UNIVERSITE DE LILLE I - SCIENCES ET TECHNOLOGIES

Ecole doctorale Science de la Matière, du Rayonnement et de l'Environnement

THESE DE DOCTORAT

Spécialité : Ingénierie des Fonctions Biologiques

Présentée par

Simon Oussama KHELISSA

Pour l'obtention du grade de

DOCTEUR DE L'UNIVERSITE DE LILLE 1

Caractérisation des propriétés physiologiques associées aux cellules détachées de biofilms et étude des interactions aux interfaces entre bactéries et matériaux : cas de *Staphylococcus aureus* et *Pseudomonas aeruginosa*

Characterization of physiological properties associated with biofilm-detached cells and study of interactions between bacteria and materials: case of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

N° Ordre 42558

Préparée au laboratoire:

Unité Matériaux et Transformations CNRS UMR 8207

Equipe : Processus aux Interfaces et Hygiène des Matériaux

Soutenue le 18 Décembre 2017 devant le jury composé de :

CHIHIB Nour-Eddine, Professeur, Université de Lille 1.	**Directeur**
JAMA Charafeddine, Professeur, Université de Lille 1.	**Co-directeur**
FEDERIGHI Michel, Professeur, Université de Nantes.	**Rapporteur**
GHARSALLAOUI Adem, Maître de conférences-HDR, Université de Lyon 1.	**Rapporteur**
FAILLE Christine, Directrice de recherche INRA, PIHM, Lille.	**Examinatrice**
WACHÉ Yves, Professeur, Université de Bourgogne.	**Examinateur**
MIDELET BOURDIN Graziella, Chercheur ANSES – HDR, Boulogne-sur-Mer.	**Invitée**

Je tiens dans un premier temps à remercier Dr. Guillaume Delaplace et Dr. Christine Faille, de m'avoir chaleureusement accueilli dans leur laboratoire et d'avoir mis à ma disposition les outils nécessaires à l'avancement de mes travaux.

J'adresse mes remerciements les plus chaleureux à mon directeur de thèse, Prof. Nour-Eddine CHIHIB, ainsi que mon co-encadrant Prof. Charafeddine JAMA pour leur soutien sans faille et les heures qu'ils ont consacrées à diriger cette recherche. Je leurs suis extrêmement reconnaissant pour les conseils, les discussions scientifiques, les corrections et de m'avoir accordé la liberté de gérer mon travail ce qui m'a permis de développer mon autonomie et mon indépendance scientifique.

Mes sincères remerciements vont également au Monsieur Michel FEDERIGHI, Professeur à l'université de de Nantes, et Monsieur Adem GHARSALLAOUI, MCF-HDR à Université Claude-Bernard-Lyon 1, pour avoir accepté d'être les rapporteurs de ce travail. Je remercie aussi Madame Christine Faille, Madame Graziella MIDELET BOURDIN et Monsieur Yves WACHÉ du jury d'avoir accepté de participer à ce jury en tant qu'examinateurs.

J'aimerais adresser un remerciement particulier à Dr. Marwan ABDALLAH, pour sa gentillesse, son aide, ses conseils et son grand soutien.

Je tiens à remercier l'ensemble de l'équipe PIHM, Christophe Dufourmantelle, Thierry Bénezech, Pascal Blanpain-Avet, Thomas Dubois, Paolo Peres de sa Peixoto, Laurent Bouvier, Pascal Debreyne, Jean-François Migdal, Anne Moreau, Annette Ronse, Gilles Ronse, Jacky Six, Thierry Six, Laurent Wauquier. Je les remercie énormément pour leur gentillesse et leur sympathie.

Et bien sûr, je remercie de tout mon cœur ma famille, ma mère, mon oncle Habib qui m'ont soutenu tout au long de mes études.

Enfin, aucun mot ne peut exprimer ma reconnaissance à mon épouse, Mariana, pour sa présence à mes côtés, pour les sacrifices qu'elle a faits et de son soutien tout au long de mes trois années de doctorat et surtout les derniers mois difficiles. Je lui adresse mes remerciements à elle ainsi qu'à notre enfant, Yanis, pour le bonheur qu'ils me procurent au fil des jours. Je vous aime.

À la mémoire de mon père et de mes grands-parents …

Scientific valorization

Publications

1. SO Khelissa, M. Abdallah, C. Jama, C. Faille, N-E Chihib (2017). Bacterial contamination and biofilm formation on abiotic surfaces and strategies to overcome their persistence. JMES 8 (9), pp. 3326-3346. **(Accepted)**

2. SO Khelissa, C. Jama, M. Abdallah, R. Boukherroub, C. Faille, N-E Chihib (2017). Effect of incubation duration, growth temperature, and abiotic surface type on cell surface properties, adhesion and pathogenicity of biofilm-detached *Staphylococcus aureus* cells. AMB express. **(Accepted)**

3. SO Khelissa, C. Jama, M. Abdallah, A. Barras, N-E Chihib (2017). A comparative study of the impact of growth conditions on the cell surface physicochemical properties and the pathogenicity of the biofilm-detached and planktonic *Pseudomonas aeruginosa* cells. J Food Prot. **(Submitted)**

4. SO Khelissa, C. Jama, M. Abdallah, R. Boukherroub, N-E Chihib (2017). Biofilm-detached and planktonic cells phenotype transitions: a comparative study of *Staphylococcus aureus* tolerance to benzalkonium chloride treatment and the resistance mechanisms on the cellular level. Appl Microbiol Biotechnol. **(Submitted)**

5. SO Khelissa, C. Jama, M. Abdallah, R. Boukherroub, C. Faille, N-E Chihib (2017). A study of biofilm-detached *Pseudomonas aeruginosa* cells susceptibility to benzalkonium chloride and associated bacterial resistance strategy **(Submitted)**

Oral communications

1. SO. KHELISSA, C. JAMA, N-E. CHIHIB. Caractérisation des interactions aux interfaces entre bactéries et matériaux pour limiter l'adhésion et la structuration des bactéries en biofilm. Journée des doctorants 19 juin UMET 2016. Polytech, Villeneuve d'Ascq

2. SO. KHELISSA, C. JAMA, N-E. CHIHIB. *Pseudomonas aeruginosa* adhesion and biofilm formation on stainless steel. International Conference on Materials & Environmental Science. December 1-3, 2016, Oujda, Morocco

Posters

1. SO. KHELISSA, C. JAMA, M. ABDALLAH N-E. CHIHIB. Characterization of interactions at the interfaces between *Pseudomonas aeruginosa* and abiotic surfaces to limit the bacterial adhesion and biofilm formation. Biofilms7. June 26-28, 2016, Porto - Portugal

2. SO. KHELISSA, C. JAMA, N-E. CHIHIB. Les biofilms et la santé publique. Les Doctoriales. 14-19 mai 2017. Lille

Characterization of physiological properties associated with biofilm-detached cells and study of interactions between bacteria and materials: case of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Abstract

The contamination of abiotic surfaces by pathogenic microorganisms in health-care and food-processing sectors leads to the establishment of biofilm. Bacterial biofilms are considered as a main cause of serious human infections, such as foodborne and nosocomial deseases. The threat of biofilm-cells comes from their higher resistance to disinfectants, when compared to their planktonic counterparts. After biofilm maturation, bacteria may detach from biofilm and colonize new surfaces. Yet, few studies have been conducted on biofilm-detached bacteria to assess their microbial risk. In this regard, the goal of the present work was to conduct a comparative study of growth conditions effect on some physiological properties of biofilm-detached and planktonic *Staphylococcus aureus* and *Pseudomonas aeruginosa* cells. The surface physicochemical properties which control the bacterial adhesion to stainless steel (SS) and polycarbonate (PC) were investigated. Moreover, the pathogenic potential of both cell populations was studied. The results showed that the bacterial growth conditions and lifestyle influenced their surface properties and therefore their adhesion on SS and PC. The growth temperature (20, 30 and 37°C), surface type (SS and PC) and incubation duration (24 and 48h) affected significantly the virulence factors production and the cytotoxicity in the supernatants recovered from biofilm and planktonic cultures. Thereafter, the effect of growth temperature (20 and 37°C) on the resistance of biofilm-detached and planktonic cells to Benzalkonium chloride (BAC) was investigated. The results showed that, in addition to the growth temperature and the lifestyle, the resistance to BAC treatment depended on the studied strain. In order to understand the mechanisms of resistance to BAC, investigations were carried out at a cellular level. In fact, the damage of bacterial membranes associated to BAC was monitored by the efflux of the intracellular potassium. In addition, the membrane fluidity of biofilm-detached and planktonic cells was investigated through the study of membrane fatty acid profiles. The results showed that biofilm-detached and planktonic bacteria were phenotypically different. Their pathogenicity and resistance response to BAC treatment depended on several parameters. The results also showed that BAC targeted and damaged the bacterial membrane. Finally, our study highlights that the modulation of bacterial membrane fluidity may be an effective strategy adopted by bacteria in response to BAC treatment.

Keywords: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, biofilm-detached cells, planktonic cells, physiology, nosocomial infections, foodborne diseases, pathogenicity, resistance to benzalkonium chloride.

Caractérisation des propriétés physiologiques associées aux cellules détachées de biofilms et étude des interactions aux interfaces entre bactéries et matériaux : cas de *Staphylococcus aureus* et *Pseudomonas aeruginosa*

Résumé

La contamination des surfaces abiotiques par des micro-organismes pathogènes dans les secteurs hospitalier et alimentaire conduit à la formation de biofilm. Le biofilm est considéré comme une cause principale d'infections humaines graves, telles que les infections nosocomiales et alimentaires. Le risque biologique élevé associé aux bactéries structurées en biofilm provient de leur plus haute résistance aux désinfectants, comparée aux cellules planctoniques. Après la maturation de biofilm, des bactéries peuvent se détacher et coloniser des nouvelles surfaces. A ce jour, peu d'études ont été menées sur les bactéries détachées de biofilm pour évaluer le risque microbiologique associé à ce type de bactérie. À cet égard, l'objectif de ce travail était de mener une étude comparative de l'effet des conditions de croissance sur les propriétés physicochimiques de surface de *Staphylococcus aureus* et *Pseudomonas aeruginosa* cultivées sous leurs formes détachées de biofilm et planctoniques. Ceci a permis d'élucider l'impact des propriétés de surface sur l'adhésion bactérienne sur l'acier inoxydable (SS) et le polycarbonate (PC). Le pouvoir pathogène des deux populations bactériennes a également été étudié. Les résultats ont montré que les conditions et le mode de croissance bactérienne influencent les propriétés de surface et par conséquent l'adhésion de *S. aureus* et *P. aeruginosa* sur le SS et le PC. De plus, la température de croissance (20, 30 et 37°C), le type de surface (SS et PC) et l'âge physiologique (24 et 48h) influencent significativement la production de facteurs de virulence et la cytotoxicité des surnageants récupérés de biofilm et de cultures planctoniques. Par la suite, l'effet de température de croissance (20 et 37°C) sur la résistance des cellules détachées de biofilm et planctoniques au chlorure de benzalkonium (BAC) a été évalué. Les résultats ont montré que, en plus de la température et du mode de croissance, la résistance au traitement BAC dépend de la souche étudiée. Les mécanismes de résistance, ont été étudiés au niveau cellulaire. En effet, les lésions des membranes bactériennes associées au BAC ont été suivies par l'efflux des ions K^+ intracellulaires. En outre, la fluidité membranaire de deux populations bactériennes a été caractérisée à travers l'étude de profils d'acides gras membranaires. Les résultats ont montré que les bactéries détachées de biofilm et celles à l'état planctonique sont phénotypiquement différentes. Leur pouvoir pathogène et leur résistance au BAC dépendent de plusieurs paramètres. Les résultats ont également montré que le BAC endommage la membrane bactérienne. Enfin, notre étude a mis en évidence que la modulation de la fluidité de la membrane bactérienne peut être une stratégie efficace adoptée pour résister au traitement antibactérien

Mots clés : *Staphylococcus aureus, Pseudomonas aeruginosa*, cellules détachées de biofilm, cellules planctoniques, physiologie, infections nosocomiales, intoxications alimentaires, pathogénicité, résistance au chlorure de benzalkonium.

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List of abbreviations

$: dollar
%: percent
€: euro
°c: degree centigrade
µl: microliter
µm: micrometer
B. cereus: *Bacille cereus*
BapA: biofilm-associated protein
BCS: biofilm-Culture-Supernatants
BSIs: blood-Stream Infections
ca.: around
CAS: chrome azurol sulphonate
CCK: cell counting kit
CDC: centers for disease control and prevention
CFU: colony-forming unit
cm2: square centimeter
CO2: carbon dioxide
CoNS: coagulase-vgative *Staphylococci*
DMEM: dulbecco's modified eagle's medium
DNA: deoxyribonucleic acid
DNase: deoxyribonuclease
E. coli: *Escherichia coli*
ECDC: european centre for disease prevention and control
EFSA: european food safety authority
EO: essential Oil
EPS: exopolysaccharides
EU: european union
FBD: food-borne diseases
FBS: phosphate buffered saline
h: hour
H2O: water
HAIs: healthcare-Associated Infections
HCL: hydrogen chloride
InVS: institut de veille sanitaire
kHz: kilohertz
KNO3: potassium nitrate
KOH: potassium hydroxide
L. monocytogenes: *Listeria monocytogenes*
LPS: lipopolysaccharide
LS: lysostaphin
M: molar
MATS: microbial adhesion to solvents
MDR: multi-drug resistant
min: minutes

ml: milliliter
mm: millimeter
mM: millimolar
MRSA: methicillin-resistant *Staphylococcus aureus*
MSSA: methicillin-sensitive *Staphylococcus aureus*
mV: millivolt
NHSN: national healthcare safety network
nm: nanometer
P. aeruginosa: *Pseudomonas aeruginosa*
PC: polycarbonate
PCBDC: polycarbonate-biofilm-detached-cells
PCS: planktonic-culture-supernatants
PEG: poly ethylene glycol
Pel: pellicle
PPB: potassium phosphate buffer
PVC: polyvinyl chloride
QACs: quaternary ammonium compounds
rpm: revolutions per minute
S. aureus: *Staphylococcus aureus*
S. epidermidis : *Staphylococcus epidermidis*
S. xylosus: *Staphylococcus xylosus*
SEA: Staphylococcal enterotoxin A
SS: stainless steel
SSBDC: stainless steel-biofilm-detached-cells
T: temperature
TMS: triMethylSilane
TSB: tryptic Soy Broth
US: united States
VAP: ventilator-associated pneumonia
ZP: zeta potential

GENERAL INTRODUCTION

General introduction

Surface contamination by pathogenic bacteria in food-processing and healthcare sectors is of great concern for the public health. In fact, the persistence of pathogenic bacteria despite the cleaning and disinfection procedures may be the origin of fatal human infections such as nosocomial and foodborne deseases. The outbreaks of these infections are responsible for high human life and critical economic losses (Hassan et al. 2010; Scharff 2012). According to a study carried out by the National Institute for Public Health Surveillance (InVS) in 2012, 5% of patient contracted an infection during their hospitalization in french hospitals. This represents about 750 000 cases of nosocomial infection per year, which would be the direct cause of 4,000 deaths each year in France (InVS 2006). It has been reported that *Staphylococcus aureus* and *Pseudomonas aeruginosa* are in the most frequently isolated bacteria from these nosocomial infections (Santajit and Indrawattana 2016). Furthermore, foodborne infections are also prevalent. In France 1,380 foodborne outbreaks were reported in 2014, affecting 12,109 people, including 649 hospitalizations and 2 deaths (Khelissa et al. 2017). The most commonly detected causative pathogens were *S. aureus, Bacillus cereus* and *Salmonella* spp. In natural and man-made environment, bacteria have a tendency to live attached to surfaces and to form a complex structure, called "biofilm" (Donlan and Costerton 2002). Hence, the planktonic growth mode is considered as transitory growth mode (Jefferson 2004). Moreover, bacteria living under the biofilm state are known to be phenotypically different from their planktonic counterparts (Lazazzera 2005), and present a higher tolerance to antimicrobial agents (Donlan and Costerton 2002). In addition, several studies have reported that biofilms formed on both food and medical equipment are to the main cause of the spread of nosocomial and foodborne infections (Donlan and Costerton 2002; Brooks and Flint 2008).

In food and medical environments, disinfectants products are constantly used in order to prevent nosocomial and foodborne infections. However, biofilms are known to enhance the microbial resistance and tolerance to disinfection treatments. Such structure constitute a potential reservoir for pathogens which serve as a continuous source of infections, cross-contaminations and material deterioration (Brooks and Flint 2008). Thus, it's of importance to understand the mechanisms that control the formation of biofilm in order to reduce the microbiological risk related to their persistence. The biofilm formation on abiotic surfaces starts with the adhesion of microbial contaminants emerging from different ecosystems. The most frequent contaminant sources of food-contact surfaces and healthcare settings are water, raw foods, dust, equipment, food handlers,

patients and healthcare workers, etc. (Donlan and Costerton 2002; Marriott and Gravani 2006; Green et al. 2006). In order to prevent the biofilm formation, several studies have been conducted to highlight the mechanisms governing the bacterial adhesion which represents the first step of the biofilm formation. In fact, this bacterial adhesion stage is known to be a reversible phenomenon and governed by nonspecific interactions such as the Lifshitz-van der Waals, electrostatic, hydrophobic and acid-base ones (Bos et al. 1999). In addition, the roughness of abiotic surfaces also has an effect on the bacterial adhesion as previously reported (Tresse et al. 2007; Mitik-Dineva et al. 2009). However, different studies underlined that non-specific interactions are not always involved in the bacterial adhesion and that this pheromone is mainly related to specific interactions between the bacterial surface structures and the abiotic surfaces. Thus, it is of importance to address this discrepancy in order to improve our knowledge regarding the mechanisms involved in the first step of the biofilm formation. Such knowledge can help to deeply understand this phenomenon and to improve strategies of the biofilm control.

Once bacteria are attached irreversibly, they start growing and multiplying within macrocolonies and producing the extracellular matrix (Donlan 2002). During the formation of such structure, adherent cells (also called sessile cells) acquire different physiological characteristics compared to their planktonic counterparts, such as increasing capacity of exopolysaccharide production, decreasing growth rate, and expressing a bacterial phenotype that increase their resistance to sanitizing agents and antibiotics (Costerton 1999; Davies 2003). During the process of biofilm development, cell detachment constitutes the last step which is essential to overcome nutrient and oxygen limitations of bacteria growing within biofilms, to allow the colonization of new surfaces (Allison et al. 1990; Sauer et al. 2002; Donlan and Costerton 2002; Kaplan et al. 2003a; Kaplan et al. 2003b; Hunt et al. 2004). Interestingly, very few investigations were carried out to elucidate the physiology of biofilm-detached cells (Boles et al. 2004; Bester et al. 2005; Ymele-Leki and Ross 2007). The majority of studies focused on mechanisms of the bacterial adhesion and changes occurring during the switch from the planktonic to the biofilm state (O'Toole et al. 2000). Thus, it seems fundamental to carry out specific studies on the biofilm-detached cells to further assess their microbiological risk and their involvement in the recontamination in ordor to optimize disinfection procedures. In this context, we have established a research project to figure out whether the biofilm-detached cells conserve the biofilm phenotype or recover the planktonic one after detachment. Therefore, we aimed to study the phenotypic characteristics of biofilm-detached cells and we

completed our work with an original comparison of these cells with their planktonic counterparts. Two bacterial strains were studied. *S. aureus* CIP 4.83 and *P. aeruginosa* CIP 103467, two bacteria commonly involved in foodborn and nosocomial infections.

Objectives

In this PhD thesis, we have studied and compared the surface physicochemical properties and adhesion behavior of biofilm-detached and planktonic cells on two abiotic surfaces commonly encountered in food and medical equipment, as function of their growth conditions. Furthermore, the goal of the study was to investigate the effect of growth temperature on the resistance of biofilm detached and planktonic cells to benzalkonium chloride, a disinfectant agent frequently used in food and medical sectors. These studies aimed to improve our knowledge regarding the biofilm-detached cell phenotype. Thus, the main targets of our study were:

1) To study the effect of the growth conditions of biofilm-detached and planktonic *Staphylococcus aureus* and *Pseudomonas aeruginosa*, such as temperature changes (20, 30 and 37°C), surface type (stainless steel and polycarbonate) and incubation duration (24 and 48 h), on:

- the cell surface physicochemical properties (Zeta potential, hydrophobicity, electron donor/acceptor character).
- the adhesion behavior on the stainless steel and polycarbonate.
- the production of virulence factors and the cytotoxicity against HeLa cells

2) To study the effect of growth temperature changes (20 and 37°C), and the growth mode (biofilm-detached or planktonic) on the resistance to benzalkonium chloride treatments.

3) To study the effect of growth temperature on the resistance/tolerance mechanisms of *S. aureus* and *P. aeruginosa* biofilm-detached and planktonic cells, to benzalkonium chloride treatment, at a cellular level (membrane fluidity).

This report consists of three parts:

a) The first part, (chapter I), is the literature review which highlights the major problems of biofilms and the environmental factors controlling their formation in the context of food and medical environment. In addition, this review aimed to highlight the different strategies used to fight against the biofilm and mechanisms which may play a role in the biofilm resistance to disinfectant agents. Moreover, chapter I reportes the major strategies adopted to make abiotic surfaces incompatible with the bacterial adhesion in order prevent the bacterial contamination and the establishment of

biofilm. This review is accepted for publication in the journal of materials and environmental science.

b) The second part presents the results obtained during this thesis work. This part is divided into two chapters. The first one, (chapter II), presents on one hand, the effect of growth conditions on the bacterial surface physicochemical properties and their impact on the adhesion behavior of biofilm-detached and planktonic *S. aureus* and *P. aeruginosa* cells to stainless steel and polycarbonate. On the other hand, the relationship between these growth conditions on the pathogenic potential (productions of virulence factors, and cytotoxicity) of the different bacterial populations was investigated. The second chapter, (chapter III), is based on the study of the impact of the growth temperature of biofilm-detached and planktonic cells on their resistance to benzalkonium chloride treatment. In addition, the invovment of the membrane fuluidity in the resistance to this disinfectant was also investigated.

c) The third part concludes this thesis with a general conclusion and perspectives of the present study.

