbiol. Methods 68, 605–612.
Choi, N.-Y., Kim, B.-R., Bae, Y.-M., Lee, S.-Y., 2013. Biofilm formation, attachment, and cell hydrophobicity of foodborne pathogens under varied environmental conditions. J. Korean Soc. Appl. Biol. Chem. 56, 207–220.
Coenye, T., Nelis, H.J., 2010. In vitro and in vivo model systems to study microbial biofilm formation. J. Microbiol. Methods 83, 89–105.
Da Silva Meira, Q.G., de Medeiros Barbosa, I., Alves Aguiar Athayde, A.J., de Siqueira-Júnior, J.P., de Souza, E.L., 2012. Influence of temperature and surface kind on biofilm formation by Staphylococcus aureus from food-contact surfaces and sensitivity to sanitizers. Food Control 25, 469–475.
Davison, W.M., Pitts, B., Stewart, P.S., 2010. Spatial and temporal patterns of biocide action against *Staphylococcus epidermidis* biofilms. Antimicrob. Agents Chemother. 54, 2920–2927.
Donlan, R.M., Costerton, J.W., 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. 15, 167–193.
Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356.
Flemming, H.C., Wingender, J., 2010. The biofilm matrix. Nat. Rev. Microbiol. 8, 623–633.
Ghafoor, A., Hay, I.D., Rehm, B.H., 2011. Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture. Appl. Environ. Microbiol. 77, 5238–5246.
Grobe, K.J., Zahller, J., Stewart, P.S., 2002. Role of dose concentration in biocide efficacy against *Pseudomonas aeruginosa* biofilms. J. Ind. Microbiol. Biotechnol. 29, 10–15.
Hostacka, A., Ciznar, I., Stefkovicova, M., 2010. Temperature and pH affect the production of bacterial biofilm. Folia Microbiol. 55, 75–78.
Ioannou, C.J., Hanlon, G.W., Denyer, S.P., 2007. Action of disinfectant quaternary ammonium compounds against *Staphylococcus aureus*. Antimicrob. Agents Chemother. 51, 296–306.
Jang, A., Szabo, J., Hosni, A.A., Coughlin, M., Bishop, P.L., 2006. Measurement of chlorine dioxide penetration in dairy process pipe biofilms during disinfection. Appl. Microbiol. Biotechnol. 72, 368–376.
Kim, S.H., Wei, C.I., 2007. Antibiotic resistance and Caco-2 cell invasion of *Pseudomonas aeruginosa* isolates from farm environments and retail products. Int. J. Food Microbiol. 115, 356–363.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 25, 402–408.
Ma, L., Conover, M., Lu, H., Parsek, M.R., Bayles, K., Wozniak, D.J., 2009. Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. PLoS Pathog. 5, 27.
Newell, D.G., Koopmans, M., Verhoef, L., Duizer, E., Aidara-Kane, A., Sprong, H., Opsteegh, M., Langelaar, M., Threfall, J., Scheutz, F., van der Giessen, J., Kruse, H., 2010. Food-borne diseases - the challenges of 20 years ago still persist while new ones continue to emerge. Int. J. Food Microbiol. 30, 22.
Nguyen, H.D.N., Yuk, H.-G., 2013. Changes in resistance of *Salmonella* Typhimurium biofilms formed under various conditions to industrial sanitizers. Food Control 29, 236–240.
Pitts, B., Hamilton, M.A., Zelver, N., Stewart, P.S., 2003. A microtiter-plate screening method for biofilm disinfection and removal. J. Microbiol. Methods 54, 269–276.
Rehm, B.A., 2009. Alginate production: precursor biosynthesis, polymerization and secretion. In: Rehm, B.H.A. (Ed.), Alginates: Biology and Applications. Springer Berlin Heidelberg, Berlin Heidelberg, pp. 55–71.
Schlisselberg, D.B., Yaron, S., 2013. The effects of stainless steel finish on *Salmonella* Typhimurium attachment, biofilm formation and sensitivity to chlorine. Food Microbiol. 35, 65–72.
Sharma, M., Anand, S.K., 2002. Characterization of constitutive microflora of biofilms in dairy processing lines. Food Microbiol. 19, 627–636.
Simoes, L.C., Lemos, M., Pereira, A.M., Abreu, A.C., Saavedra, M.J., Simoes, M., 2011. Persister cells in a biofilm treated with a biocide. Biofouling 27, 403–411.
Smith, K., Hunter, I.S., 2008. Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates. J. Med. Microbiol. 57, 966–973.
Theraud, M., Bedouin, Y., Guiguen, C., Gangneux, J.P., 2004. Efficacy of antiseptics and disinfectants on clinical and environmental yeast isolates in planktonic and biofilm conditions. J. Med. Microbiol. 53, 1013–1018.
Toté, K., Horemans, T., Berghe, D.V., Maes, L., Cos, P., 2010. Inhibitory Effect of Biocides on the Viable Masses and Matrices of Staphylococcus aureus and Pseudomonas aeruginosa Biofilms. Appl Environ Microbiol 76, 3135–3142.
Vazquez-Sanchez, D., Habimana, O., Holck, A., 2013. Impact of food-related environmental factors on the adherence and biofilm formation of natural *Staphylococcus aureus* isolates. Curr. Microbiol. 66, 110–121.
von Holy, A., 2006. Food Safety: Thinking Globally Acting Locally. Emerald Group Publishing.
Xu, X., Stewart, P.S., Chen, X., 1996. Transport limitation of chlorine disinfection of *Pseudomonas aeruginosa* entrapped in alginate beads. Biotechnol. Bioeng. 49, 93–100.
Yang, L., Hu, Y., Liu, Y., Zhang, J., Ulstrup, J., Molin, S., 2011. Distinct roles of extracellular polymeric substances in *Pseudomonas aeruginosa* biofilm development. Environ. Microbiol. 13, 1705–1717.