CHAPTER: I

ARTICLE I - LITERATURE REVIEW

Bacterial contamination and biofilm formation on abiotic surfaces and strategies to overcome their persistence

S.O. KHELISSA [1, 2], M. ABDALLAH [2,] C. JAMA [1], C. FAILLE [2], N-E CHIHIB [2],*

[1] Université de Lille1, CNRS, ENSCL, UMR 8207-UMET-PSI, Avenue Dimitri Mendeleïev, CS 90108 59652 Villeneuve d'Ascq, France

[2] Université de Lille1, CNRS, INRA, UMR 8207-UMET-PIHM, 369 rue Jules Guesde, CS 20039, 59651 Villeneuve d'Ascq, France

Abstract

Abiotic surfaces are vulnerable to bacterial adhesion and to biofilm formation. Therefore, it is necessary to understand the parameters that influence the bacterial adhesion to find out solutions against cell adhesion and biofilm formation. The ability of pathogenic bacteria to adhere and to form biofilms on abiotic surfaces represents a major health safety problem. Bacteria embedded in biofilms are more resistant to sanitizing agents than those growing under planktonic state. In fact, surface contamination by these pathogens is enhanced by favourable environmental conditions encountered in food and health sectors. Thus, the understanding of bacterial adhesion and biofilm formation on abiotic surfaces is of interest to setup efficient anti-biofilm strategies. In this context, this review highlights the main factors controlling the bacterial adhesion and biofilm formation on abiotic surfaces. It also describes the current and emergent strategies used to eradicate and prevent the biofilm formation on the most frequently used abiotic surface.

Keywords: Biofilm resistance, adherent cells, abiotic surface, anti-biofilm strategies

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* Corresponding author: Dr. Nour-Eddine CHIHIB.

Tel: +33 320435443

Email: nour-eddine.chihib@univ-lille1.fr

Introduction

Microbial adhesion onto abiotic surfaces and therefore the biofilm formation are considered serious issues, regarding their economic and public health consequences in many sectors, such as food-processing and health-care ones. The presence of pathogenic microorganisms on food sector facilities represents a severe potential health risk to consumers. Contaminated food contact surfaces promote contamination of food products which leads to Food-Borne Diseases (FBDs) [1]. In 2014, 864 FBD outbreaks were reported in the United States (US) resulting in 13,246 illnesses, 712 hospitalizations, 21 deaths, and 21 food recalls [2].

According to the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), a total of 5,251 food-borne outbreaks, including water-borne outbreaks, were reported in the European Union (EU) in 2014. Overall, 45,665 human cases, 6,438 hospitalizations and 27 deaths were reported. The evidence supporting the link between human cases and food vehicles was strong in 592 outbreaks [3]. In healthcare sector, orthopedic implant surface bacterial contamination is responsible for nosocomial infections also called Healthcare-Associated Infections (HAIs). Such infections are defined as infections that occurred during a hospitalization and are not present prior to hospital admission. Generally, nosocomial infections appear after prosthetic and implant surgery by handling contaminated or non-sterile devices. In France, from 1999 to 2006, 14,845 surgical site infections were reported involving 964,128 patients in 838 participating hospitals [4]. HAIs and FBDs are responsible for high critical economic losses. In fact, the direct cost of the HAIs was up to $16.6 billion in the US hospitals [5]. It has been reported that the resulting aggregated annual cost of FBD was $77.7 billion [6]. Generally, microorganisms live attached to surfaces and form biofilm [7]. When bacteria grow within a biofilm they gain several advantages, including enhanced resistance to antimicrobial agents [8]. Biofilms represent a threat to public health when found in food [9] and medical sector [10]. In addition, biofilms are also of concern in different other sectors such as maritime environment [11], water systems [12] and in oil pipes industries [13]. Their formation results in heavy costs in cleaning and maintenance. The persistence of biofilm in both food and medical sectors may constitute a reservoir for pathogens which increase the occurrence of HAIs and FBDs. Therefore, it is necessary to investigate different strategies in order to reduce the bacterial adhesion and the formation of biofilm. Disinfection is an important used strategy to control biofilm formation and

to avoid infection transmission. Other strategy requires designing abiotic surfaces able to hamper the bacterial adhesion and therefore the biofilm formation.

Rather than developing new materials, another promising way is surface modification of existing surfaces [14] by grafting functional chemical groups or antibacterial molecules inhibiting bacterial adhesion [15]. However, the main challenge here is the durability of the treated surface [16]. Thus, setting up antimicrobial surfaces could be very useful for food processing equipment to enhance the food safety and in biomedical sector to prevent microbial colonization on hospital surfaces. To achieve such challenge, the choice of appropriate antimicrobial molecules and surface modification techniques is required. In addition, a deep understanding of the interaction between three main components: the bacterial cell, the attachment surface, and the environmental parameters is needed. In this regard, the goal of this review is to discuss the impact of bacterial adhesion and biofilm formation on abiotic surfaces. In addition, we attempt to highlight the strategies and approaches commonly applied in order to prevent bacterial adhesion and by the way biofilm formation.

Main pathogenic bacteria associated with FBDs and HAIs

Food-borne diseases

Bacteria are all around us, in the air, on surfaces and in/on the human body. Bacteria are often harmless but some of them can be pathogenic for humans. In natural, industrial, hospital and domestic environments, there are many persistent pathogenic bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* spp., *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus cereus* which have serious economic and public health consequences. The contamination of abiotic surfaces with these pathogens leads to human infections worldwide [17]. *L. monocytogenes* has been involved as causative agent of FBDs due to its ubiquitous nature and its ability to grow under hostile conditions [18, 19]. This bacterium is frequently associated with FBDs outbreaks that are characterized by widespread distribution and relatively high mortality rates. Listeriosis, a serious infection, is usually caused by eating contaminated food. The disease primarily affects older adults, pregnant women, newborns, and adults with weakened immune systems. According to the EFSA and the ECDC, the number of confirmed human listeriosis cases in the EU increased slightly to 1,642 in 2012 compared with 2011 [20]. This number includes 198 death cases, which represents the highest number of fatal cases reported since 2006. According to

this study, France is the most affected country with up to 63 fatal reported cases. Thus, the EU fatality case rate was 17.8 % among the 1,112 confirmed cases (67.7 % of all confirmed cases) [20]. The CDC estimates that about 1,600 illnesses and 260 death cases due to listeriosis occur annually in the US [21]. The worst listeriosis outbreak in the US history has occurred in 2011 and it was associated with consumption of cantaloupe from a single farm. In fact, 147 illnesses, 33 deaths, and 1 miscarriage were reported in 28 states [22]. This psychotropic microorganism is able to grow at refrigeration temperatures as low as 2 to 4°C [23] and to contaminate the food-processing environment. Contamination of food with *L. monocytogenes* seems to occur most frequently during the food-processing due to the ability of this bacterium to attach to Stainless Steel (SS) and other abiotic surfaces [24] and form biofilm [25]. In addition, *L. monocytogenes* has been isolated from various surfaces in dairy and meat processing environments [26]. FBDs are also commonly caused by Gram-positive enterotoxigenic *S. aureus* [27]. *S. aureus* is an ubiquitous bacterium which can be found in the air, dust, sewage, humans and animals. In France, food poisoning cases associated with *S. aureus* have been listed in 2012 as the first cause of food-borne outbreaks [28]. In fact, 300 of 1,288 reported food-borne outbreaks (23%) were due to this pathogen [28]. *S. aureus* is able to adhere and form biofilm in food processing plants [29]. Despite the inactivation of *S. aureus* by heating the food prior to consumption, this bacterium can still induce intoxication. In fact, staphylococcal enterotoxins remain stable since they resist to extreme environmental conditions (freezing, drying, heat treatment, low pH and proteolytic enzymes) [27, 30, 31]. According to the EFSA and ECDC [32], in 2011, 6.1 % of all food-borne outbreaks in the EU were caused by staphylococcal toxins. This represents an increase of 25.9 % compared to 2010 (274 outbreaks) and was mainly due to the fact that France has reported 290 outbreaks in 2011 compared with 220 in 2010. In France, *S. aureus* represents the second cause of FBDs after *Salmonella* with 1,361 cases [33]. *Salmonella* spp. is the major food-borne pathogen for humans and animals worldwide. It has been reported that about 1.4 million human salmonellosis cases occur in the US leading to more than 16,000 hospitalizations with nearly 600 deaths and resulting in a high cost amounting to several billion dollars annually [34]. In 2012, the number of salmonellosis cases in humans decreased by 4.7% compared with 2011. A statistically significant decreasing trend in the EU was observed over the period 2008-2012. A total of 91,034 confirmed human salmonellosis cases were reported in 2012 [20]. The two most common *Salmonella* serovars, involved in food poisoning outbreaks, are *Typhimurium* and *Enteritidis* [35]. *Salmonella Enteritidis* was the predominant

serovar associated with the *Salmonella* outbreaks accounting for 66 % of human cases involved in these outbreaks followed by *Salmonella Typhimurium* which has been associated with 16.9 % of cases. The persistence of *Salmonella* in food processing environment, despite the cleaning procedures, could lead to microbial cross-contamination and to biofilm formation [36, 37]. In fact, several studies have demonstrated the ability of *Salmonella* to form biofilms on abiotic surfaces such as SS [38], plastic [39] and rubber [40]. Generally, once attached, these pathogens may produce resistant biofilms constituting a reservoir for cells which, once detached, contaminate food products continuously. In addition, it is now established that in natural and man-made ecosystems, more than 99.9% of micro-organisms live attached to surfaces and form a specific and complex structure called biofilm. *E. coli* strains are common bacteria of the gastrointestinal tract [41]. Some *E. coli* strains are able to produce toxins that induce serious human infections [41]. Grass-fed cattle are the main reservoir of such *E. coli* strains. Their faeces might contaminate the meat during slaughter and thus act like microbial carrier which might end up contaminating other foods (e.g. milk, vegetables) and water. Outbreaks due to *E. coli* 0157:H7 have been associated primarily with consumption of undercooked beef meat, but also other foods have been involved as contamination carrier [42]. In fact, cross-contamination of foods can occur in food-processing plants and during subsequent handling and preparation, resulting in a wide range of foods being involved in *E. coli* O157:H7 outbreaks [43, 44]. In 2011, the ECDC have reported 2,495 food-borne outbreaks caused by the pathogenic *E. coli* including 54 deaths in the EU [32].

Nosocomial infections

According to the ECDC the most frequently reported HAI type was pneumonia and other lower respiratory tract infections, representing 25.7% of all reported HAIs [45]. The second most frequently reported type of HAI was surgical site infection (18.9%) followed by urinary tract infection (17.2%), bloodstream infection (14.2%) and gastro-intestinal infection (7.8%) [45]. *S. aureus* and *P. aeruginosa* are in the top four microorganisms most frequently isolated from these HAIs in the EU [45]. *P. aeruginosa* is found in various environmental niches including soil, water, plants, and hospital environments [46]. Despite the advances in health care and the improvement of strict disinfection procedures, *P. aeruginosa* is among the most dreaded Gram-negative pathogens in hospital setting and is the one of main causes of nosocomial infections [47]. According to the National Healthcare Safety Network (NHSN), *P. aeruginosa* was involved in 8%

of HAIs in the US hospitals [48]. Moreover, in 2013, the CDC reported that about 51,000 health-care-associated *P. aeruginosa* infections occur in the US each year. More than 6,000 (13%) of these are multi-drug-resistant with roughly 400 deaths per year [49]. In the EU, *P. aeruginosa* represents 8.9% of total pathogens associated with nosocomial infections [45]. *P. aeruginosa* is an important cause of infection among patients with impaired immune systems. In 2012, high percentages of Multi-Drug Resistant (MDR) *P. aeruginosa* isolates were reported in several countries, especially in Southern and Eastern Europe. Combined resistance was common, with 14% of the isolates reported as resistant to at least three different antimicrobials [50]. Another bacterium causing similar problems is the Gram-positive *S. aureus.* Besides to being responsible for food poisoning outbreaks, this species has been recognized as an important pathogen which causes different serious human diseases [51]. *S. aureus* in its methicillin-resistant form (MRSA) is a major cause of anti-microbial resistant health-care associated infections worldwide. MRSA remains a public health priority in the EU, as the percentage of MRSA is still above 25% in seven of 29 reporting countries [52]. In the EU the number of patients acquiring health-care-associated infections in acute care hospitals has been estimated at 4.1 million each year [53]. *S. aureus* is the most involved pathogen in bloodstream infections in the US. According to the NHSN, this bacterium is associated with 15% of total HAIs reported between 2011 and 2012 in the US [54].

How bacteria adhere to surfaces and form biofilms?

Biofilm formation is a complex process which gives bacteria a better resistance to cleaning agents than bacteria growing under planktonic form [7]. Biofilm is a community of microorganisms in which cells stick to a surface and to each other (Figure 1).

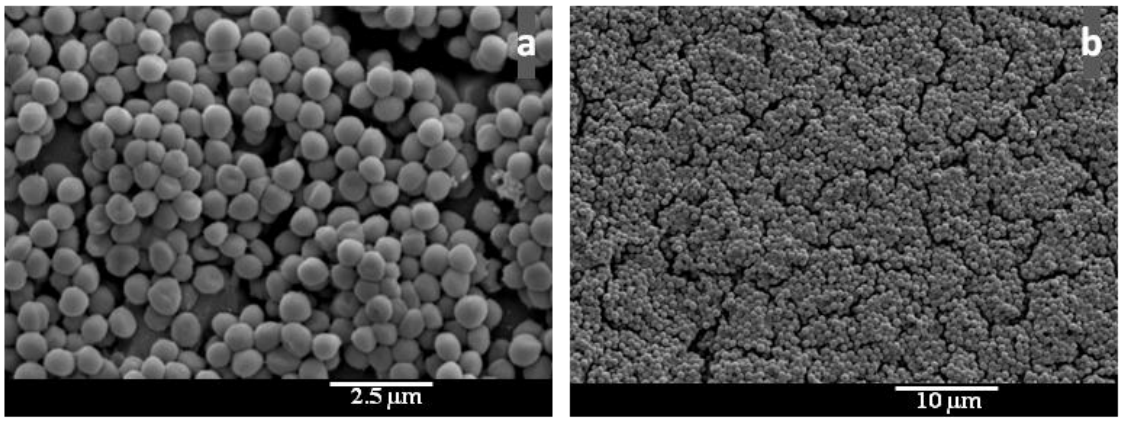

Figure 1: Scanning electron microscopy image of biofilm produced by *Staphylococcus aureus* CIP 4.83 on 316L stainless steel after 24 h incubation at 37°C. The scale bars in the images are 2.5 µm (a) and 10 µm (b).

This cell cluster is marked by the secretion of extracellular matrix (Figure 2) with adhesive and protective properties [1, 7].

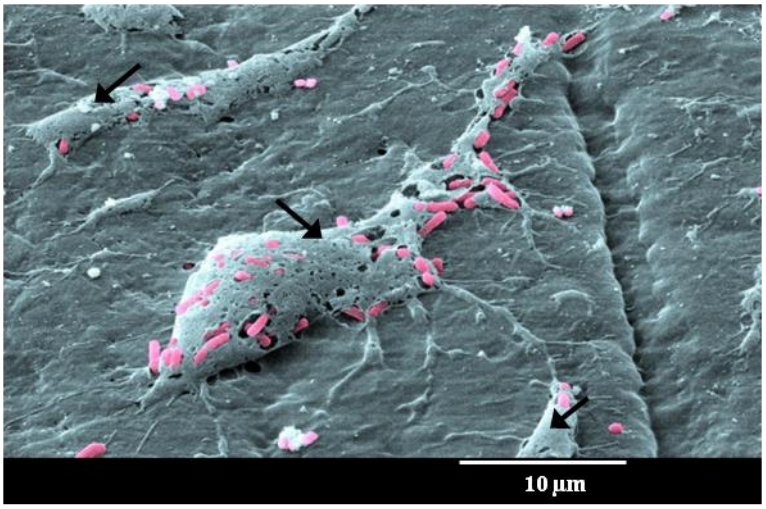

Figure 2: Scanning electron microscopy image of *Escherichia coli* biofilm on Teflon. The arrows in the image point to the extracellular matrix enclosing the *Escherichia coli* biofilm bacteria (colored in pink). The scale bar in the image is 10 µm.

Biofilm formation requires different steps and there are a number of mechanisms by which many microbial species may come closely in contact with a surface, attach and promote cell-cell interactions in order to grow and form biofilms. These mechanisms have been widely described [55]. The different steps leading to biofilm formation are now well understood. The adsorption of bacteria or reversible adhesion to the surface is the first step of biofilm formation. It is triggered when the microorganisms approach the surface over 50 nm, through van der Waals interactions. Then, when the distance is between 10 and 20 nm, more non-covalent forces such as hydrophobic, acid-base and electrostatic interactions get involved in the adhesion process. As the distance decreases the adhesion becomes irreversible, and at less than 0.5 nm other specific interactions, also called short-range interactions, are needed to attach bacteria to abiotic surfaces (Figure 3). In fact, bacteria have some structural adhesins which are a part of the cellular envelope such as pili, Fimbria and flagella that enhance the cellular adhesion. These structures create bridges between cells and surfaces and allow overcoming unfavourable conditions in order to strongly anchor bacteria to abiotic surfaces [56].

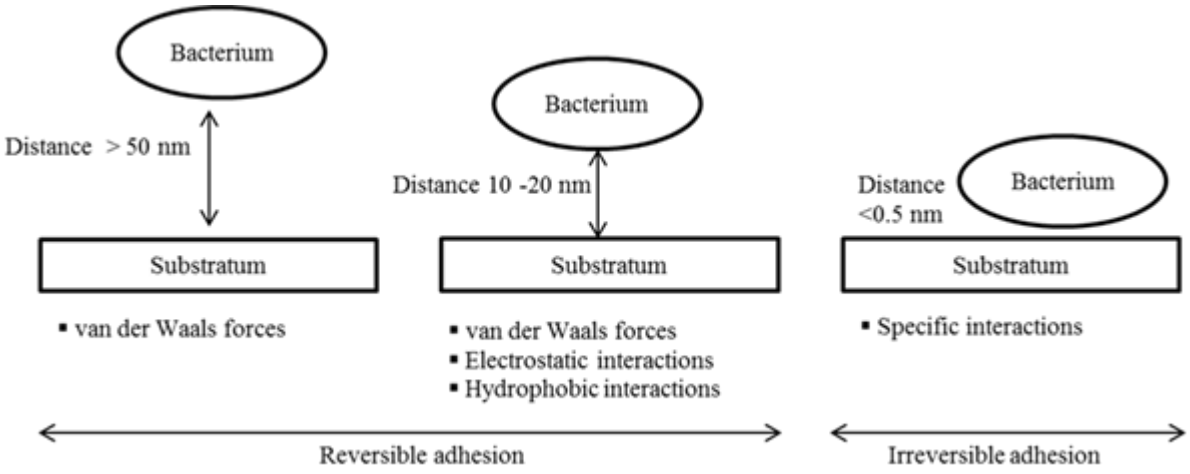

Figure 3: The reversible bacterial adhesion consists in the initial attraction of the bacterial cells to the surface through the effects of non-specific physical forces (distance > 50 nm between bacterial cells and surfaces). The irreversible adhesion is achieved through the effects of the specific (short-range) interactions (distances < 5 nm, with involvement of hydrogen bonding, ionic and dipole interactions, hydrophobic interactions and bacterial structural adhesins).

Once the irreversible adhesion is established, bacteria start synthesizing insoluble exopolysaccharides (EPS). Within hours of EPS accumulation, bacteria get entrapped in a complex protecting extracellular matrix and form a mature biofilm that provides protective environments against antibacterial agents and antibiotics [57]. Indeed, this EPS matrix makes traditional surface cleaning procedures and application of detergents or biocides on materials in contact with food not fully efficient to eliminate mature biofilms [58]. Therefore, one of the most effective strategies to limit biofilm formation is to prevent or restrict bacterial adhesion on surfaces. Bacterial adhesion on abiotic surfaces and subsequent biofilm formation constitute a serious issue in several sectors such as food industries, water canalizations and medical facilities. Indeed, bacteria find favourable conditions to colonize surfaces and establish biofilms [7]. The persistence of biofilm in food, medical and other sectors constitutes reservoirs for pathogens which increase the occurrence of HAIs and FBDs. Thus, it is necessary to investigate different strategies in order to reduce the bacterial adhesion and the formation of biofilm.

Food-borne infections and adherent cells

Food contact surfaces and equipment are considered a serious factor contributing to contamination of foods if not properly cleaned [59]. In addition, surface contamination may lead to biofilm formation which enhances the capacity of food-borne bacteria to survive stress conditions encountered within food processing environments [60]. Surface contamination by pathogenic

bacteria results in serious food-borne outbreaks generating a considerable disease burden and also economic losses [61]. The economic cost of food-borne outbreaks is highly affecting the US economy at a cost of 50 to 80 billion US dollar annually [62]. Other statistics has estimated that the total burden of FBDs was 152 billion US dollar [63]. In Australia and New Zealand, the cost of food-borne outbreaks has been estimated at 1,289 billion and 86 million US dollar respectively per year [64, 65]. In Sweden, the annual cost of food-borne outbreaks was estimated to be 171 million US dollar [66]. In this regard, globalizing of food market with worldwide transportation makes food safety a major priority in order to prevent spreading of pathogenic bacteria and the emergence of food poisoning outbreaks worldwide. In England and Wales, FBDs cause more than 2 million cases, 21,138 hospitalizations and 718 deaths per year [67]. Pathogenic bacteria are able to adhere and form biofilms on various food contact surfaces [68, 69]. It is now established that the persistence of pathogenic bacteria on food contact surfaces, equipment and processing environments, is a contributing factor in food-borne outbreaks, especially those involving *L. monocytogenes, B. cereus, S. aureus, E. coli* and *Salmonella* spp. [70]. Equipment, utensils and cutting boards are likely to be the key cross contamination routes as they become contaminated with pathogens from the handlers, sewage, water and condensation caused by the faulty ventilation [71–73]. Therefore, it has been reported that in the United Kingdom, 14 % of all food-borne illnesses involving *S. aureus, E. coli, Salmonella enterica* and *L. monocytogenes,* may be due to inadequately cleaned cutting boards and knives [74]. According to the French national health monitoring institute (InVS), 1,380 FBD outbreaks were reported in 2014, affecting 12,109 people, including 649 hospitalizations and 2 deaths. The three most frequently suspected pathogens were *S. aureus* (30%), *B. cereus* (22%) and *Salmonella* spp. (15%). The French available data showed also that food contact surfaces and equipment were up to 60 % involved in FBD outbreaks (2011) in collective and home catering [75]. In fact, food industries represent a favourable environment for bacterial adhesion and biofilm formation [76]. In the dairy, meat and sea-food industries food contact surfaces are often contaminated by pathogenic bacteria including *L. monocytogenes, S. aureus, Salmonella* spp., *B. cereus* and *E. coli* [77–80]. Moreover, it has been reported that even after cleaning, *E. coli* bacterial densities up to 10^5 CFU/cm^2 could be recovered on food processing surfaces [81]. It has been mentioned that in small-scale facility producing traditional dry sausage, sixteen *L. monocytogenes* strains and nine *Salmonella* spp. subspecies were isolated from the stuffing machines [82]. Moreover, many pathogenic bacteria such as *B. cereus* and *S. aureus* are

often isolated from the dairy, meat and sea-food industries surfaces [79]. In addition, a highest prevalence of *sea* gene encoding for Staphylococcal Enterotoxin A (SEA) have been reported. The *sea* gene is the most common in *Staphylococcus*-related food poisoning [83].

Nosocomial infections and adherent cells

Nosocomial infections contracted during hospitalization can lead to high morbidity and even mortality of immune-depressed patients. Bacterial adhesion to medical devices surfaces and surgical sites is considered the base of the pathogenic mechanism [84]. Bacterial risk is of major concern in the medical sector because of the high rate of contamination of materials which are inserted into or in contact with the human body. Medical implants such as urinary catheters, central venous catheters and implanted prosthetic devices are prone to biofilm formation and represent a serious nosocomial infection source [85–87]. The issue starts when an indwelling medical device is contaminated with pathogenic bacteria which may develop a biofilm. Once these microorganisms irreversibly attach to devices introduced into a body, they start producing extracellular polysaccharides to develop an infectious biofilm. Such infections are known nowadays as chronic polymer-associated infection [88]. According to the National Nosocomial Infection Surveillance system of the CDC, Blood-Stream Infections (BSIs) represent 90% of all nosocomial blood infections and they are always considered to be device related if they happen after the insertion of an intravascular catheter [89]. Moreover, intravascular catheters are one of the most common causes of nosocomial bacteremia. In fact, catheter-related BSIs are affecting over 250,000 patients per year in US [90]. In this context, it has been shown by scanning and transmission electron microscopy that almost all indwelling catheters are colonized by microorganisms embedded in a biofilm matrix [91]. These biofilms may be located either on the lumen or on the outer surface of the catheter [92]. The colonizing microorganisms may originate either from patient's skin micro-flora or other micro-flora from health-care staff and contaminated facilities. Furthermore, staphylococci are recognized as the most frequent causes of biofilm-associated infections [93]. The percentage of implant failure, due to infection by three different groups of staphylococci: MRSA, Methicillin-Sensitive *S. aureus* (MSSA), and Coagulase-Negative Staphylococci (CoNS), is of *ca* 2% of all implants, representing an average of 4500 incidents per year [94]. Moreover, the prevalence of Ventilator-Associated Pneumonia (VAP) is 8 to 28% among patients who received prolonged mechanical ventilation [95]. This results from the respiratory system colonization by the endogenous flora or by exogenous pathogens acquired from

the intensive care environment [96,97]. *P. aeruginosa* is also considered one of the most frequently associated pathogen with HAIs. It has been identified that healthcare water systems are associated with patient infections with *P. aeruginosa* in intensive care units [98]. In fact, *P. aeruginosa* biofilms are likely to represent a potential reservoir source of nosocomial infection when it colonizes water systems in healthcare facilities [98]. Besides to their fatality towards human, HAIs represents a high economic cost. The annual direct medical cost of HAI to the US hospitals ranges from $28.4 to $33.8 billion [99]. In France the total cost of nosocomial infections in acute care units was estimated to be up to €3.2 million per year [100].

Parameters controlling biofilm formation

Abiotic surfaces are vulnerable to biofilm formation. Therefore, it seems to be necessary to understand the parameters that influence bacterial adhesion in order to find solutions against biofilm formation. Bacterial adhesion to surfaces is likely to be related to three main parameters which are the physiochemical characteristics of the bacterial cell and abiotic surfaces and finally the environmental conditions.

Role of the physiochemical characteristics of the bacterial cell surface in biofilm formation

The attachment of bacterial cells to abiotic surfaces is a process tightly related to several physiochemical forces such as van der Waals, electrostatic, steric forces and hydrophilic ⁄ hydrophobic. Moreover, the physicochemical surface properties of bacterial cells are determined by structures and molecules that are exposed on the cell surface which control the attachment and biofilm formation. Here, the major bacterial cell structures will be highlighted.

Role of bacterial cell surface structures

Flagella

Flagella have been generally considered major virulence factors mainly because of their motility property. However, flagella are getting recognized to play other roles with more functions besides motility and chemotaxis. Recent studies have defined flagella as an effective bacterial surface compound in many additional processes including adhesion, biofilm formation and virulence factor secretion [101]. Motility is considered a virulence factor facilitating the colonization of abiotic surfaces by pathogenic bacteria. According to different studies the flagellar motility is important for initial cell-to-surface contact leading to biofilm formation and development [102,103]. Flagella

can facilitate the attachment of bacteria to surfaces by overcoming the repulsive forces that might hamper cell- to-surface contact. Thus, flagella are not only required for motility but also plays an important role in surface sensing and the earliest steps of surface adhesion that leads to the formation of a biofilm. *E. coli* and *L. monocytogenes* use flagella, pili, and membrane proteins to initiate attachment [104]. The loss of these cell appendages changes their surface properties which may lead to decreased attachment ability on some abiotic surfaces [105].

Fimbriae or pili

Fimbriae (or pili) are a group of rigid, straight, and filamentous proteinaceous structures composed of protein subunits called pilin associated to the outer bacterial membrane surface [106]. Their role in biofilm formation on abiotic surfaces is considered critical in the early stable cell-to-surface attachment. It has been showed that Type 1 and Type 3 fimbriae on *Klebsiella pneumoniae* strain surface are the main factors facilitating adherence and the formation of a full-grown biofilm on abiotic surfaces [107,108]. Moreover, fimbriae have a critical role in *P. aeruginosa* adhesion to SS, polystyrene and Polyvinyl chloride (PVC) [109]. Type 1 fimbriae of *E. coli* facilitate attachment on abiotic surfaces and promote biofilm formation. In fact, it has been reported that the expression level of type 1 fimbriae had a direct effect on *E. coli* adhesion to surfaces [110]. Furthermore, it has been reported that the presence of type I pili is essential for the initial attachment of *E. coli* to PVC [111]. In fact, cells carrying lesions in genes encoding for the regulation or biogenesis of type I pili did not attach [111].

Extracellular polymeric substances (EPS)

The main composition of bacterial EPS includes polysaccharides, proteins, nucleic acids, lipids and phospholipids [112]. The lipopolysaccharide (LPS) outer layer of Gram-negative bacteria affects the bacterium's susceptibility to disinfectants and influences the biofilm formation [113]. The *pel* genes encode proteins with similarity to components involved in *P. aeruginosa*'s polysaccharide biogenesis. The *pel* gene cluster is conserved in other Gram-negative bacteria and was previously identified in the *P. aeruginosa* PA14 strain as required for the production of a glucose-rich matrix material involved in the formation of a thick pellicle and resistant biofilm. Indeed, mutation in *pel* genes may lead to an adherence defect [114]. For *E. coli*, truncation of LPS affects the biosynthesis of Type 1 fimbriae and flagella resulting in a reduced adherence [115]. Alterations in the peptidoglycan structure exposed at the surface of *L. monocytogenes* can also have an effect on attachment [116]. Many bacteria produce EPSs which are an important constituent of

the biofilm extracellular matrix. Overproduction of EPS can even inhibit initial attachment of *E. coli* O157:H7 to SS [117]. Several studies targeting the cell-surface proteins have revealed the existence of a large group of cell-surface protein called biofilm-associated proteins (Bap) on *S. aureus*. Recently, BapA was reported as necessary for biofilm formation by *Salmonella Enteritidis* [118]. Moreover, in *Salmonella* biofilms, cellulose is the main matrix EPS and represents the second component of EPS after the curli fimbriae. Cellulose is a β-1→4-D-glucose polymer which is biosynthesized by the *bcsABZC-bcsEFG* genes (bacterial cellulose synthesis) [119], two operons that are involved in cellulose biosynthesis in both *Salmonella Enteritidis* and *Salmonella Typhimurium* respectively [120,121]. Colanic acid, another EPS belonging to capsular extracellular polysaccharide, is essential for *Salmonella Typhimurium* biofilm. The importance of colanic acid in the biofilm formation capacity of *Salmonella* strains unable to produce either curli fimbriae or cellulose have also been confirmed [122].

Role of bacterial surface hydrophobicity in bacterial adhesion

In addition to the influence of the type of molecules expressed on the bacterial cell surface on the attachment to solid surfaces, there is a correlation between bacterial surface hydrophobicity and adhesion. In general, bacteria behave as hydrophobic particles. However, the degree of hydrophobicity depends on many parameters such as the pH, the ionic strength of growth medium and the bacterial species [123]. It has been reported that *S. epidermidis* strains with higher surface hydrophobicity adhered more than the ones with less surface hydrophobicity to polyethylene [124]. Hydrophobicity of bacteria can be evaluated by contact angle measurements, such as the sessile drop method or by their ability to adhere to hexadecane [125].

Role of bacterial surface charge in bacterial adhesion

The surface charge of bacteria is another important physical factor for their adhesion [126]. Depending on their surface groups' ionization, bacteria acquire a surface electric charge in aqueous suspension. In fact, bacteria have a net negative surface charge. The surface charge of bacteria varies according to bacterial species and is influenced by the growth medium, the pH and the ionic strength of the suspending buffer, bacterial age and bacterial surface structure [123]. The surface charge is usually characterized by the electrophoretic mobility (zeta potential) [125]. However, the contribution of bacterial surface charge to bacterial adhesion has not been clearly understood. The adhesiveness of *S. epidermidis* correlates directly with surface electro-negativity and

hydrophobicity while the adhesion of *E. coli* is inversely proportional to the degree of negative surface charge but is not influenced by hydrophobicity [105].

Role of bacterial membrane potential in bacterial adhesion

Bacterial membrane potential is a physical characteristic that plays a dominant role in the adhesion of microorganisms to abiotic surfaces. Surface potential mapping using Kelvin probe force microscopy showed that the bacterial membrane potential is not the same on different material substrates [127]. The changes in bacterial membrane potential have been considered a direct result of changes in cellular metabolism and motility [127]. Adhesion has been shown to depend mainly on the pH, ionic strength of the suspending solution and of material surface properties. Some studies had also established that the membrane potential plays an important role in the bacterial adhesion on surfaces too [128].

Role of the physiochemical characteristics of the abiotic surface in biofilm formation

The main factors influencing bacteria adherence to abiotic surfaces include the physiochemical properties such as surface energy and hydrophobicity, chemical composition of the solid surface and surface roughness [126].

Chemical composition of the solid surface

Bacterial adhesion to surface and biofilm formation depend on the solid surface chemistry. Surfaces can have different functional groups that influence the bacterial attachment which depends also on the hydrophobicity and charge of material [123]. *S. aureus* was found to adhere preferentially to metals and *S. epidermis* to polymers [129]. This result may explain why *S. epidermidis* often causes polymer implant infection while *S. aureus* is often the major pathogen in metal implant infections. The surface chemistry might be modified with different types of coating. The most current is plasma coatings that considerably reduce bacterial adhesion to surfaces [130]. Different studies have shown that the hydrophilicity of the native PVC was altered after thiocyanation of PVC surface, resulting in the decrease of bacterial adhesion to this material [131]. It has been reported that nisin-coated surfaces also inhibited the bacterial adhesion [132].

Surface topography and roughness

The relationship between bacterial adhesion and the surface topography was studied intermittently for 45 years [133]. Thus different opinions on the effect of the surface roughness on bacterial adhesion and biofilm formation have emerged. The food hygienic quality is closely related to the cleanability degree of equipment used in the production lines. The roughness of SS is considered a primary factor in the attachment of bacteria and biofilm formation [134]. The influence of material roughness on the bacterial adhesion has been investigated closely. Many studies focusing on the topography of different types of surfaces have found that the irregularities of abiotic surfaces enhance bacterial adhesion and biofilm formation whereas the smooth surfaces decrease the ability of bacterial adhesion [135]. In fact, rough surfaces have a greater surface area and provide for bacteria protective shelter against cleaning agents and more favorable sites for colonization (Figure 4) [76].

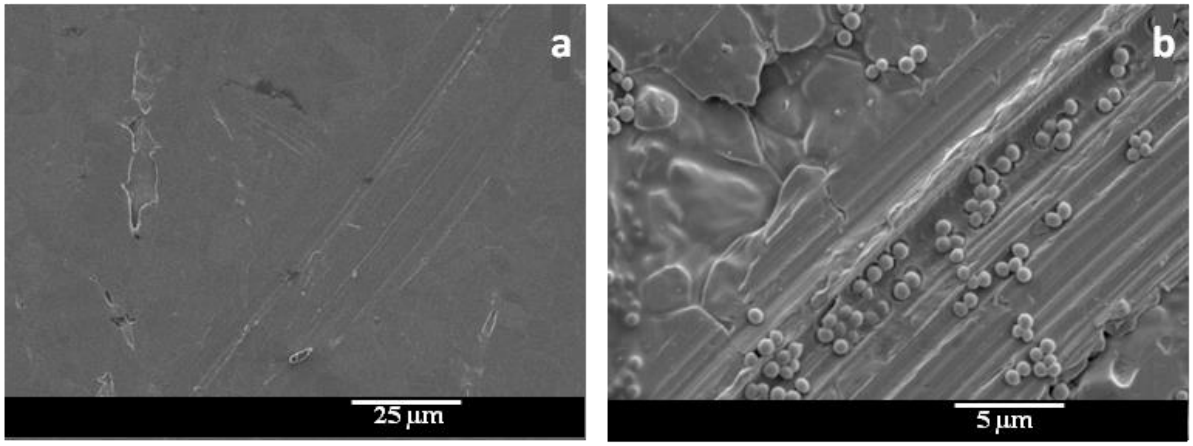

Figure 4: Scanning electron microscopy image of *Staphylococcus aureus* CIP 4.83 adhesion on 316L stainless steel. Stainless steel surface before (a) and after (b) the bacterial adhesion. Bacteria attach to the crevices and align often along longitudinal scratches. The scale bars in the images are 25 µm (a) and 5 µm (b).

Moreover, porosity of materials has a significant effect on the bacterial attachment. It has been found that implant site infection rates are different between porous and dense materials with porous materials having a much higher rate. This shows that bacteria adhere and colonize the porous surface preferentially. Indeed, bacteria adhere more to porous and grooved surfaces compared to dense and flat ones because of their larger contact surface [135].

Surface energy and hydrophobicity degree

The physicochemical properties of abiotic surfaces in food processing industry are suspected to significantly influence the biofilm formation mainly via the initial attachment of bacteria. In fact, the attachment of the bacteria depends on the critical surface tension of the solid surface [136]. The surface energy of a solid surface is a direct indicator for interfacial attractive forces. The modification of the surface energy of surfaces has a direct influence on the bacterial adhesion [137]. It has been reported that the adhesion of *S. xylosus* depends on the physicochemical properties of the surface and ionic strength of the surrounding medium [138]. It has been defined that hydrophobic interactions are the strongest of all non-covalent interactions in biological systems [139]. Physicochemical forces involved in adhesion are dependent of each other. The relationships between surface hydrophobicity and charge have been observed. A decrease in surface charge is often accompanied by an increase in hydrophobicity [56]. Surface hydrophobicity has been considered a determinant factor for microbial cell adhesion [140]. The concept of hydrophobicity opposes that of surface wettability since hydrophobic surfaces present low wetting. Furthermore, hydrophilic surfaces generally allow greater bacterial attachment and biofilm formation than hydrophobic ones [141]. Indeed, it has been found that initial attachment of *L. monocytogenes* Scott A to SS was more rapid than to rubber [142]. Moreover, several studies have investigated the relationship between the hydrophobicity degree and the bacterial adhesion rate. The relationship between the hydrophobicity degree of different abiotic surfaces and the number of attached *S. epidermidis* and Alcaligenes *denitrificans* cells have been assessed and results showed that the adhesion rates increased with the surface hydrophobicity [143]. In the same context, Sheng et al. (2008) [144] have reported that bacterial adhesion is lower on metal surfaces with reduced hydrophobicity.

Environmental conditions influencing bacterial adhesion

The physicochemical properties of both cell and material surfaces are very critical proprieties affecting the adhesion of bacteria and the formation of biofilm [145]. Moreover, bacterial adhesion is an extremely complicated process that is affected by many other factors including the environmental conditions (pH, temperature, bacterial concentration, nutrient availability and the associated flow conditions) that need to be controlled in order to find strategies against biofilm formation [68]. The number of attached bacteria is significantly affected by the flow conditions

and generally the number of attached bacteria decreases when shears rates are high. Moreover, variations in pH value in the culture environment also influence bacterial adhesion and the growth of biofilm [146]. The pH influences the cell surface hydrophobicity and better adhesion to hydrophobic surfaces was found at pH in the range of the isoelectric point when bacteria are uncharged [147]. Therefore, pH influences bacterial adhesion by influencing the surface charge and changing surface characteristics of the bacteria [55]. Moreover, variations in external pH can disturb the trans-membrane electrochemical gradient and have a biocidal effect on the microorganisms. The growth temperature is also an important condition for bacterial adhesion and biofilm formation as well as the presence of nutrient [1,148]. High growth temperature was found to increase the biomass and the attachment ability of bacteria probably, due to the production of heat stress proteins associated with the cell surface [149,150]. Otherwise, different studies concerning *S. aureus* biofilm formation have shown that temperature variation has no clear effect on the biomass [151]. Thus, optimum temperature enhances the biofilm formation. Temperature also affects the bacterial surface polymer composition which decreases at low temperature and reduces the adhesive properties of bacteria [152]. Another important factor in biofilm formation is nutrient availability. In fact, nutrients influence the surface charge of bacteria. For instance, glucose and lactic acid in the growth medium decreased the bacterial cell wall electro-negativity through the neutralization of the surface charge [153]. Thus, a synergistic effect between the environmental factors may occur and affect biofilm formation.

Strategies to control biofilm formation and development

Virulence of microorganisms is often enhanced when embedded into biofilm [154]. Unfortunately, in the industrial fields, the availability of nutrient and water promotes the biofilm formation. In this regard, several strategies have been proposed to control biofilm formation and to avoid biofouling. Ideally, preventing biofilm formation would be a more logical option than treating it once established. Thus two major ways to control biofilm formation can be adopted. The first one is based on the use of antimicrobial agents, physical forces, enzymes, plant extracts, etc. to eradicate or disrupt already formed biofilms. The second strategy aims to anticipate and prevent bacterial adhesion and therefore biofilm formation by modifying the physiochemical properties of abiotic surfaces.

Eradication of biofilms

Cleaning and disinfecting of abiotic surfaces

In food processing industry, effective cleaning and disinfecting of equipment and surfaces is required to reduce the bacterial contamination and produce safe products with acceptable shelf life and quality [155]. Cleaning is the first step of sanitizing intended to reduce the number of pathogenic bacteria on surfaces before disinfecting [155]. An efficient cleaning and disinfection procedure consists of a sequence of rinses using good quality water with application of detergents and disinfectants [155]. Cleaning frequency must be clearly defined for each process line (daily, after production runs, or more often, if necessary). Cleaning is an important step to minimize microbial colonization of industrial food processing equipment. It seems to be of great importance to eliminate as many micro-organisms as possible before applying a disinfectant [156]. In food and health sectors, disinfectants are used for decontamination and to reduce the surface population of viable cells left after cleaning in order to prevent microbial growth and biofilm formation on surfaces [112]. There are different kinds of commercialized disinfectants such as alcohol-based one, hypochloric solutions, aldehydes, hydrogen peroxide, ozone and quaternary ammonium compounds (QACs) [1]. These disinfectants can be used in different sectors at different concentrations [157]. The particularity of these antimicrobial agents is that they have more than one target site. In fact, they can target the cytoplasmic constituents, the outer cell components and the cell cytoplasmic membrane [158]. The activity and the efficiency of disinfectants against biofilms depend on several chemical and physical factors such as concentration, pH, temperature and contact duration. Moreover, the surface type may also affect the efficacy of biocides against biofilms [1]. The involvement of surface type is mostly related to the nano-scale surface morphology which affects the biofilm architecture and weakens the effectiveness of cleaning and sanitizing procedures [159].

The biofilm resistance to disinfectants

Micro-organisms are generally adhered to surfaces under a biofilm state. Disinfectants are often used at very high concentrations relative to their minimal inhibitory concentrations in order to make it impossible for bacteria to overcome the massive damage and develop resistance [160]. Many studies have shown that bacteria exposed to disinfectant levels lower than those required to deliver a lethal insult might develop resistance. In fact, the cells living under a biofilm state can be up to

1000 fold more resistant to disinfectant agents than their planktonic counterparts [1]. Thus, the disinfectant agents are frequently inefficient in the eradication of biofilms and increase the risk of severe health problems and economic losses. In fact, there are many strategies evolved by biofilm cells to achieve or increase their resistance: (1) Diffusion limitations of disinfectants in biofilms, (2) The phenotypic adaptations of biofilm cells to sub-lethal concentrations of disinfectants and (3) presence of disinfectant-adapted and persister cells.

Diffusion limitations of disinfectants in biofilms

A mature biofilm is characterized by the production of an extracellular matrix composed of exopolysaccharides (EPS), proteins and lipids [161,162]. The multiple layers of cells and EPS may constitute a complex and compact structure which prevents disinfectants from penetrating and reaching the internal layers, thus hampering their efficacy. It has been shown, that the disinfectant's diffusion and reaction limitations are involved in the biofilm resistance [163]. In fact, several studies have found that the restricted diffusion of disinfectant molecules was related either to the size exclusion or the electrostatic interactions. The interactions between antimicrobials and biofilm components seem more likely to explain the limitations of penetration into the biofilm [164]. Moreover, the electrostatic interactions of the biofilm matrix seem to have an important role in the resistance to biocides [165].

The phenotypic adaptations of biofilm cells

Different studies have illustrated the role of extracellular matrix in the resistance of biofilms. Nevertheless, other investigations have shown that despite an effective penetration of disinfectants into biofilm, only a low level of resistance was achieved [166]. Thus, other mechanisms based on the phenotypic adaptation such as reduced growth rate and metabolism [167,168], membrane permeability/fluidity [169], phenotypic adaptation and gene expression [170,171], could be involved in the resistance of biofilms to biocide agents.

Presence of disinfectant-adapted and persister cells

Food and medical environments constitute a reservoir of bacteria which have developed tolerance to disinfectants misused at lower concentrations than that recommended by the manufacturer [172]. Moreover, bacteria may develop cross-resistance to different disinfectants [173]. The involvement of a subpopulation of persister cells in the biofilm may account for the observed resistance to

biocides. Persisters are highly tolerant to disinfectants and may have adopted a highly protected, perhaps spore-like, state [174].

Treatment with plant extracts

The use of bio-based antimicrobial agents can be an effective alternative for the control of biofilm formation. One approach may be the use of plant essential oils (EO) which have been used since many centuries to fight against different pathogens including bacteria, fungi and viruses [175]. The cumin seed EO was found to reduce the *K. pneumonia* biofilm formation. Fadli et al. (2012) [176] have demonstrated the synergistic effect of ciprofloxacin and EOs of endemic Moroccan thyme species, on antibiotic-resistant bacteria involved in nosocomial infections. Essential oils may damage the cell wall and membrane, leading to cell lysis and leakage of cell contents [177]. In addition to their high ability to kill bacteria, essential oils do not promote the acquisition of resistance unlike antibiotic and chemical disinfectant [178]. It has been shown that selected antimicrobial essential oils can eradicate bacteria within biofilms with higher efficiency than certain important antibiotics, making them interesting candidates for the treatment of biofilms [179]. Moreover, other plant extracts seem to have highly effective anti-biofilm activity [180] and represent promising strategies to overcome resistant biofilm formation.

Mechanical and enzymatic treatments

Chemical based agents used for disinfection possess several disadvantages such as their toxicity, generation of chemical wastes, reaction with materials and promotion of the bacterial resistance. In order to overcome these disadvantages, new approaches including applying mechanical forces and enzyme have been proposed. Mechanical cleaning of surfaces is probably the simplest and most successful way to remove biofilms and maintain surfaces clean [7,181]. The newer physical methods used for the control of biofilms include super-high magnetic fields [182] and ultrasound treatment [183]. Enzymes can be used to effectively eradicate biofilms in the food industry. Several studies have demonstrated that DNase1 reduced biofilm biomass by approximately 40% among all tested Gram-positive (*S. aureus* and *Streptococcus pyogenes*) and Gram-negative bacteria (*Acinetobacter baumannii*, *Haemophilus influenza*, *K. pneumoniae*, *E. coli*, and *P. aeruginosa*) [184,185]. Lysostaphin (LS) is a naturally occurring enzyme that can effectively penetrate into biofilms [186]. The LS was found to be capable of eradicating biofilms of all *S. aureus* and *S. epidermidis* strains [187]. Different enzymes, such as, protease, α-amylase, b-glucanase, and

endoglycosidase have been reported to be effective in the removal of biofilm of different pathogens [182]. Nevertheless, a combination of different enzymes and antimicrobials/disinfectants is a promising, highly effective method for removing and controlling biofilms.

Bacteriophage treatments

Bacteriophages treatment is a nowadays major strategy of the biofilm control and removal. Bacteriophages are naturally occurring viruses that infect bacteria within biofilms [188]. Phages have been used for the treatment of human infectious diseases [189]. The use of phages to control biofilms has potential for several reasons. Phages can replicate at the site of an infection. During the lytic replication cycle, the infection of a bacterial host cell by a single phage virion will result in the production of other progeny phage, depending on the particular phage and host strains. Some engineered enzymatic bacteriophage produce enzymes that degrade the biofilm EPS matrix which represents promising tool of biofilm control [190]. Moreover, biofilm removal by enzymatic bacteriophage has been found to be more efficient than the classical enzymatic treatment [191]. It has been reported that a combined use of the bacteriophage K and a novel DRA88 bacteriophage has showed successful effect in reducing the *S. aureus* biofilm formation [192]. The phage mixture may form the basis of an effective treatment for infections caused by *S. aureus* biofilms. Similarly, lytic bacteriophages were found to be efficient in the prevention and eradication of biofilms of different pathogenic bacteria [193].

Prevention of biofilm formation by the modification of abiotic surfaces properties

In food industry, all surfaces are subjected to bacterial contamination since exposed to air, humidity or diverse environmental conditions. To overcome these problems, several strategies involving the modification of surface physicochemical properties have been used in order to set up antimicrobial surfaces which reduce bacterial adhesion and prevent biofilm formation.

Bactericidal/Bacteriostatic coating

Modifying the surface properties of food contact surface or indwelling medical devices is one of the main focuses to prevent or decrease bacterial colonization and biofilm-related infections. Coating the material surface with bactericidal/bacteriostatic substances seems to be an innovative approach to make surfaces resistant to bacterial adhesion and biofilm formation. It has been shown that *S. epidermidis* biofilm formation was significantly inhibited on titanium implant surfaces

coated covalently with vancomycin [194]. However, the use of antibiotics can lead to antibiotic resistance and even induce biofilm formation [195]. The effectiveness of a nisin coating onto low-density polyethylene in reducing the population of *L. innocua, L. monocytogenes, B. cereus* and *S. aureus* has been demonstrated in a recent study [132]. Moreover, implant surfaces such as titanium have acquired antibacterial properties after being coated with hydroxyapatite [196]. Silver particles, well-known as one of the strongest bactericidal agents, were also used as an anti-biofilm coating on polymer and metal surfaces [197]. It has been demonstrated that biofilm formation by a number of pathogens on silver nanoparticle coated catheters was almost completely prevented [198]. It has been reported, also, that silver-based coatings are widely used in medical implants due to the bactericidal effect of the released silver ions from the surface, against both Gram-positive and Gram-negative bacteria [199,200]. Surfaces possessing chemically bonded hydrophobic QACs have shown bactericidal properties [201]. Glass surfaces were coated with QACs functionalized silica nanoparticles and exhibited inhibition of growth and accumulation of Gram-negative and Gram-positive bacteria [202]. QACs coated surfaces have been shown to damage bacteria by the disruption of their cellular membranes [201]. The perturbation in the lipid bilayers occurs when the positively charged nitrogen in the ammonium group interacts with the negatively charged groups of acidic phospholipids in the bacterial cellular membrane [203]. This causes the perturbation of low-molecular-mass solutes efflux. In fact, under the action of QACs, bacterial cells release their potassium ion, which in turn causes the cell to lose its ability to undergo osmoregulation and other physiological functions, resulting in the cell death [204]. Unlike silver ions which have a release-based antibacterial mechanism [205], QACs coatings possess a long-lasting contact-based antibacterial mechanism [206]. Despite these properties, it has been reported that bacteria are able to develop resistance against these modified surfaces [207]. To improve the antibacterial effect of coated surfaces, many studies have investigated combinatorial approaches of different antibacterial molecules. The combined release of silver and the contact-killing abilities of QACs have shown a synergistic antibacterial effect [208]. Nitric oxide loaded nanoparticles have also been reported to be bactericidal [209]. It has been suggested that the antibacterial effect of nanoparticles arises from their physiochemical properties. In fact, due to their nano-metric size, these particles are capable to carry the antimicrobial molecules and accumulate near the cytoplasm, which kills the cells. Moreover, some of these nanoparticles might possess oxidizing power by generating reactive oxygen species [210,211].

Immobilization of bioactive compounds

Bioactive molecules can be attached onto polymers in two different ways. The choice of the antimicrobial agent immobilization technique depends on the expected behaviour of the modified surface. Indeed, for setting up active antimicrobial surfaces, the immobilization may be done either chemically in a covalent manner or physically by a simple adsorption [15]. In case of chemical immobilization, the antimicrobial agent is strongly fixed onto the surface providing a long-term action and does not migrate from material surface to the food such as modified polymers used in food transformation platforms. When the bioactive molecules are immobilized by adsorption the antimicrobial effect is achieved with migration. Thus, such antimicrobial surfaces may be intended for biomedical applications and not only for food sector [15].

Non-covalent adsorption is mainly governed by hydrogen bonding, van der Waals forces, electrostatic and hydrophobic interactions between the antimicrobials and the polymer surfaces [15]. Non-covalent methods provide short-term applications because antimicrobials are released from the polymer. The factors affecting bioactive molecules adsorption on surfaces depend on the surface physiochemical properties, the characteristics of the bioactive molecule itself and the environmental factors [15]. However, covalent immobilization provides the most stable linkage between the antimicrobial molecule and the functionalized polymer surface that usually requires the use of cross-linkers or "spacer" molecules that link the functionalized polymer surface to the bioactive agent [15]. In fact, covalent binding may alter the conformational structure and the active site of the bioactive molecules such as enzymes and thus, may affect their activity. This lack of activity can disturb the effectiveness of the modified surfaces. Thus, the parameters affecting the antimicrobial performance of immobilized bioactive agents (concentration of bound antimicrobials, spacer choice, length and flexibility) need to be controlled. Spacers or cross-linkers are hydrophilic molecules used for attaching bioactive compounds, such as, enzymes which may lose activity when linked directly to a solid surface because of steric constraints. For example, Poly Ethylene Glycol (PEG) is often used to cross-link enzymes to substrates. Indeed, PEG may shield the enzymes from denaturation and maintains their bioactivity by keeping their active site in the appropriate conformation [212]. PEG was used to tether trypsin and lysozyme onto SS in order to prevent biofilm formation [213]. In another study, the anticoagulation properties of immobilized heparin were improved by using a PEG spacer when compared to heparin immobilized directly to the polymer surface. Using PEG seems to be an interesting process to increases the bio-specificity

of tethered bioactive compounds [214]. Furthermore, using poly-functional reagent allows increasing the number of reactive sites available on a surface for immobilization of bioactive compounds [214]. The major drawback in utilizing a highly poly-functional agent tether is overcrowding of the functional groups which may reduce the immobilization of bioactive compounds, or that bioactive compounds are sterically hindered [214].

Initial surface modification and anti-biofouling effect of antibacterial surfaces

The anti-biofouling surface could also be achieved by depositing a thin layer of anti-adhesion coating on the surface to reduce attachment of pathogenic bacteria. The physicochemical properties of the surface have a direct effect on the ability of microorganisms to adhere to abiotic surfaces. Thus, it is believed that the surface chemistry and/or surface architecture and topography of the surface control their anti-biofouling behaviour [215–217]. The sub-nanometre and nanometre roughness scales of metallic surface have shown differential anti-biofouling properties against bacteria. It has been reported that *P. aeruginosa* cells are unable to trigger their attachment on such surfaces. Recently, it has been shown that slippery liquid-infused porous surfaces prevented 99.6% of *P. aeruginosa*, 97.2% of *S. aureus*, and 96% of *E. coli* biofilm attachment over a seven days period under both static and physiologically realistic flow conditions [218]. Surface properties of materials or medical devices including chemical composition and reactivity, hydrophilicity/hydrophobicity [219], roughness [220] and charge can be modified by introducing a variety of coating, or surface modification to setup the desired anti-biofouling characteristics without altering the bulk properties of materials. The surfaces of SS and titanium have been coated using TriMethylSilane (TMS) plasma nano-coatings based on low-temperature plasma technology [130]. These TMS plasma coated materials have significantly reduced the *S. epidermidis* adhesion and biofilm formation. In fact, the decreased bacterial adhesion to the coated surfaces can be associated to the decreasing protein adsorption after surface properties modification. Anti-biofouling coatings prevent biofilm formation at early stages which should be more desirable in food and medical settings. However, it is necessary to understand the mechanism by which adhesion is hindered to improve the efficiency of the coatings. Moreover, different techniques can be used to modify the surface properties, depending on the material application.

Applications

The different processes and techniques discussed above have been investigated with the goal of developing specific applications of bound bioactive molecules to surfaces within a wide range of scientific disciplines. Several applications in different sectors are cited below.

Food industry and other field application

In food processing industry, antimicrobial polymers such as active packaging can be used to improve food safety [221]. Immobilized lysozyme, glucose oxidase, and chitosan have been applied to set up antimicrobial packaging films. These packaging technologies could play a role in extending shelf-life of foods and reduce the risk of growth of pathogenic microorganisms by direct contact of the package with the food product [222]. Several compounds have been proposed and tested for antimicrobial activity in food packaging including organic acids, antibacterial peptides and fungicides [132,223–225]. In addition, antimicrobial food-contact surfaces include cutting boards and dishcloths which contain triclosan are found to reduce effectively the bacterial contamination [225]. It is important to optimize, rather than simply maximize, the density of the surface immobilized bioactive compound. In the case of enzyme immobilization, too many surface functional groups can lead to overcrowding attachment of the enzyme, which result in reduced overall bioactivity after denaturation [226]. Moreover, it is necessary to exercise responsibility in using bioactive compounds in order to set up antibacterial surfaces. Indeed, several studies have shown the impact of antimicrobial agents in promoting development of resistant strains [227,228]. When surface modification strategies are applied to obtain antibacterial food processing surfaces, they can help reduce biofouling and cross-contamination. Fouling of process equipment in the dairy industry is one of the main issues to be solved. Despite, the corrosion resistance of SS, still today, when exposed to chloride solutions, localized corrosion can appear [229]. Many strategies have been taken in consideration to bend the corrosion of metallic material [230]. The effectiveness of coating SS with anticorrosion undercoat paint was investigated in several studies [231].

Biomedical application

Modified abiotic surfaces expected to be used inside or in contact with human body have to meet the demands required for both their surface and bulk properties. For the medical purpose, modified materials are not recommended if the substances will leach out causing cytotoxicity [232]. Metal

ions release from metallic materials implanted into human body may cause various health problems such as metal accumulation in organs, allergy, and carcinoma [233–235]. The most important property that a modified abiotic surface must involve is biocompatibility. The biocompatibility of antibacterial QACs that are commonly used as disinfectants in hand solutions, cosmetics, and environmental treatment plants have been recently reviewed [236]. Biocompatibility can be divided into two kinds. One concerns the bulk property of the biomaterial, the other its surface property. The bulk biocompatibility is critical for the implantation of biomaterials. In fact, the rigidity of modified implants must match with that of the adjacent tissue, otherwise, hyperplasia or absorption of the tissue will prevail, resulting in failure of implantation. The second kind includes interfacial biocompatibility between the biomaterial and the living adjacent tissue which may induce rejection reactions towards the foreign-body. Biomaterial surface can be modified to influence the interactions between the material and the biological environments. For example, general biocompatibility can be imparted by immobilizing a hydrophilic polymer such as PEG to reduce protein adhesion since the pathway leading to blood coagulation begins with surface protein adhesion [237]. Several studies have mentioned different applications of a variety of modified biomedical devices [238,239].

Appropriate controls

When polymer surfaces are modified or grafted with bioactive compound, it is important to include appropriate controls. Surface functionalization is a multi-step process during which surface properties are often modified. It is not only important to compare bioactivity of the modified polymer, but also to evaluate bioactivity of the surface modified polymer to which the bioactive compound has not yet been attached. By this control, one can identify whether the change in bioactivity is due to the presence of the bioactive compound or simply a change in polymer surface chemistry. In some applications, the bond between the bioactive compound and the polymer surface must be covalent. Alternatively, in applications where a covalent linkage is necessary, comparing the quantity of biomolecule bound to unmodified polymer surface, functionalized polymer surface with or without the use of cross-linker may add value to the drawn conclusions as well as the potential commercial applicability. The design of materials intended to be in contact with food must comply with rules of food compatibility. Food-contact materials are intended to come in contact with food. Thus, there is the possibility of the chemical substances migrating from the material to the food, which could be potentially harmful to human health [240]. Indeed,

regulation must involve the antimicrobial substances in food packaging or modified food transforming devices, since, they are considered food additives if they migrate to food [241]. In response to this issue, many countries have implemented food contact regulations to ensure food safety [242,243]. Therefore, packagings and materials intended to come into direct contact with food are highly regulated around the world and must comply with several requirements that have been laid down at the European level. Within that context, regulation (EU) No 10/2011 on plastic materials and articles intended to come into contact with food was published on the 14th of January 2011 [244]. In the US, antimicrobials in food packaging that may migrate to food are considered food additives and must meet the food additive standards. Packaging forms include bulk food storage containers, paperboard cartons, plastic or paper food wraps, jars and bottles [222]. Examples of antimicrobial uses include surface sanitizing solutions for milk containers, hydrogen peroxide uses in aseptic packaging, and antimicrobials impregnated into food packaging to protect either the package, or to extend the shelf-life of the food. It is possible that compounds that are not approved food additives could be transformed into approved additives during the migratory process. In food processing industries, it is also of great importance that these materials be easy to clean in order to limit their contamination with pathogenic bacteria. In fact, in food industries, particularly the open working surfaces, the environment is propitious for contamination by microorganisms. Thus, the choice of materials excepted to be in contact with food is crucial. These materials have to withstand the potentially harsh environmental conditions such as high pressure, high concentrations of alkalis and acids, high temperatures, while remaining cleanable. Moreover, these materials must have qualities such as: corrosion resistance, non-toxicity, mechanical stability. Further considerations must concern the cost of such process to set up antimicrobial surfaces which may be susceptible to be expensive. Thus, this may have impact on their commercialization. The approval of surface modified medical devices by regulatory agencies like the International Organization for Standardization (ISO) requires that biocompatibility assessment be conducted to assure safety of the device or material. The primary guidance for the US, EU and Japan and associated countries has formally become the ISO 10993 standards, with each having reference to their own regulations only in special cases. The concern with devices and biomaterials is what migrates from the material into the body. It should be noted that there is ISO guidance (ISO 10993) for medical device risk assessment by the identification and quantification of chemical substances that can be extracted from a device over a period of time after the device would be prolonged (or

introduced) into internal patient contact. The potential biological risks to patients must be assessed and allowable limits of exposure established. Thus, modified medical material must comply with the tests mentioned in ISO 10993 standards [245]. ISO 10993 concerns the following points, under the general title: biological evaluation of medical devices [245]:

- Tests for cytotoxicity, genotoxicity, sensitization, carcinogenicity and reproductive toxicity.
- Tests for interactions with blood.
- Tests for local effects after implantation.
- Identification and quantification of potential degradation of medical material (polymers, ceramics, metals and alloys).
- Toxicokinetic study design for degradation products and leachables in order to establish allowable limits for leachable substances.

Conclusion

Biofilm formation is one of the main concerns that demand to elaborate effective strategies for their prevention or eradication in the food and medical sectors. In both sectors, several factors may enhance the bacterial colonization and biofilm formation on food contact surfaces and medical devices. Therefore, it is of great importance to understand the mechanisms of bacterial adhesion to these surfaces in order to reduce the surface contamination. Furthermore, biofilm is an adaptive form of bacterial cells to hostile environments which allow developing high resistance to disinfection treatments. Thus, it is of interest to understand the relationship between the environmental conditions of biofilm formation such as temperature, surface type, and biofilm age, and the biofilm resistance, in order to control the issues related to biofilms and improve the anti-biofilm treatments.

Furthermore, antibacterial surface development is nowadays an expending research field. This review gives an overview of the current approaches that aim to design antibacterial surfaces for food and medical applications. Antibacterial surfaces are expected to provide two distinct performances. Either they are capable of repelling bacterial cells, preventing their attachment and the initialization of biofilm formation or they inactivate/kill cells that do come into contact with them. Several antibacterial agents have been used to obtain antibacterial surfaces. Therefore, their mechanisms of action must be understood beforehand. Moreover, the durability, specificity and the procedure of the modification needs to be thoroughly evaluated in order to minimize costs. Since,

these bactericidal mechanisms rely on the surface structure modification, they may help reduce the chemical wastes generated by the traditional, chemical-based approaches.

In the same context, as the old adage goes "an ounce of prevention is worth a pound of cure", it is wiser to act at the source of the problem by hindering bacterial adhesion to abiotic surfaces instead of lately fighting already established biofilm. In fact, more consideration should be given to the design of anti-biofouling surfaces by focusing on the impact of the surface topography, charge and hydrophobicity on the initial adhesion of bacteria. Biocompatibility is an important point to take into consideration when we deal with materials that are susceptible to be in direct contact with human body or food. Thus, it is necessary to investigate the toxicological effect of the antibacterial surface employed in both health and food sectors.

Acknowledgments

This work was carried out within the framework of an ALIBIOTECH (Agroalimentaire et Biotechnologie, Nord Pas-de-Calais region) program. The authors also thank the Haut de France region and FEDER (Fonds européen de développement régional) for their financial support.

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CHAPTER: II

ARTICLE II AND III – RESULTS

Introduction

Despite the advances in the cleaning and disinfection procedures in both food and medical sectors, pathogenic bacteria are still involved in foodborne and nosocomial infections (EFSA 2009; Klevens et al. 2007). The most frequent contaminant sources in food and medical sectors are equipment, food handlers, raw materials, healthcare workers and processing water (Donlan and Costerton 2002; Todd et al. 2009). Furthermore, the persistence of pathogens in these sectors result in the biofilm formation on food and medical equipment. The pathogens living under biofilm state are known to be more tolerant to the antimicrobial treatments than planktonic cells. This high tolerance to antimicrobial often leads to the failure of disinfection procedures and to the spread of contaminations.

Staphylococcus aureus is a versatile pathogen which may cause serious human diseases (EFSA 2009; Rosenthal et al. 2012). *S. aureus* growing in foods may produce many virulence factors including the thermostable toxins that cause illness (Schelin et al. 2011). *P. aeruginosa* is an ubiquitous and an opportunist human pathogen, able to survive nutrient limitation and environmental stresses and therefore to persist in both food and hospital settings. Moreover, *P. aeruginosa* has been isolated from different medical devices such as the respiratory assistance devices and catheters (Rosenthal et al. 2012). To reduce the microbiological risk related to surface contamination by these pathogens, it's of interest to understand the mechanisms that control interactions, between both bacterial and abiotic surfaces, which govern the first step of the biofilm development (i.e. bacterial adhesion). Such study will help to improve strategies of biofilm control and to reduce issues associated with this structure. The adsorption of free floating bacteria to abiotic surfaces has been described as a reversible phase, which involves nonspecific interactions such as the electrostatic, hydrophobic and acid-base ones. However, under certain stress conditions, pathogens may detach from the biofilm and recolonize other areas, which leads to the spread and the persistence of contamination. Thus, there is a need to focus on the biofilm-detached bacteria grown under different environmental conditions, as the majority of studies deal with bacteria imbedded in biofilm. In fact, the environmental conditions commonly met in food and medical sectors may influence the surface properties of bacterial cells and therefore their adhesion behavior to abiotic surfaces (Cappello and Guglielmino 2006; Gordesli and Abu-Lail 2012).

In this context, the first part of this thesis was conducted on the study of the effect of growth conditions, on the surface physicochemical properties of *S. aureus* and *P. aeruginosa* biofilm-detached cells and on their ability to adhere to the stainless steel and the polycarbonate, two surfaces commonly encountered in the food and medical equipment. The biofilm-detached cells were also compared with their planktonic counterpart in order to check out whether bacteria recover their planktonic phenotype after detaching from biofilm or they maintain the sessile cell one. Moreover, our investigation highlighted the pathogenic potential of cells growing within biofilms and compared to that of their planktonic counterparts. The temperatures used for this study were 20, 30 and 37°C. In fact, 20°C represents the room temperature of several areas of food and medical sectors. The 30°C is also frequently met in food sectors, such as in smoked food and cheese processing. The 37°C represent the body temperature of food handlers, which are constantly in contact with food-contact-surfaces and food products, and healthcare providers.

Article II

Effect of incubation duration, growth temperature, and abiotic surface type on cell surface properties, adhesion and pathogenicity of biofilm-detached *Staphylococcus aureus* cells

Simon Oussama KHELISSA[a, b], Charafeddine JAMA[a], Marwan ABDALLAH[a], Rabah BOUKHERROUB[c], Christine FAILLE[b], Nour-Eddine CHIHIB[b]*

[a] CNRS, ENSCL, UMR 8207-UMET-PSI, Université de Lille 1, Avenue Dimitri Mendeleïev, CS 90108 59652 Villeneuve d'Ascq, France

[b] CNRS, INRA, UMR 8207-UMET-PIHM, Université de Lille1, 369 rue Jules Guesde, CS 20039, 59651 Villeneuve d'Ascq, France

[c] CNRS, IEMN, UMR 8520, Université de Lille1, Cité Scientifique, Avenue Poincaré, 59652 Villeneuve d'Ascq, France

* Corresponding author: Dr. Nour-Eddine CHIHIB.

Tel: +33 320435443

Email: nour-eddine.chihib@univ-lille1.fr

Abstract

The goal of this study was to investigate the effect of growth conditions such as the temperature (20, 30 and 37°C), incubation duration (24 and 48 h) and surface type (stainless steel and polycarbonate) on the cell surface physicochemical properties and adhesion to abiotic surfaces of biofilm-detached and planktonic *Staphylococcus aureus* cells. This study tested also the hypothesis that *S. aureus* planktonic cells exhibit distinct pathogenic properties compared with their sessile counterparts. The results showed that the changes of the growth conditions promoted changes in the zeta potential, hydrophobicity, electron donor/acceptor character of the studied cell populations. Biofilm-detached cells showed a greater adhesion to stainless steel and polycarbonate compared with planktonic cells. Compared with planktonic cells, sessile ones showed higher cytotoxic effect against HeLa cells, DNase activity, and siderophore levels. The higher cytotoxic effect and production of DNase and siderophore increased with the increase of temperature and duration of incubations. Based on the obtained data, the *S. aureus* biofilm-detached cells were found to be distinct in many physiological properties from their planktonic counterparts.

Keywords: *Staphylococcus aureus*, physiology, planktonic cells, biofilm-detached cells, surface properties, pathogenicity

Introduction

Staphylococcus aureus is an important Gram-positive human pathogen frequently associated with numerous forms of human infections (Harris et al. 2002; Khelissa et al. 2017; Valaperta et al. 2010). *S. aureus* represents the main cause of hospital acquired infections such as infections associated with indwelling medical devices and surgical wounds (Percival et al. 2015). The pathogenesis of such bacterium correlates with several virulence factors including hemotoxins, pore forming toxins, super antigens (e.g. toxic shock syndrome toxin-1, staphylococcal enterotoxin) and several secreted enzymes that result in tissue destruction and bacterial dissemination (Normanno et al. 2007). The ability of this bacterium to produce iron acquisition factors (siderophores), such as staphyloferrins A and B, staphylobactin and aureochelin, is also likely important to its pathogenesis (Dale et al. 2004; Oogai et al. 2011). Furthermore, the ability of *S. aureus* to form biofilms and colonize medical devices is regarded as an important virulence determinant in the pathogenesis of this bacterium.

Biofilm is a community of microorganisms attached to abiotic or biotic surfaces and embedded in a protective extracellular polymeric matrix (Donlan 2002). The biofilms are formed on abiotic surfaces through multiple steps, including the adhesion of planktonic cells, maturation, and dispersion of attached cells. Sessile *S. aureus* cells are particularly problematic and their physiology differ distinctly from that of planktonic ones. In fact, sessile cells are much more resistant to the host immune response, antibiotics, biocides and hydrodynamic shear force (Lewis 2001; Garrett et al. 2008). The bacterial adhesion to a surface constitutes the first and the essential step of the biofilm formation (Abdallah et al. 2014a). It has been reported that the physicochemical properties of bacterial and abiotic surfaces, such as the hydrophobicity, the electrostatic charge, and the electron donor/acceptor characters, play a key role in the bacterial attachment to abiotic surfaces (Abdallah et al. 2014a). However, another study has underlined that the physicochemical properties have only a minor role and the correlation between the surface properties and the bacterial adhesion were poor (Teixeira et al. 2008). The bacterial detachment is a main part of the biofilm life cycle (Wilson et al. 2004). The phenomenon is involved in the dissemination of infection and contamination in the healthcare and food settings (Nickel et al. 1994; Poulsen 1999). Moreover, Fux et al. (2004) reported that the mechanical biofilm detachment by flushing a colonized catheter provokes sepsis. The erosion of biofilm also results spontaneously, either in the detachment of single cells or clumps of thousands bacteria which contaminate and colonize other

surfaces. Thus it is of importance to understand *S. aureus* phenotype changes related to bacterial growth under planktonic and biofilm states. Such investigations might yield important information regarding the virulence and the pathogenicity required for certain acquired human infections.

The purpose of the current work is to investigate the impact of *S. aureus* growth conditions on the physicochemical properties of the biofilm-detached-and the planktonic-cells and on their ability to adhere to the stainless steel (SS) and to the polycarbonate (PC). The planktonic and the biofilm cells were recovered from cultures incubated at different growth temperatures and ages commonly encountered in the medical environments. This work also investigated the effect of these growth conditions on the expression of some virulence factors, involved in the pathogenesis of *S. aureus*, and the cytotoxicity against HeLa cells.

Materials and methods

Bacterial strain and culture conditions

The bacterial strain used in this study was *Staphylococcus aureus* CIP 4.83. The strain was stored at -80°C in Tryptic Soy Broth (TSB; Biokar Diagnostics, Pantin, France) containing 40% (v/v) of glycerol. Pre-cultures were done by inoculating 100 µl from frozen stock tubes into 5 ml of TSB and then incubated at 20, 30 or 37°C. The 30 and 37°C pre-cultures were incubated for 24 h, whereas that of 20 °C was incubated for 48 h. The main cultures were done in 500-ml sterile flasks containing 50 ml of TSB. The cultures of 20, 30 and 37°C were prepared by inoculating 10^4 CFU/ml from the 20, 30 and 37°C pre-culture tubes, respectively. The cultures were then incubated under shaking (160 rpm) at 20, 30 or 37°C. The cultures were stopped at the late exponential phase.

Coupons preparation

The SS (304L, Equinox, Willems, France) and PC (Plexilux, Vaux-le-Pénil, France) slides were soaked in ethanol 95° (Fluka, Sigma-Aldrich, Saint-Quentin-Fallavier, France) for an overnight and then rinsed twice with distilled water. Then the slides were soaked in 500 ml of DDM ECO detergent (1%) for 15 min at room temperature (20°C) under agitation (ANIOS, Villeneuve d'Ascq, France). Slides were thoroughly rinsed five times, for one minute under agitation, in 500 ml of distilled water and three times in ultrapure water (Milli-Q® Academic, Millipore, Guyancourt, France) at 20°C to eliminate detergent residues. SS slides were air-dried and sterilized by autoclaving at 121°C for 20 min. PC slides were sterilized in the ethanol 95° for 15 min.

Cell suspension preparation

Staphylococcus aureus cells, grown at 20, 30 and 37°C, were harvested by centrifuging cultures at 5000 g for 5 min at 20°C. Bacteria were washed twice with 20 ml of 100 mM Potassium Phosphate Buffer (PPB; pH 7) and finally resuspended in 20 ml of PPB. In order to disperse cells, bacterial suspensions were subjected to a sonication at 37 kHz for 5 min at 20°C (Elmasonic S60H, Elma®). The bacterial suspensions at 10^8 CFU/ml were then prepared by adjusting the optical density to $OD_{620\ nm} = 0.110 \pm 0.005$ using a Jenway 6320D UV/visible light spectrophotometer. Standardized cell suspensions (10^8 CFU/ml) were diluted tenfold for the biofilm formation and the bacterial adhesion assays (10^7 CFU/ml).

Biofilm formation assays

Sterile coupons (90 × 90 × 1 mm) were placed in the horizontal position in cell culture dishes (140 mm in diameter). The upper face of slides was covered by 12 ml of 20, 30 and 37 °C cell suspensions (10^7 CFU/ml) and incubated at 20°C for 1 h to allow bacterial adhesion. Thereafter, the 12 ml were removed and slides were gently rinsed twice with 12 ml of PPB to remove loosely attached cells. The upper face of slides was covered by 12 ml of TSB and the biofilm formation was started by incubating slides, at the same temperature of bacterial-cell-cultures (20, 30 or 37°C), for an incubation duration of 24 or 48 h. For the biofilm grown for 48 h, the culture medium was changed after 24 h of biofilm growth, except for DNase, cell cytotoxicity, and siderophore quantification assays where the culture medium was not changed. After 24 and 48 h, supernatants were removed and used for the DNase, the cell cytotoxicity, and siderophore quantification assays. The slides were rinsed twice with 12 ml of PPB in order to remove loosely attached cells. Attached cells were then recovered into 10 ml of PPB by surface scraping. Attached cells were harvested by centrifuging suspensions at 5000 g for 5 min at 20°C and then washed once with 20 ml of PPB. In order to remove the biofilm matrix, attached cells were resuspended in 20 ml of PPB and suspensions were sonicated at 37 kHz for 5 min at 20°C. Finally, the attached cells were recovered in 20 ml of PPB. The bacterial suspensions were adjusted to a cell concentration of 10^7 CFU/ml for the bacterial adhesion assays.

Adhesion assays

The adhesion of planktonic cells was performed on both SS and PC discs (41 × 1 mm). The adhesion of bacteria detached from biofilms grown on SS and PC was performed respectively on sterile SS and PC using the *NEC Biofilm system* (Abdallah et al. 2015). Sterile coupons of SS and PC were placed in the horizontal position in sterile *NEC Biofilm system*. The upper face of each slide was covered with 3 ml of corresponding-cell-suspensions (10^7 CFU/ml) and statically incubated at 20°C for 60 min to allow bacterial adhesion. After 1 h, the slides were removed using sterile forceps and rinsed twice by gently dipping into 30 ml of PPB to remove excess liquid droplets and loosely attached cells. Cells were then stained for 15 min in the dark using Acridine Orange 0.01% (w/v) (Sigma Aldrich, Saint-Quentin Fallavier, France) and then rinsed once by gently dipping in 30 ml of ultrapure water. The attached cells were quantified using epifluorescence microscope (Nikon Optiphot-2 EFD3). A total of 30 fields per coupon was scanned and the stained cells were enumerated. The adhesion rates were presented as a number of bacteria per microscopic field. The results present the average of three independent experiments and in each experiment, two slides were studied.

Microbial adhesion to solvents (MATS)

The hydrophobicity and the electron donor (basic) or acceptor (acidic) properties of planktonic and biofilm-detached *S. aureus* were determined using the MATS method as described by Bellon-Fontaine et al. (1996). This method is based on the comparison of bacterial affinity to four solvents (Sigma Aldrich, Saint-Quentin Fallavier, France) with different physicochemical properties. The following pairs of solvents were used: chloroform (electron acceptor solvent)/hexadecane (a nonpolar solvent); ethyl acetate (an electron donor solvent)/decane (a nonpolar solvent). Due to the similar Lifshitz–van der Waals components of the surface tension in each pair of solvents, differences between the affinities to solvents would indicate the electron donor and electron acceptor characters of the bacterial surfaces. The affinity of cells to hexadecane was used as a measure of cell surface hydrophobicity.

Experimentally, bacterial suspensions of 10^8 CFU/ml were prepared in PPB by adjusting the optical density to $OD_{400\ nm} = 0.8$ (A_0). Then 2.4 ml of each bacterial suspension were added to 0.4 ml of each solvent and then vortexed for 90 s. The mixture was allowed to stand for 30 min to ensure the complete separation of the two phases. Then the optical density of the aqueous phase (A_1) was

measured at 400 nm using a Jenway 6320D UV/visible light spectrophotometer. The affinity of cells to each solvent was subsequently calculated using the following equation: Affinity % = $[1 - (A_1/A_0)] \times 100$. The results represent the average of three independent experiments.

Measurement of zeta potential

The electrostatic properties of *S. aureus* were determined by measuring the zeta potential (ZP) which is derived from the electrophoretic mobility, using the Helmotz–Smoluchowski equation (Bayoudh et al. 2009). The electrophoretic mobility of bacteria cells suspended in PPB was measured using a laser Zeta Compact zetameter (CAD Instruments, Les Essarts-le-Roi, France), by tracking bacteria with a coupled device camera, under an electric field of 80 V. Each bacterial suspension was diluted in PPB to obtain about 70 bacteria per reading. A 1 mM of the KNO_3 solution was used as the electrolyte and KOH (1 mM) and HNO_3 (1 mM) were used to adjust the pH to 7.2 (Sigma-Aldrich, Saint-Quentin-Fallavier, France). For each sample, the ZP measurements were repeated five times. Each experiment was performed in duplicate by using two independent cultures.

Cytotoxicity assay

Supernatants were recovered from biofilms grown on SS and PC, and planktonic cultures, after 24 and 48 h of incubation. Supernatants of planktonic and biofilm cultures, grown at 20, 30 and 37°C for 24 and 48 h, were collected and the pH was adjusted to 7.2 ± 0.05 using 1 M hydrochloric acid (HCl) (Sigma-Aldrich, Saint-Quentin-Fallavier, France). Next, supernatants were filtered through sterile 0.2 µm Millipore filters. Both planktonic and sessile *S. aureus* supernatants were diluted after being adjusted to similar cell densities based on optical density (620 nm) measurements. The HeLa cell line, derived from cervical carcinoma from a 31-year-old female (ATCC® CCL-2™, ECACC), were cultured and maintained in Dulbecco's Modified Eagle's medium (DMEM, Gibco®, Thermo Fisher Scientific, Illkirch, France) supplemented with 10% Fetal Bovine Serum (FBS, Gibco®) and 1% penicillin–streptomycin (Gibco®) in a humidified incubator at 37 °C and 5% CO_2. Cells were seeded at a cell density of 10^4 cells/well in a 96-well plate and grown for 48 h before assay. For cytotoxicity assay, the culture medium was replaced with 100 µl of 10% FBS or TSB (pH 7.2) for the negative control or with 100 µl of *S. aureus* culture supernatants. After 3 h of contact, the mixture was aspirated and cells were washed with Phosphate Buffered Saline (PBS,

pH 7.4, ThermoFisher Scientific, Illkirch, France). The cell viability was evaluated using Cell Counting Kit-8 (CCK-8, Sigma-Aldrich, Saint-Quentin-Fallavier, France) assay. Briefly, 10 µl of the CCK-8 solution were added to each well containing 100 µl of DMEM with 10% FBS and the plate were incubated for 1 h in the humidified incubator. The absorbance of each well at 450 nm was measured using a microplate reader (PHERA star FS, BMG LABTECH GmbH, Germany). The mean absorbance value of cells non-treated with supernatants was taken as 100% cellular viability. The results represent the average of three independent experiments and each experiment was done in triplicate.

Deoxyribonuclease (DNase) activity assay

Bacterial supernatants were collected as described above. Enzyme production was tested on DNA agar (Thermo Fisher Scientific, Illkirch, France) by the deposition of 100 µl of each supernatant in 6 mm diameter well. Supernatant volume was allowed to diffuse for 2 h at 4°C. The plates were incubated at 37°C overnight. After incubation, wells were flooded with 1 M HCl. DNase production was identified by a halo zone of clearance (DNA degradation) around the supernatant deposition well. The halo zone diameters correlated with the DNase activity in the corresponding supernatant. The results represent the average of three independent experiments and each experiment was done in duplicate.

Quantitative spectrophotometric assay for siderophore production

The siderophore quantification of *S. aureus*-culture-supernatants is based on Chrome Azurol Sulphonate (CAS assay) according to Schwyn and Neilands (1987). All reagents were purchased from Fluka Sigma-Aldrich (Saint-Quentin-Fallavier, France). Briefly, in order to prepare the CAS assay solution, 6 ml of 10 mM hexadecyltrimethylammonium bromide, 1.5 ml of iron (III) solution (1 mM $FeCl_3 6H_2O$, 10 mM HCl), 7.5 ml of 2 mM aqueous CAS solution and 20 ml of 2.5 mM piperazine buffer in H_2O (pH 5) were mixed in a 100-ml volumetric flask which was then filled with water to afford 100 ml of CAS assay solution. Then, 100 mg of 5-sulfosalicylic were added to the CAS assay solution and stored in the dark.

In order to quantify the siderophores, 0.5 ml of the culture supernatant was mixed with 0.5 ml from the prepared CAS assay solution. After 1 h of incubation at 20°C, the absorbance ($A_{630\ nm}$) is measured by a Jenway 6320D UV/visible light spectrophotometer. The CAS-iron complex color changes from dark blue to orange after the iron chelation by siderophores. The TSB was used as

the blank (reference sample). The percentage of siderophore units was estimated as the proportion of CAS color shift using the formula [(Ar − As)/(Ar)] × 100, where Ar is the $A_{630\,nm}$ of the reference sample (TSB + CAS assay solution + shuttle solution) and As is the $A_{630\,nm}$ of the sample (supernatant + CAS assay solution + shuttle solution).

Statistics

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) to determine the significance of differences. Results were considered significant at a *P* value of < *0.05*.

Results

Effect of growth conditions on the Zeta potential of biofilm-detached and planktonic *S. aureus* cells

This investigation aimed to study the electronegativity of planktonic and biofilm-detached cells in response to different bacterial growth temperatures (20, 30 and 37°C) and incubation durations (24 and 48 h). For the biofilm formation, two abiotic surfaces, the SS and the PC were used. Figure 1 presents the zeta potential (ZP) values of bacterial surfaces as a function of *S. aureus* growth conditions. The results indicated that *S. aureus* cells were negatively charged, with negative ZP values, whatever the growth conditions (Fig. 1).

Figure 1a showed that the growth temperature and the incubation duration had a significant effect on the ZP of planktonic cells ($P < 0.05$). The increase of growth temperature from 20 to 37°C significantly increased the ZP of the 24 h planktonic cells from − 26.3 to − 13.6 mV ($P < 0.05$) and the ZP of 48 h planktonic cells from − 22.7 to − 18.7 mV ($P < 0.05$) (Fig. 1a). When cells were grown at 20°C, the results underlined that the increase of incubation time from 24 to 48 h increased by 1.2-fold the ZP of planktonic cells ($P < 0.05$). However, the increase of the incubation duration of 37 °C planktonic cultures from 24 to 48 h significantly decreased by 1.4-fold the ZP of planktonic cells ($P < 0.05$).

Furthermore, the results showed in Fig. 1 indicated that planktonic cells were significantly more negatively charged than their biofilm-detached counterparts whatever the studied conditions ($P < 0.05$), except for planktonic cells grown at 37°C for 24 h where the electronegativity of planktonic cell surfaces was lower than that of 24 h biofilm-detached cells ($P < 0.05$). In addition,

our findings underlined that the abiotic surface type had a significant effect on the electronegativity of the biofilm-detached cells (Fig. 1b, c). When the growth temperature increased from 20 to 37 °C, the ZP of 24 h and 48 h Polycarbonate-Biofilm-Detached-Cells (PCBDCs) significantly decreased from − 16.4 to − 19.4 mV and from − 12.4 to − 18.4 mV, respectively ($P < 0.05$) (Fig. 1b).

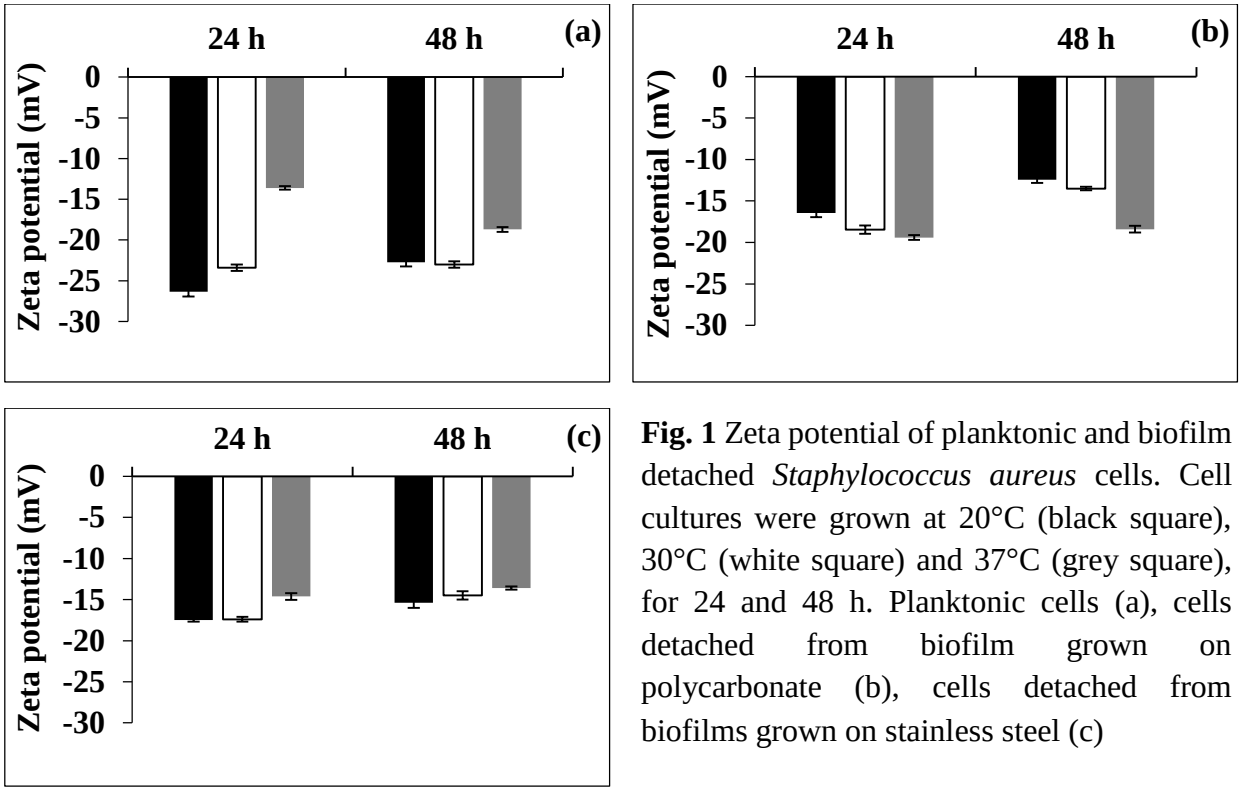

Fig. 1 Zeta potential of planktonic and biofilm detached *Staphylococcus aureus* cells. Cell cultures were grown at 20°C (black square), 30°C (white square) and 37°C (grey square), for 24 and 48 h. Planktonic cells (a), cells detached from biofilm grown on polycarbonate (b), cells detached from biofilms grown on stainless steel (c)

The increase of incubation duration from 24 to 48 h had only a significant effect on the electronegativity of PCBDCs grown at 20 and 30°C. The increase of incubation duration of the biofilm cultures from 24 to 48 h increased by 1.4-fold the ZP of 20 and 30°C PCBDCs ($P < 0.05$) (Fig. 1b). The stainless steel-biofilm-detached-cells (SSBDCs) showed an opposite electronegativity trend regarding the effect of growth temperature. Fig. 1c showed that the increase of the biofilm growth temperature from 20 to 37°C significantly increased the ZP of SSBDCs by 1.2-fold ($P < 0.05$) whatever the incubation durations (Fig. 1c).

Effect of growth conditions on the cell surface hydrophobicity and electron donor/acceptor characters of biofilm-detached and planktonic *S. aureus* cells

This study investigated the physicochemical surface properties of planktonic and biofilm-detached *S. aureus* cells, using the microbial adhesion to solvents (MATS), in response to different incubation durations (24 and 48 h), growth temperatures (20, 30 and 37°C) and abiotic surfaces (SS and PC). The results related to the hydrophobicity (affinity to hexadecane) and the acceptor/donor character of planktonic and biofilm-detached *S. aureus* cells are shown in Fig. 2 and Table 1.

The results underlined that the increase of growth temperature of *S. aureus* significantly increased the hydrophobic character of planktonic cell surfaces ($P < 0.05$) (Fig. 2a, b). When cells were grown at 20°C, the increase of the incubation duration from 24 to 48 h significantly increased the affinity of planktonic cells to hexadecane from 61.9 to 73.2% ($P < 0.05$) (Fig. 2a, b). However, the surface hydrophobicity of planktonic cells grown at 30 and 37°C was not influenced by the increase of the incubation duration of *S. aureus* cultures ($P > 0.05$). Table 1 showed that planktonic cells have low relative electron acceptor character whatever the growth conditions. However, the electron donor character of planktonic cells grown for 24 h decreased from 31.2 to 0.7 with the increase of growth temperature from 20 to 37°C. Similar results were observed for planktonic cells grown for 48 h (Table 1). Our findings also showed that, in addition to the incubation duration and the growth temperature, the surface type, had a significant effect on the hydrophobicity as well as the acceptor/donor character of *S. aureus* biofilms-detached cells (Fig. 2a, b). After an incubation duration of 24 h, the surface hydrophobicity of SSBDCs decreased with the increase of the biofilm growth temperature. The affinity of 24 h SSBDCs to hexadecane decreased from 96 to 77% when the biofilm growth temperature increased from 20 to 37°C ($P < 0.05$) (Fig. 2a). However, an opposite profile was observed for cells recovered from biofilms grown on the SS for 48 h. The affinity of 48 h SSBDCs to hexadecane increased from 80 to 98% when the biofilm growth temperature increased from 20 to 37°C (Fig. 2b). The affinity of 48 h PCBDCs to the hexadecane increased from 78 to 99% ($P < 0.05$) when the biofilm growth temperature increased from 20 to 37°C (Fig. 2b). Furthermore, the results showed that the electron donor characters of 24 h SSBDCs increased from 1.9 to 21.6 with the increase of the biofilm growth temperature from 20 to 37°C. The electron donor character of 24 h PCBDCs increased from 2.2 to 4 when the growth temperature increased from 20 to 37°C (Table 1). After 48 h of incubation, the electron donor character of

SSBDCs and PCBDCs decreased from 18.6 to 1.3 when the growth temperature of biofilms increased from 20 to 37°C whatever the surface type of the biofilm formation (Table 1). Table 1 also showed that the increase of biofilm growth temperature from 20 to 37°C significantly decreased the electron acceptor character of 24 h and 48 h SSBDCs from 17.4 to − 22.3 and from 7.2 to − 17.7, respectively (Table 1). The results of Table 1 also showed that PCBDCs presented low relative electron acceptor character whatever the growth conditions.

When cells were grown at 20°C, the result showed that the electron donor characters of 24 h and 48 planktonic cells were 16 and 1.3-fold higher than those of 24 and 48 h biofilm-detached cells, respectively (Table 1). However, the electron donor character of SSBDCs, grown at 30 and 37°C, was twofold higher than that of their planktonic counterparts whatever the biofilm incubation duration, except for the 24 h SSBDCs grown at 37 °C where the electron donor character was of 30-fold higher. The results also showed that the electron donor characters of 30 and 37°C SSDBCs were significantly higher than that of their PCBDCs counterparts whatever the biofilm incubation duration.

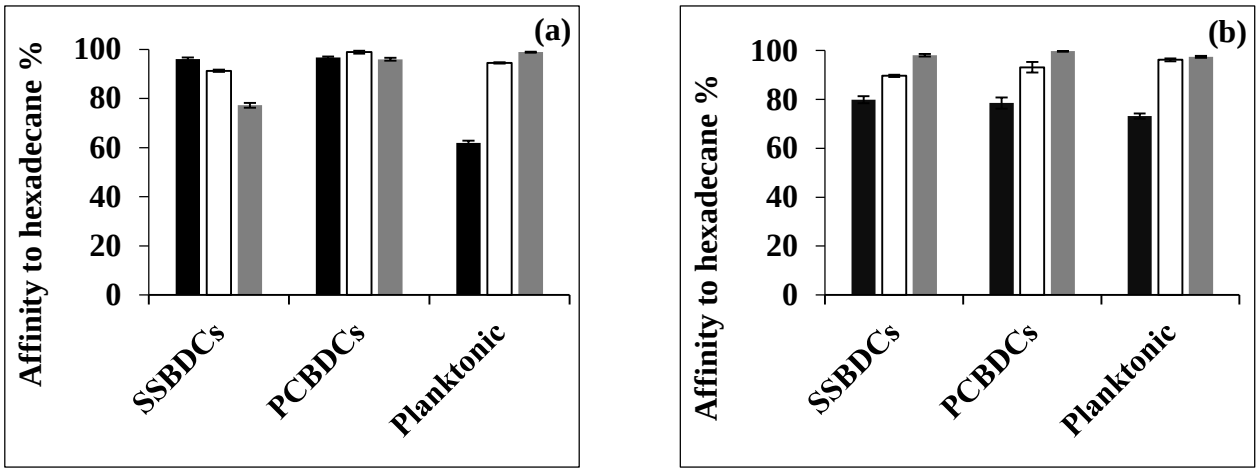

Fig. 2 Affinity of planktonic and biofilm detached *Staphylococcus aureus* cells to hexadecane. Cells grown, at 20°C (black square), 30°C (white square) and 37°C (grey square). SSBDCs represents the stainless steel-biofilm-detached-cells. PCBDCs represents the polycarbonate-biofilm-detached-cells. Cells grown during 24 h (a) and 48 h (b)

Table 1 Electron donor/acceptor character of biofilm-detached and planktonic *Staphylococcus aureus* cells, grown at 20, 30 and 37°C, during 24 and 48 h

		Electron donor [a]			Electron acceptor [b]		
	T°C [c]	SSBDCs [d]	PCBDCs [e]	Planktonic	SSBDCs	PCBDCs	Planktonic
24 h	20°C	1.9 ± 0.1	2.2 ± 0.5	31.2 ± 1.1	17.4 ± 0.5	-49.6 ± 3.2	3.9 ± 0.6
	30°C	7.8 ± 0.6	0.9 ± 0.7	4.8 ± 0.5	-1.5 ± 0.2	-19.5 ± 2.4	-31.8 ± 0.2
	37°C	21.6 ± 0.6	4 ± 0.6	0.7 ± 0.1	-22.3 ± 0.9	-17.9 ± 0.7	-63.6 ± 0.2
48 h	20°C	18.6 ± 1.6	18.6 ± 2.2	25.2 ± 0.5	7.2 ± 1.3	-23.5 ± 1.8	-70.3 ± 2.8
	30°C	9.8 ± 0.2	6.2 ± 1.8	3.7 ± 0.7	7.1 ± 0.3	-22.6 ± 2.7	-38.5 ± 2.6
	37°C	1.3 ± 0.3	-1 ± 0.2	0.6 ± 0.1	-17.7 ± 0.2	-14.3 ± 3.6	-25.4 ± 1.7

[a] The differences between the chloroform and hexadecane affinities of cells suspended in 100 mM PPB (pH 7) presents the electron donor character

[b] The differences between the ethyl acetate and decane affinities of cells suspended in 100 mM PPB (pH 7) presents the electron acceptor character

[c] T°C represents the growth temperature.

[d] SSBDCs represents the Stainless Steel-Biofilm-Detached-Cells

[e] PCBDCs represents the Polycarbonate-Biofilm-Detached-Cells

Effect of growth conditions on the adhesion of biofilm-detached and planktonic *S. aureus* cells to stainless steel and polycarbonate

This investigation aimed to study the effect of the *S. aureus* growth conditions on the adhesion behavior of planktonic *S. aureus* cells on SS and PC. The adhesion assays have been done using planktonic cells recovered from cultures grown under different growth temperatures (20, 30 and 37°C) and durations (24 and 48 h).

Our results showed that the increase of the growth temperature of *S. aureus* from 20 to 37°C slightly increased by 1.2-fold the adhesion of planktonic cells on the SS whatever the incubation duration ($P > 0.05$) (Fig. 3a). The adhesion experiments performed on the PC showed that the adhesion rate of 24 h planktonic cells increased by 1.2-fold when the growth temperature of *S. aureus* increased from 20 to 37 °C ($P > 0.05$) (Fig. 3b). Figure 3a, b showed that the adhesion rate of 24 and 48 h planktonic cells was respectively 1.4- ($P < 0.05$) and 1.2-fold ($P > 0.05$) higher on the SS than on

the PC whatever the growth temperature, except for the planktonic *S. aureus* cells grown for 48 h at 20°C where the adhesion rates were similar on both studied abiotic surfaces.

This study also investigated the adhesion behaviour of biofilm-detached cells, recovered from biofilms grown under different incubation temperatures (20, 30 and 37°C), durations (24 and 48 h) and surface types (SS and PC), on the SS and PC. For this study, the adhesion of SSBDCs and PCBDCs was investigated respectively on the SS and the PC.

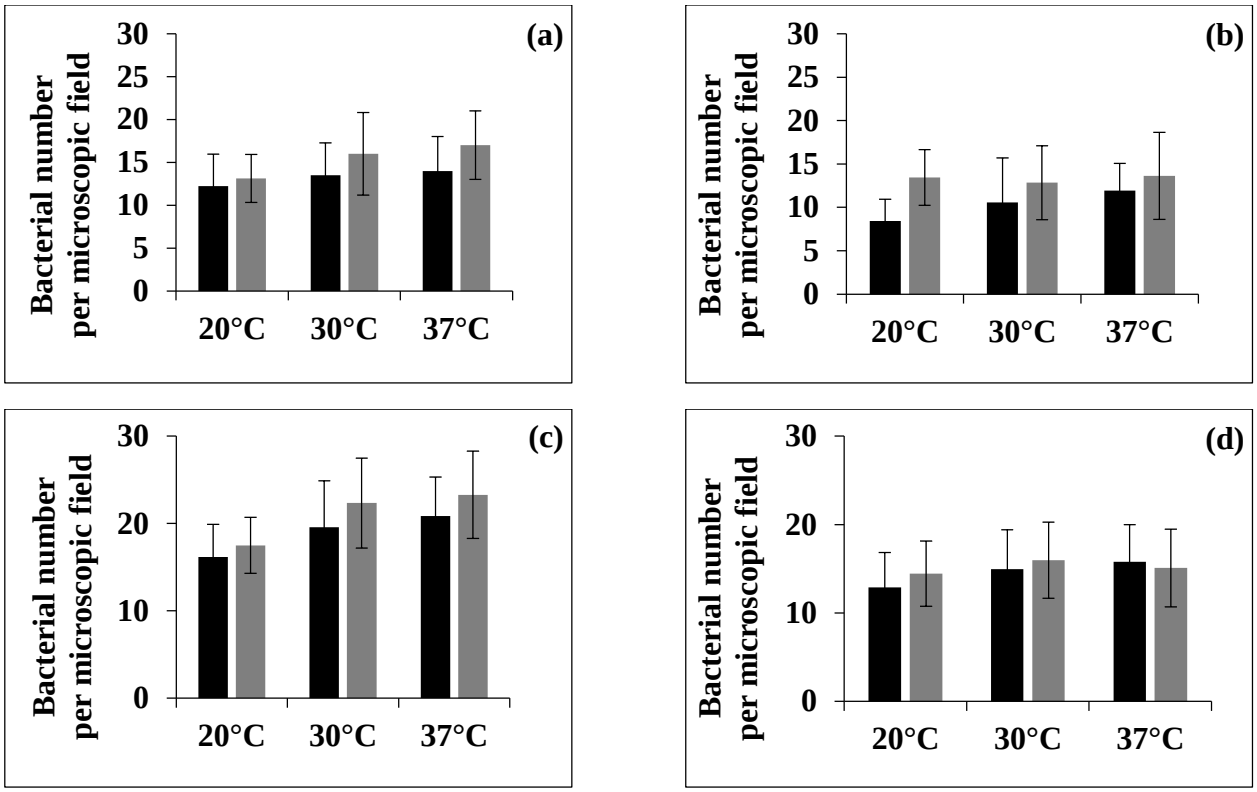

Fig. 3 Adhesion of planktonic and biofilm-detached *Staphylococcus aureus* cells on stainless steel and polycarbonate. Cell cultures were grown at 20, 30 and 37°C, during 24 h (black square) and 48 h (grey square). Planktonic cells adhesion on stainless steel (a) and polycarbonate (b). Adhesion of stainless steel-biofilm-detached-cells on stainless steel 24 (c) and polycarbonate-biofilm-detached-cells on polycarbonate (d)

The results underlined that the abiotic surface type and the temperature of the biofilm formation had an effect on the adhesion behavior of SSBDCs on the SS. Figure 3c showed that the increase of the biofilm growth temperature from 20 to 37°C increased by 1.3-fold the adhesion rate of 24 and 48 h SSBDCs on the SS (Fig. 3c). However, the Fig. 3d showed that neither the time nor the temperature of biofilm growth had a significant effect on the adhesion rate of PCBDCs on the PC ($P > 0.05$). Furthermore, our data showed that the adhesion rate of SSDBCs on the SS was 1.3-fold

higher than the adhesion rate of their PCBDCs counterparts on the PC whatever the studied conditions (Fig. 3c, d). Moreover, Fig. 3a, c showed that the bacterial adhesion rate of SSBDCs on the SS was 1.4-fold higher than the adhesion rate of their planktonic counterparts on the same surface whatever the growth temperature and incubation durations ($P < 0.05$). The adhesion rate of 24 h PCBDCs on the PC was 1.5-fold higher than that of 24 h planktonic cells on the same surface whatever the growth temperature ($P < 0.05$). However, the adhesion rates of 48 h PCBDCs on the PC was similar to that of 48 h planktonic cells on the same surface whatever the growth temperature ($P > 0.05$) (Fig. 3b, d).

Effect of growth conditions on the production of DNase by *S. aureus* biofilm and planktonic cultures

The assessment of the nuclease activity was realized on supernatants recovered from planktonic cultures and biofilm grown on SS and PC at different growth temperatures (20, 30 and 37°C) and incubation durations (24 and 48 h). The TSB has been used as a negative control and the results showed that it had no DNase activity (data not shown).

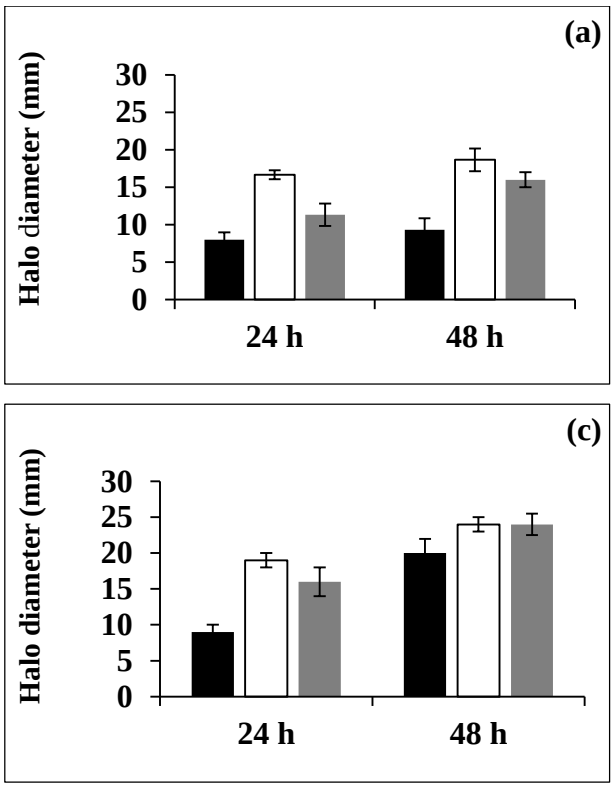

Fig. 4 DNase activity in supernatants recovered from *Staphylococcus aureus* planktonic and biofilm cultures. Cell cultures were grown at 20°C (black square), 30°C (white square) and 37°C (grey square), during 24 and 48 h. Planktonic cultures **(**a**)**, biofilms grown on polycarbonate (b**)** and biofilms grown on stainless steel (c)

The results showed that the DNase activity of the planktonic-culture-supernatants (PCSs) seems to be dependent on the temperature and the incubation duration of growth. The increase of the growth temperature from 20 to 30°C significantly ($P < 0.05$) rose the DNase activity of PCSs by twofold whatever the incubation duration (Fig. 4a). When the incubation temperature increased from 20 to 37°C, the DNase activity of 24 and 48 h PCSs increased respectively by 1.2- and 1.8-fold ($P > 0.05$) (Fig. 4a). The results also showed that the increase of the incubation duration of planktonic cultures from 24 to 48 h significantly increased the DNase activity of 30 and 37°C PCSs respectively by 1.2- and 1.6-fold ($P < 0.05$) (Fig. 4a).

Our results also showed that the abiotic surface type had a significant effect on the DNase production by sessile *S. aureus* cells and this effect is dependent on the duration and the temperature of biofilm growth. When biofilm growth temperature increased from 20 to 37°C, the DNase activity of supernatants recovered from 24 h and 48 h SS-biofilms increased respectively by 1.9- and 1.2-fold ($P < 0.05$) (Fig. 4c). The increase of the PC biofilm incubation temperature from 20 to 37 °C increased the DNase activity of 24 and 48 h biofilm-cultures-supernatants (BCSs) respectively by 1.4- and 1.2-fold (Fig. 4b). Furthermore, the results showed that the increase of the incubation duration from 24 to 48 h increased the DNase activity of 20, 30 and 37°C BCSs respectively by 1.6, 1.1 and 1.4-fold when the biofilms were grown on the PC ($P < 0.05$) and respectively by 2.2, 1.2 and 1.5-fold when the biofilms were grown on the SS ($P < 0.05$) (Fig. 4c). Furthermore, our data underlined that BCSs of *S. aureus* seem to have higher DNase activity than that of PCSs whatever the studied conditions ($P < 0.05$) (Fig. 4a–c).

Effect of growth conditions on the cytotoxicity of *S. aureus* biofilm and planktonic cultures

The planktonic and biofilm culture supernatants, used for the DNase analysis, have been used to test their cytotoxic effects against HeLa cells. This study willed to evaluate the supernatant cytotoxicity of *S. aureus* cells as a function of their growth conditions. The viability of HeLa cells, after an incubation of 3 h with appropriate supernatants, is shown in Fig. 5. The TSB has been used as a negative control. The results showed that TSB, used as a negative control, slightly reduced the viability of HeLa cells by 5% whatever the studied conditions (Fig. 5a–c).

Our findings also underlined that planktonic and biofilm-culture supernatants had a significant effect on HeLa cells viability ($P < 0.05$) and this effect seems dependent on the temperature and the incubation duration of planktonic cultures. The results showed that the PCSs did not affect the

HeLa cell viability when cultures are incubated at 20 and 30°C for 24 and 48 h (Fig. 5a). However, the 37°C PCSs reduced by twofold ($P < 0.05$) the HeLa cell viability whatever the incubation duration of planktonic cultures (Fig. 5a).

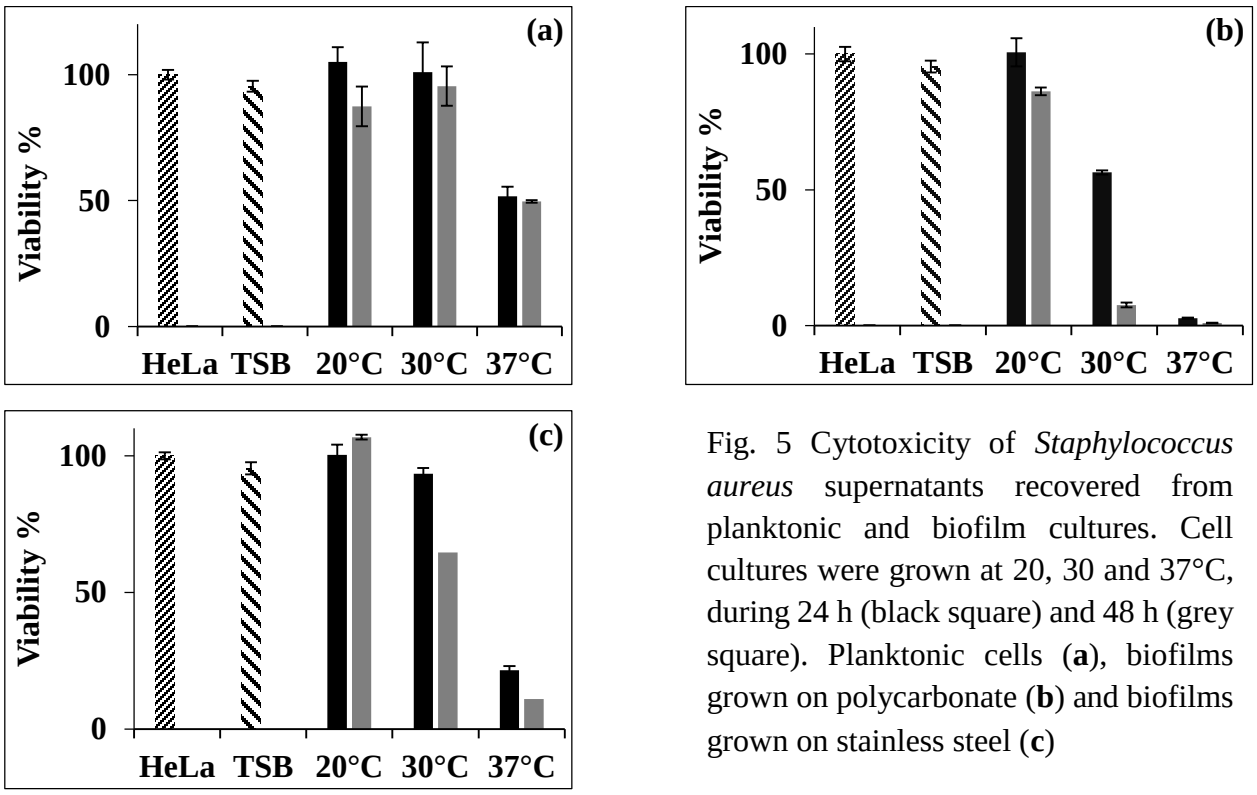

Fig. 5 Cytotoxicity of *Staphylococcus aureus* supernatants recovered from planktonic and biofilm cultures. Cell cultures were grown at 20, 30 and 37°C, during 24 h (black square) and 48 h (grey square). Planktonic cells (**a**), biofilms grown on polycarbonate (**b**) and biofilms grown on stainless steel (**c**)

Figure 5b, c also showed that the surface type of the biofilm formation had a significant effect on the cytotoxicity of the BCSs ($P < 0.05$). After an incubation time of 24 h, the cytotoxicity of PC and the SS-BCSs significantly decreased the viability of HeLa cells respectively by 36.4- and 4.6-fold when the biofilm growth temperature increased from 20 to 37°C ($P < 0.05$) (Fig. 5b, c). Similar data were observed for the 48 h BCSs (Fig. 5b, c). At 20 °C, the biofilm supernatants have not shown a significant cytotoxic effect against HeLa cells whatever the studied conditions (Fig. 5a–c). Furthermore, the PC-BCSs seem to be more cytotoxic than their SS and planktonic counterparts (Fig. 5a–c). After 24 h of incubation, the supernatants of 20, 30 and 37°C PC-biofilms was respectively 1.3-, 1.2- and 3-fold more cytotoxic than that of their SS counterparts (Fig. 5b, c). After 24 h, the cytotoxicity levels of the 20, 30 and 37°C PC-BCSs was 1.3, 2 and 24-fold higher ($P < 0.05$) than those of 20, 30 and 37°C planktonic supernatants, respectively (Fig. 5a, b). This trend was more pronounced when comparing the supernatant cytotoxicity of 48 h aged biofilm to the 48 h planktonic ones ($P < 0.05$) (Fig. 5a, b). In fact, the cytotoxicity of the PC and SS culture supernatants were respectively 57 and fivefold higher than that of planktonic cultures (Fig. 5a–c).

Effect of growth conditions on siderophore production by *S. aureus* biofilm and planktonic cultures

The goal here is to investigate the effect of incubation duration, growth temperature and surface type on the siderophore production by planktonic and biofilm *S. aureus* cells (Fig. 6).

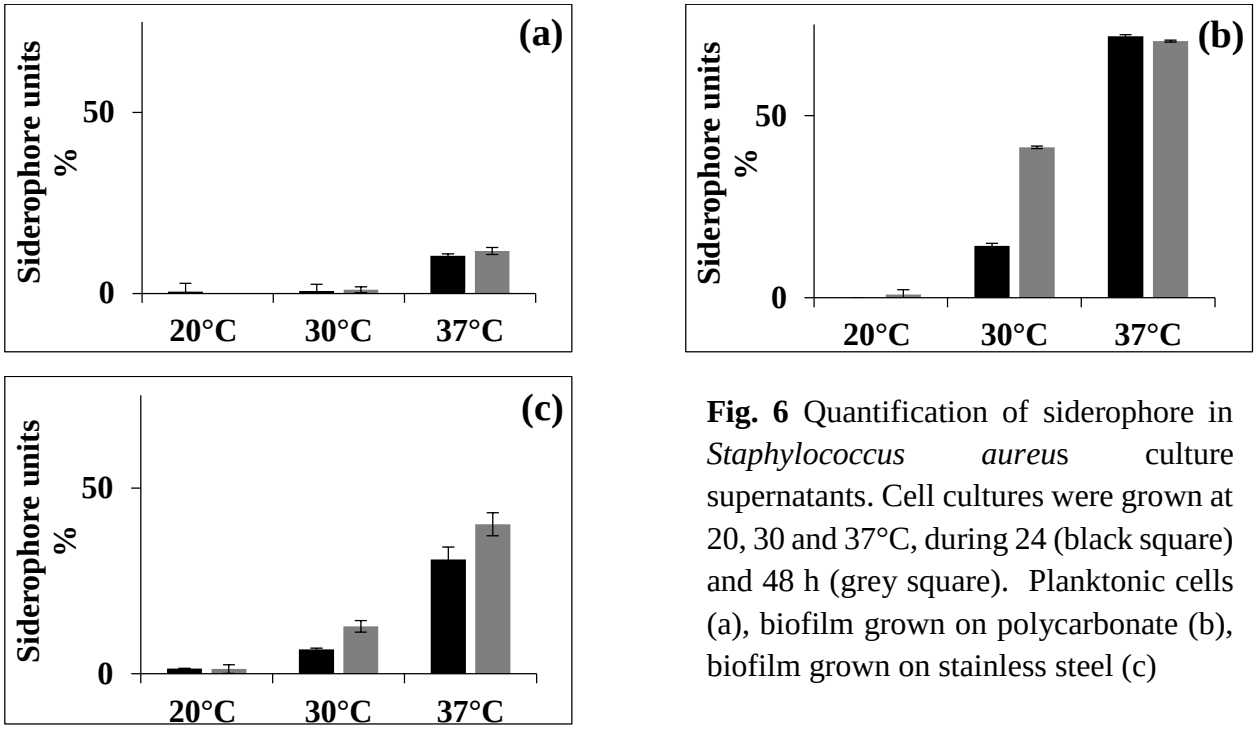

Fig. 6 Quantification of siderophore in *Staphylococcus aureu*s culture supernatants. Cell cultures were grown at 20, 30 and 37°C, during 24 (black square) and 48 h (grey square). Planktonic cells (a), biofilm grown on polycarbonate (b), biofilm grown on stainless steel (c)

Results of Fig. 6a showed no detectable siderophore production when planktonic cells were grown at 20 and 30°C whatever the incubation durations. However, the planktonic cells grown at 37 °C exhibited 11% of siderophore units whatever the incubation duration of planktonic cultures (Fig. 6a). Our findings also showed that the surface type, the growth temperature, and the incubation duration had a significant effect ($P < 0.05$) on siderophores production by sessile *S. aureus* cells (Fig. 6b, c). When grown on the SS, the increase of the biofilm growth temperature from 20 to 37°C significantly increased the percentage of siderophores units of 24 h and 48 h biofilm supernatants from 1.4 to 30.8% and from 1.3 to 40.2%, respectively ($P < 0.05$) (Fig. 6c). When *S. aureus* biofilms are grown on the PC, the percentage of produced siderophore units increased from an undetectable level to 71% ($P < 0.05$) when the biofilm growth temperature increased from 20 to 37°C whatever the incubation duration of the biofilm formation (Fig. 6b). In addition, our data showed that the amount of produced siderophore by sessile cells grown on PC

was significantly higher than that of their planktonic and SS counterparts whatever the studied conditions ($P < 0.05$) (Fig. 6a–c).

Discussion

Bacterial adhesion and biofilm formation have become a serious problem in healthcare and food sectors, and much investigations have been done for better understanding of the processes involved. However, most of studies have focused on the bacterial adhesion of planktonic cells but have not considered the biofilm-detached cells which may be involved in contamination spread. It has been reported that the physiology of planktonic and biofilm-detached cells are deeply different (Stewart and Costerton 2001; Donlan and Costerton 2002). In this context, our study investigated, in particular, the impact of growth conditions on the physicochemical properties of biofilm-detached and planktonic *S. aureus* cells and on their ability to adhere to the SS and PC. Overall, our results showed that the increase of temperature and the incubation duration slightly increased the adhesion of *S. aureus* to the SS and the PC. These results are in agreement with previous studies which highlighted the effect of these parameters on the adhesion of *S. aureus* (Abdallah et al. 2014b), *Listeria monocytogenes* (Gordesli and Abu-Lail 2012) and *Escherichia coli* (Tsuji and Yokoigawa 2012) to different surfaces. Furthermore, our results showed that biofilm-detached cells had a higher adhesion rate than that of their planktonic counterparts. The same trend was reported by Berlanga et al. (2015), who underlined the greater ability of biofilm-detached *Halomonas venusta* cells to colonize new surfaces compared to their planktonic counterparts. By contrast, other studies (Allison et al. 1990), reported that there were no significant differences between the adhesion of biofilm-detached and planktonic *E. coli* cells to abiotic surfaces. Furthermore, we investigated the effect of growth conditions on the surface physicochemical properties of *S. aureus* cells. Overall, we showed that the hydrophobicity of biofilm-detached and planktonic *S. aureus* cells increased when the growth temperature increased from 20 to 37°C. These findings seem to be consistent with those of Abdallah et al. (2014a), who found that the hydrophobicity of *S. aureus* increased with the increase of the growth temperatures. Therefore, this result may explain the increase of *S. aureus* adhesion onto SS and PC. However, and if we consider, particularly, the results related to the bacterial surface hydrophobicity we could suggest that cell adhesion should be greater on hydrophobic supports such as the PC which is not the case under our experimental conditions. In accordance with a previous study (Abdallah et al. 2014a), our results showed a greater adhesion

rate of *S. aureus* cells on SS than on the PC. Such results highlight that the hydrophobic interactions cannot always explain the bacterial adhesion onto abiotic surfaces. It has been reported that the acid–base interactions are the main forces governing the bacterial adhesion to abiotic surfaces (Bos et al. 1999). Our study highlighted the decrease of the electron donor character of 48 h-biofilm-detached cells with the increase of growth temperature. This may result in a decrease of repulsive acid–base interactions between the cells and the abiotic surfaces. Such decrease may, therefore, explain the increase of the bacterial adhesion of 48 h-biofilm-detached cells on the SS. By contrast, our results also showed that the electron donor characters of *S. aureus* did not always explain the differences found in the experimental results. In fact, the increase of electron donor character of 24 h-biofilm-detached-cells, with the increase of growth temperature from 20 to 37°C, was accompanied by an increase of the bacterial adhesion on both surfaces. Furthermore, we investigated the involvement of electrostatic interactions in the *S. aureus* adhesion to the SS and the PC. Our results showed that the ZP of *S. aureus* cells was negative whatever the studied conditions. Our findings also showed that biofilm-detached cells are less negatively charged than their planktonic counterparts, probably due to the up-regulation of cationic staphylococcal poly-*N*-acetylglucosamine surface polysaccharide (Otto 2008). Therefore, the low relative negative charge of biofilm-detached cells may result in a decrease of repulsive electrostatic forces between cells and negatively charged abiotic surfaces, which may explain their greater adhesion rates on abiotic surfaces as compared to that of their planktonic counterparts. Furthermore, our results showed that the increase of the growth temperature may result in a decrease of repulsive electrostatic interactions, between negatively-charged bacterial cells and abiotic surfaces. Therefore, this may explain the enhanced adhesion of the biofilm-detached *S. aureus* cells onto the SS. By contrast, our data showed that the increase of the growth temperature resulted in a decrease of the zeta potential of the biofilm-detached cells and simultaneously in an increase of the bacterial adhesion to the PC. Hence, we suggest that the electrostatic interactions may not always explain the bacterial adhesion to abiotic surfaces which involves other factors related to the cell envelope in this process (Hori and Matsumoto 2010). This work also investigated the effect of the growth conditions on the pathogenicity and cytotoxicity of the different studied *S. aureus* cultures. The DNase activity of biofilm cultures was greater than that of the planktonic cultures. In addition, the results showed that the DNase activity increased with the increase of the growth temperature and the incubation duration. These results are in disagreement with other studies (Resch et al. 2005; Wang et al. 2011),

which underlined that the virulence factor production by planktonic *S. aureus* was greater compared to that of biofilm cultures. However, our results seem in line with those of Coenye et al. (2007), who stated that the sessile *Propionibacterium acnes* cells produced more virulence factors than the planktonic ones and this production increased with the increase of the incubation time. The present findings also appear to be in agreement with those of Mahoney et al. (2010), who underlined that the bacterial virulence regulation is influenced by the growth temperature. Furthermore, our findings showed that BCSs had a higher cytotoxic effect, on HeLa cells, than the PCSs whatever the studied condition. The cytotoxic effect of BCSs and PCSs increased in response to the increase of the temperature and the incubation duration. Taken together, our results may explain the influence of growth conditions on the bacterial metabolism controlling the production of virulence factors (Holler et al. 1998). According to Secor et al. (2011), the different metabolic states in planktonic and biofilm cultures likely have a large impact on the pathogenic effects on human cells. Thus, in our case, the important cytotoxic effect of *S. aureus* BCSs compared to that of PCSs could be related to the presence of higher amounts of virulence factors including exoenzymes such as DNase, which may disturb the biological activity of human cells (Modun and Williams 1999; Pancholi and Chhatwal 2003; Jarosław et al. 2005; Secor et al. 2011). Nevertheless, our results showed that BCSs recovered from biofilms grown on the PC surface were more cytotoxic to HeLa cells than those of biofilm grown on SS. Interestingly, our investigation showed that the siderophore production, which is enhanced under iron-limiting conditions (Vasil and Ochsner 1999; Gaonkar 2015), in the supernatant of biofilm grown on PC were higher than that of biofilm grown on SS. It has been reported that iron and nickel could be released from the SS into solution (Ortiz et al. 2011). Therefore, the limited availability of iron in the medium of biofilm grown on the PC, which is a plastic surface, could enhance the production of siderophores (Gaonkar 2015). In *S. aureus*, the greater production of siderophores correlated with higher virulence and more resistant (Rozalska et al. 1998; Dale et al. 2004). Taken together, our findings and previous studies may explain the greater cytotoxicity and pathogenicity of supernatants recovered from *S. aureus* biofilms grown on the PC when compared to those recovered from biofilms grown on the SS.

In conclusion, this study showed that biofilm-detached-cells are phenotypically distinct from planktonically grown cells. Moreover, our results showed that the bacterial history and the growth conditions affect the adhesion of *S. aureus* to abiotic surfaces by influencing the bacterial surface

physicochemical properties. Our investigations also underlined the hazardous characters of biofilm-detached cells which appeared to be abler to adhere to abiotic surfaces than their planktonic counterparts. Such results highlight the importance of considering cell detachment as a serious stage in the process of biofilm development. These results should contribute to more effective management of disinfection strategies, especially by ensuring a rapid removal and killing of cells detached from contaminated surfaces to prevent the persistence and the spread of contamination. However, our findings underlined that the bacterial physicochemical properties cannot always fully explain the bacterial adhesion. An interesting perspective would consist in focusing on the quantification of bacterial adhesion forces using atomic force microscopy in order to extend the knowledge of the mechanisms mediating bacterial adhesion to abiotic surfaces and to develop new strategies for the prevention of the biofilm formation. In addition, our results showed that sessile cells produce higher amounts of different virulence factors which represent a serious threat in case of human infection by *S. aureus.* Interestingly, growth temperatures close to that of the human body increased the cells virulence potential and cytotoxicity to human cells. Moreover, biofilm formed on plastic surfaces, such as PC, showed higher pathogenic risk than those formed on metallic ones, such as SS. Thus, our results highlight that the presence biofilm on plastic indwelling medical devices such as catheters, may increase the risk of severe infections. Our work offers a novel insight into the infectious potential of *S. aureus*, which suggests that a virulent strains may increase their virulence by forming a biofilm and achieve persistent infection in vivo.

Acknowledgements

This work was carried out within the framework of an ALIBIOTECH (Agroalimentaire et Biotechnologie, Nord Pas-de-Calais region) program. The authors also thank the Haut de France region and FEDER (Fonds européen de développement régional) for their financial support.

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Article III

Study of growth conditions impact on the adhesion to food contact surfaces and the pathogenicity of the biofilm-detached *Pseudomonas aeruginosa*

Simon Oussama KHELISSA [a, b], Charafeddine JAMA [a], Marwan ABDALLAH [a], Nour-Eddine CHIHIB [b] *

[a] CNRS, ENSCL, UMR 8207-UMET-PSI, Université de Lille 1, Avenue Dimitri Mendeleïev, CS 90108 59652 Villeneuve d'Ascq, France

[b] CNRS, INRA, UMR 8207-UMET-PIHM, Université de Lille 1, 369 rue Jules Guesde, CS 20039, 59651 Villeneuve d'Ascq, France

*Corresponding author: email: nour-eddine.chihib@univ-lille1.fr, Tel: +33320435443

Abstract

Aims: The majority of studies is associated with planktonic or biofilm-structured cells and very few investigations have been conducted on biofilm-detached cells in order to elucidate their behavior. The present experiment was conducted in order to study the effect of the growth temperature, incubation duration and surface type on the pathogenicity and the ability of biofilm-detached *Pseudomonas aeruginosa* cells to contaminate the food contact surfaces**.**

Methods and Results: The growth conditions had a significant effect on the physicochemical properties of *P. aeruginosa* cells surfaces. Biofilm-detached cells showed a greater adhesion to stainless steel and polycarbonate compared with planktonic cells. In addition, the results showed that the cytotoxic effect against HeLa cells, proteasic activity, and siderophore levels increased with the increase of growth temperature and the incubation duration. Their production levels were higher in supernatants recovered from biofilms than those recovered from planktonic cultures.

Conclusion: This study highlights that *P. aeruginosa* biofilm-detached cells have distinct physiological properties compared with the planktonic ones. *P. aeruginosa* biofilm-detached cells represent a serious microbiological quality and safety risks in the food sector.

Significance and Impact of the Study: This study focused on *P. aeruginosa* biofilm-detached cells to investigate their specific phenotype. The results might be used to assist risk assessment studies and to establish more effective and adapted control measures.

Keywords: *Pseudomonas aeruginosa*, physiology, pathogenicity, biofilm-detached bacteria, planktonic bacteria

Introduction

Pseudomonas aeruginosa is an ubiquitous environmental Gram-negative bacterium that grows in soil, water, plants, animals and human environment (Abdallah et al. 2015a). The emergence of such an opportunistic pathogen in the healthcare sector is a significant risk for the public health (Khelissa et al. 2017a). In fact, *P. aeruginosa* is the main cause of hospital-acquired-infections worldwide (Mesaros et al. 2007; Khelissa et al. 2017a; Abdallah et al. 2015a). The pathogenesis of this bacterium correlates with a large arsenal of pathogenicity factors which interfere with host defenses (Gellatly and Hancock 2013). A number of *P. aeruginosa* pathogenicity factors include exotoxins which have been shown to induce host cell death and tissue destruction by apoptosis, in addition to lipases and phospholipases which break down the phospholipids of host cell membranes (Gellatly and Hancock 2013; Al-Wrafy et al. 2016; Gellatly et al. 2012; Bender and Flieger 2010). Proteases secreted by *P. aeruginosa* have been shown to contribute to sepsis and tissue damage (Ołdak and Trafny 2005; Sadikot et al. 2005). The ability of this bacterium to produce low-molecular-weight iron-chelating agents such as pyoverdin and pyochelin is likely important to establish infections and the progression to a chronic infection (Lau et al. 2004; Gellatly and Hancock 2013). The ability of the bacterium to form biofilms offers further protection from antibiotics and from the host immune system (Donlan and Costerton 2002; Mulcahy et al. 2014; Hoiby et al. 2010). Nevertheless, the regular occurrence of *P. aeruginosa* infections is often linked to biofilm formation on contaminated medical devices (Donlan 2001). Biofilm is defined as a structured community of microorganisms, adherent to an inert or living surface, and embedded in a self-produced extracellular polymeric matrix (Donlan and Costerton 2002). Biofilm formation includes different steps including the bacterial adhesion on a surface, extracellular matrix production and biofilm maturation then the dispersion of cells to spread in the environment (Khelissa et al. 2017b). The bacterial adhesion to a surface is the first step of the biofilm formation (Garrett et al. 2008). It has been reported that certain physiochemical parameters of both bacterial and abiotic surfaces play a crucial role in the bacterial attachment (Abdallah et al. 2014). Once the adhesion is accomplished, bacteria start multiplying and synthesizing insoluble exopolysaccharides (EPS). Within hours of EPS accumulation, bacteria get entrapped in a complex protecting extracellular matrix and form a mature biofilm (Khelissa et al. 2017a). The biofilm dispersal in the environment is a fundamental step of the biofilm life cycle that contributes to biological dissemination, bacterial survival, and disease transmission (Kaplan 2010). In fact, several factors

can explain the causes and the mechanisms of the biofilm cells dispersal (Kaplan 2010; Wrangstadh et al. 1989; Allison et al. 1998; Jang et al. 2017). It has been suggested that biofilm-detached cells are physiologically different their sessile and planktonic counterparts (Liu et al. 2013). Thus it is of importance to understand *P. aeruginosa* phenotype changes in correlation with its life phases.
The goal of this work is to investigate the impact of the growth conditions on the physiochemical properties of biofilm-detached and the planktonic- *P. aeruginosa* cells, and on their ability to adhere to the stainless steel (SS) and the polycarbonate (PC). The biofilm-detached and the planktonic cells were recovered from cultures incubated at different growth temperatures and physiological ages commonly encountered in the medical environments. This work also investigated the effect of these growth conditions on the expression of some virulence factors, involved in the pathogenesis and cytotoxicity of *P. aeruginosa*.

Materials and methods

Bacterial culture

Pseudomonas aeruginosa CIP 103467 pre-cultures were first grown from frozen stock (−80°C), maintained in Tryptic Soy Broth (TSB; Biokar Diagnostics, Pantin, France) with 40 % (v/v) glycerol), for 24 h at 30 and 37°C, or for 48 h at 20°C. Cultures were initiated in 50 ml of TSB using 10^4 CFU.ml^{-1} from pre-cultures then incubated at 20, 30 or 37 °C until late exponential growth phase.

Cell suspension preparation

A 5 min at 20°C centrifugation (5000 *g*) was used to harvest *Pseudomonas aeruginosa* cultures, then cells were washed twice with 20 ml of 100 mM Potassium Phosphate Buffer (PPB; pH 7) before being resuspended in 20 ml of PPB then sonicated (ElmasonicS60H, Elma ®, 37 kHz) for 5 min at 20°C. A 10^8 CFU.ml^{-1} bacterial suspensions were prepared by adjusting the optical density to OD_{620nm}= 0.110 ± 0.005 (Jenway 6320D UV/visible light spectrophotometer).

Coupon preparation

Coupons of SS (304L, Equinox, France) and PC (Plexilux, France) were overnight soaked in ethanol 95° (Fluka, Sigma-Aldrich, France) and then rinsed with distilled water. Then coupons

were put in a bath of 1 % DDM ECO detergent solution (ANIOS, France) for 15 min at 20°C. Coupons were then thoroughly rinsed first with distilled then with in ultrapure water (Milli-Q® Academic, Millipore, France) at 20°C and sterilized either by autoclaving at 121°C for 20 min (SS) or in the 95° ethanol for 15 min (PC).

Biofilm-detached-cells preparation

Sterile coupons were placed in cell culture Petri dishes then 12 ml of cell suspension (10^7 CFU.ml^{-1}) were used to cover the upper face of coupons. The bacterial suspension was left to stand undisturbed at 20°C for one hour to allow the bacterial adsorption. Then the deposited volume was removed and coupons were gently rinsed (PPB). The coupons were covered with 12 ml of TSB and incubated at 20, 30 and 37°C for 24 or 48 h to allow biofilm formation. Biofilm supernatants were recovered, filter sterilized (0.2 µm sterile filters) and stored in aliquots at -80°C until cytotoxicity and virulence factors quantification assays. The slides were rinsed with PPB before being scraped to detach and collect biofilm cells into 10 ml volume of PPB. Detached biofilm cells were harvested by centrifugation at 5000 g for 5 min at 20°C. Cell pelt was resuspended in 20 ml of PPB and sonicated at 37 kHz (5 min at 20°C). The sonicated cells were recovered in 20 ml of PPB after being centrifuged at 5000 g for 5 min.

Bacterial adhesion assay

Discs of SS and PC were used to perform adhesion experiments. The *NEC Biofilm system* (Abdallah et al. 2015b) was used for the adhesion tests of biofilm-detached bacteria grown on SS and PC. Sterile discs were placed in the sterile well of each *NEC Biofilm System* reactor. 3 ml of corresponding-cell-suspensions, adjusted to 10^7 CFU.ml^{-1}, were used to cover the upper face of each slide, then the *NEC Biofilm System* reactor was incubated at 20°C for 60 min to allow bacterial adhesion. Thereafter, the 3 ml were removed and the discs were rinsed twice by gently dipping in PPB, using sterile forceps, to remove loosely attached cells. Adherent cells were stained with 0.01 % Acridine orange for 15 min. Following staining, the cells were rinsed in distilled water and allowed to air dry. A total of 50 fields per slide were viewed using epifluorescence microscope (Nikon Optiphot-2 EFD3). The adhesion rates were presented as the average of enumerated stained bacteria per field. The results present the average of three independent experiments and in each experiment, three coupons were used.

Microbial adhesion to solvents (MATS)

The Bellon-Fontaine et al. (1996) MATS method was used to assess the hydrophobicity and the electron donor or acceptor properties of *P. aeruginosa* cells. Chloroform (electron acceptor solvent); hexadecane (a nonpolar solvent); ethyl acetate (an electron donor solvent) and decane (a nonpolar solvent) were the used solvents. Bacterial cell suspensions (10^8 CFU.ml^{-1}) were prepared in PPB by adjusting the optical density to OD_{400nm}= 0.8 (A_0). Then 2.4 ml of each bacterial cell suspension were added to 0.4 ml of each solvent and then vortexed (90 s). The mixed volume was allowed to stand for 30 min after which the OD_{400nm} of the aqueous phase (A_1) was measured using a Jenway 6320D UV/visible light spectrophotometer. The cell affinity of to each solvent was calculated using the following equation: Affinity % = $[1 - (A_1/A_0)] \times 100$. Due to the similar Lifshitz–van der Waals components of the surface tension in each pair of solvents, differences between the affinities to chloroform and hexadecane and those between ethyl acetate and decane would indicate respectively, the electron donor and electron acceptor character of the bacterial surface. The affinity of cells to hexadecane was used as a measure of cell surface hydrophobicity. The results represent the average of three independent experiments.

Bacterial surface charge measurement

The surface charge of *P. aeruginosa* was assessed by measuring the Zeta Potential (ZP), according to the equation of Helmotz-Smoluchowski (Bayoudh et al. 2009). A laser Zeta Compact Zetameter (CAD Instruments, Les Essarts-le-Roi, France) was used to determine the electrophoretic mobility of bacteria cells suspended in PPB, under an electric field of 80V. Each bacterial suspension was diluted in PPB to obtain about 70 bacteria per reading. A 1 mM of the KNO_3 solution was used as the electrolyte and KOH (10^{-3} mol.l^{-1}) and HNO_3 (10^{-3} mol.l^{-1}) were used to adjust the pH to 7.2. For each sample, the ZP measurements were done five times. Each experiment was performed in triplicate by using three independent cell cultures.

Supernatant cytotoxicity assay

Supernatants recovered from biofilm and planktonic cultures were pH neutralized with hydrochloric acid (1 mol.l^{-1}). Both planktonic and biofilm-detached *P. aeruginosa* supernatants were adjusted to similar cell densities based on optical density (620 nm) measurements. The supernatant cytotoxicity assays were performed against HeLa cell line, derived from cervical

carcinoma from a 31-year-old female (ATCC® CCL-2™, ECACC, Sigma Aldrich, Saint-Quentin Fallavier, France) according to (Khelissa et al. 2017). The results represent the average of three independent experiments and each experiment is done in triplicate.

Protease activity assay

Bacterial supernatants were collected and prepared as described above. Protease activity was studied in pH-indicator solid media (2 % agar, 0.01 % bromocresol purple; adjusted to pH 5.2) containing 1 % skimmed milk. 100 µl of each supernatant were deposited in 6 mm diameter wells. Supernatant volume was allowed to diffuse for 2 hours at 4°C. The plates were, then, incubated at 37°C overnight. The diameters of clear zones around the wells, resulting from the degradation of milk proteins, correlated with the Protease activity in the corresponding supernatant. The results represent the average of three independent experiments.

Siderophore activity quantification

The quantification of siderophores present in *P. aeruginosa*-culture-supernatants is based on Chrome Azurol Sulphonate (CAS) assay according to Schwyn and Neilands (1987). The CAS assay solution was prepared as reported by Khelissa et al (2017b). In order to quantify the siderophores, 0.5 ml of the corresponding supernatant was mixed with 0.5 ml from the prepared CAS assay solution. The mixture is left to react for 1 hour at 20°C, then the absorbance ($A_{630\ nm}$) was measured by a Jenway 6320D UV/visible light spectrophotometer. The CAS-iron complex color changes from dark blue to orange after the iron chelation by siderophores. The TSB was used as the blank (reference sample). The percentage of siderophore units is estimated as the proportion of CAS color shift using the formula [(Ar – As)/ (Ar)] x 100, where Ar is the $A_{630\ nm}$ of the reference sample (TSB + CAS assay solution) and As is the $A_{630\ nm}$ of the sample (supernatant + CAS assay solution). The results represent the average of three independent experiments.

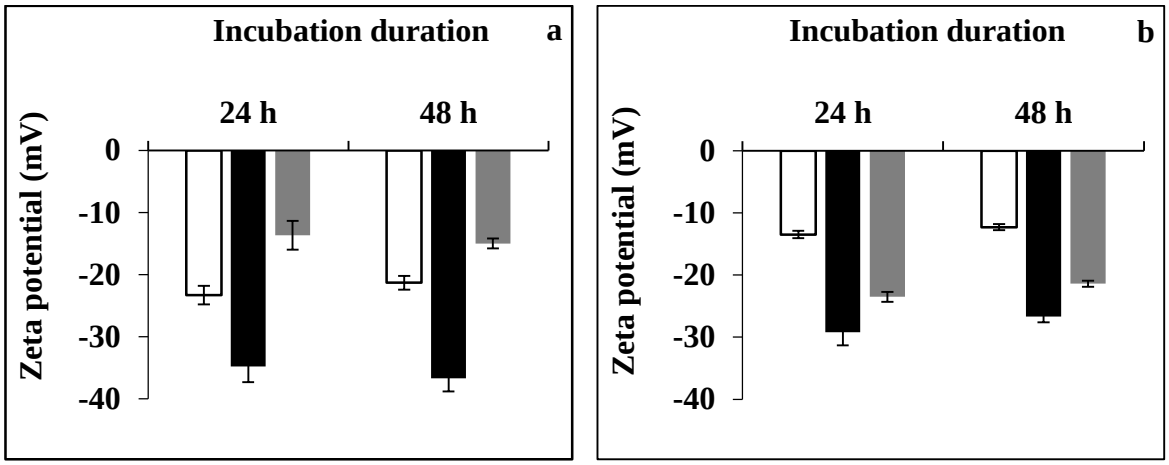
Statistic analysis

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

Results

Impact of growth conditions on the surface charge of *P. aeruginosa* cells

This study aimed to assess the effect of the incubation duration (24 and 48 h) and the growth temperature (20, 30 and 37°C) on the Zeta Potential (ZP) of biofilm-detached *P. aeruginosa* cells grown on the SS and the PC as compared to their planktonic counterparts grown under the same conditions. The results showed that after 24 h of incubation, the ZP of planktonic cells decreased, from -23.3 to -34.8 mV *(P < 0.05)* then increased from -34.8 to -13.7 mV *(P < 0.05)* when growth temperature increased respectively from 20 to 30°C and from 30 to 37°C (Fig. 1a). After 48 h of incubation, the ZP of planktonic cells decreased, from -21.3 to -36.7 mV *(P < 0.05)* then increased from -36.7 to -15 mV *(P < 0.05)* when growth temperature increased respectively from 20 to 30°C and from 30 to 37°C (Fig. 1a). Furthermore, our findings showed that the surface type had a significant effect *(P < 0.05)* on biofilm-detached cells ZP (Fig. 1b and c). In fact, the ZP of 24 h Polycarbonate-Biofilm-Detached-Cells (PCBDCs) decreased from -13.5 to -29.2 mV *(P < 0.05)* and increased from -29.2 to -23.5 mV *(P < 0.05)* when the incubation temperature increased respectively from 20 to 30°C and from 30 to 37°C (Fig. 1b). The same trend was observed after 48 h for PCBDCs. The Stainless-Steel-Biofilm-Detached-Cells (SSBDCs) showed stable ZP values of *ca* -15.4 whatever the studied condition (Fig. 1c).



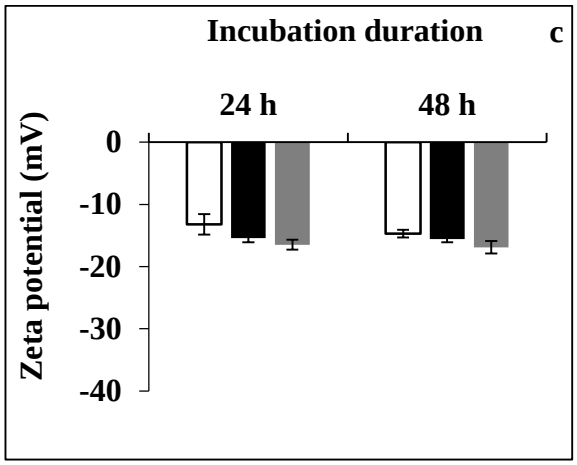


Figure 1 Zeta potential of *Pseudomonas aeruginosa* cells grown at 20 (white column), 30 (black column) and 37°C (grey column), for 24 and 48 h. Planktonic cells (a), cells detached from biofilm grown on polycarbonate (b), cells detached from biofilms grown on stainless steel (c).

Effect of growth conditions on *P. aeruginosa* affinity to solvents

This study investigated the physicochemical surface properties of biofilm-detached and planktonic *P. aeruginosa* cells by the Microbial Adhesion To Solvents (MATS) method, in response to growth conditions mentioned above. The results related to the hydrophobicity and the acceptor/donor character of biofilm-detached and planktonic *P. aeruginosa* cells are shown respectively in Fig. 2 and Table 1.

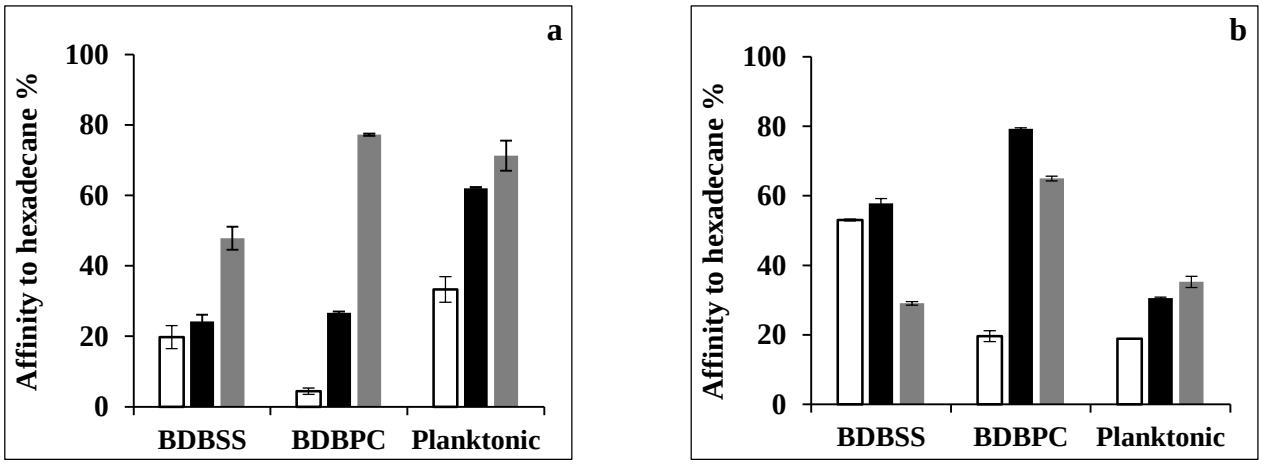


Figure 2 Affinity of Stainless-Steel-Biofilm-Detached (SSBDC), Polycarbonate-Biofilm Detached (PCBDC) and planktonic, *P. aeruginosa* cells to hexadecane. Cells were grown, at 20 (white column), 30 (black column) and 37°C (grey column), for 24 (a) and 48 h (b).

The increase of the growth temperature from 20 to 37°C increased the affinity of 24 and 48 h aged planktonic cells to hexadecane respectively from 33 to 71 % and from 19 to 35 % *($P < 0.05$)* (Fig. 2a and b). The affinity to hexadecane of 24 h-planktonic *P. aeruginosa* was *ca* 2-fold higher *($P < 0.05$)* than that of 48 h-planktonic cells whatever the temperature studied (Fig. 2a and b).

Furthermore, Table 1 showed that when the growth temperature increased from 20 to 37°C, the electron acceptor character of planktonic cells grown for 24 h and 48 h decreased respectively from 29.1 to 9.9 *(P < 0.05)* and from 52.8 to 39.8 *(P < 0.05)* (Table 1). However, the electron donor character of planktonic cells grown for 24 h and 48 h decreased respectively from 51.5 to 24.4 *(P < 0.05)* and from 71.5 to 58.1 *(P < 0.05)* when the growth temperature increased from 20 to 37°C (Table 1). Furthermore, our findings showed that the affinity of 24 h-SSBDCs to hexadecane increased from 19.8 to 47.8 % *(P < 0.05)* when the growth temperature increased from 20 to 37°C (Fig. 2a). The affinity of 48 h-SSBDCs to hexadecane decreased from 53 to 29.1 % *(P < 0.05)* under the same growth temperature conditions (Fig. 2b). Figure 2 showed that the affinity to hexadecane of 24 and 48h-PCBDCs increased respectively from 4.4 to 77.2 % *(P < 0.05)* (Fig. 2a) and from 19.7 to 65 % when the biofilm growth temperature increased from 20 to 37°C (Fig. 2b). Furthermore, the results showed that the electron donor character of 24 h-SSBDCs decreased from 65.2 to 36.4 then increased from 36.4 to 50.8 when the biofilm growth temperature the increased respectively from 20 to 30°C then from 30 to 37°C (Table 1). The electron donor character of 24 h-PCBDCs significantly decreased from 91.1 to 10.6 *(P < 0.05)* when the growth temperature increased from 20 to 37°C (Table 1). After 48 h of incubation, the electron donor character of SSBDCs increased from 25.7 to 42.8 *(P < 0.05)* and that of PCBDCs decreased from 47 to 24 (*P < 0.05*) when the growth temperature of biofilms increased from 20 to 37°C (Table 1). Table 1 also showed that the increase of biofilm growth temperature from 20 to 37°C significantly decreased the electron acceptor character of 24 h-SSBDCs from 45.2 to 26.1 (*P < 0.05*) (Table 1). Our results also showed that the electron acceptor character of 24 and 48 h -PCBDCs decreased respectively from 25.7 to - 0.1 and from 31.3 to 19.4 when the temperature increased from 20 to 37°C. The electron donor character of the 24 h planktonic cells was lower (*P < 0.05*) than that of 24 h PCBDCs at 20 and 30°C (Table 1). However, at 37°C, the electron donor character of the 24 h planktonic cells was 2.4-fold higher (*P < 0.05*) than that of 24 PCBDCs (Table 1). In addition, the electron acceptor character of the 24 h planktonic cells was higher (*P < 0.05*) than that of 24 h whatever the studied temperature, except at 30°C, where it was 4.4-fold lower (Table 1). The results also showed that the electron donor and the electron acceptor characters of the 48 h planktonic cells were significantly higher (*P < 0.05*) than those of biofilm detached cells whatever the studied condition (Table 1).

Table 1 Electron donor/acceptor character of planktonic and biofilm-detached *Pseudomonas aeruginosa* cells from stainless steel and polycarbonate surfaces, grown at 20, 30 and 37°C, during 24 and 48 h.

		Electron donor [a]			Electron acceptor [b]		
	T°C [c]	SSBDCs [d]	PCBDCs [e]	Planktonic	SSBDCs	PCBDCs	Planktonic
24 h	20°C	65.2 ± 2.1	91.1 ± 0.8	51.5 ± 0.7	45.2 ± 0.8	25.7 ± 0.4	29.1 ± 2.4
	30°C	36.4 ± 3.2	49.1 ± 0.4	33.6 ± 0.2	27 ± 1.2	49.8 ± 0.2	-11.3 ± 2.7
	37°C	50.8 ± 2.6	10.6 ± 0.4	24.4 ± 4,4	26.1 ± 0.5	-0.1 ± 0.9	9.9 ± 3.2
48 h	20°C	25.7 ± 0.5	47 ± 2.4	71.5 ± 0.9	-14.6 ± 1.6	31.3 ± 1.1	52.8 ± 0.7
	30°C	20.3 ± 1.2	9.3 ± 0.7	58.2 ± 1.6	33.1 ± 0.7	-0.1 ± 0.8	36.3 ± 0.7
	37°C	42.8 ± 0.7	24 ± 2	58.1 ± 1.3	29.3 ± 5	19.4 ± 0.7	39.8 ± 1.6

[a] The differences between the chloroform and hexadecane affinities of cells suspended in 100 mM PB (pH 7) presents the electron donor character
[b] The differences between the ethyl acetate and decane affinities of cells suspended in 100 mM PB (pH 7) presents the electron acceptor character
[c] T°C represents the growth temperature.
[d] SSBDCs represents the Stainless Steel-Biofilm-Detached-Cells
[e] PCBDCs represents the Polycarbonate-Biofilm-Detached-Cells

Effect of conditions effect on the adhesion of *P. aeruginosa* to abiotic surfaces

The goal here is to study the effect of the *P. aeruginosa* growth conditions on the adhesion behavior of biofilm-detached and planktonic *P. aeruginosa* cells on SS and PC.

Our findings showed that the adhesion of planktonic cells after 24 h of incubation is significantly higher than after 48 h *($P < 0.05$)* whatever the growth conditions, except on the SS at 20°C where the adhesion was similar (Fig. 3a and b). When the growth temperature increased from 20°C to 37°C, the planktonic cells adhesion on the SS increased by 2.2 fold *($P < 0.05$)* and 1.5 *($P < 0.05$)* fold respectively after 24 and 48 h of incubation (Fig. 3a). Moreover, the adhesion rate of 24 h planktonic *P. aeruginosa* cells to the PC increased by 2.3 fold *($P < 0.05$)* when the growth temperature increased from 20°C to 37°C (Fig. 3b). Moreover, the adhesion of planktonic cells on SS was significantly higher *($P < 0.05$)* than on the PC whatever the studied condition (Fig. 3a and b). Our results showed that the abiotic surface type and the temperature of the biofilm formation had an effect on the adhesion behavior of SSDBCs on the SS. Fig. 3c showed that the adhesion rates of 24 h-SSDBCs on the SS were similar *($P > 0.05$)* at 30 and 37°C but 1.6 fold lower than at

20°C. The same trend was observed for the PCDBCs adhesion on the PC. Our results showed also that the increase of incubation duration from 24 to 48 h decreased the adhesion of SSDBCs on the SS by 4 fold *(P < 0.05)* at 20°C (Fig. 3c). However, the incubation duration showed no significant effect *(P > 0.05)* on the adhesion of SSDBCs on the SS at 30 and 37°C (Fig. 3c). The increase of the incubation duration from 24 to 48 h decreased significantly *(P < 0.05)* the adhesion of PCDBCs on the PC by 11, 3 and 1.6 fold respectively at 20, 30 and 37°C. The adhesion of 48 h PCDBCs on the PC increased by 4 fold when the temperature increased from 20 to 37°C. Furthermore, we observed that the adhesion of SSDBCs on the SS surface was higher than PCDBCs adhesion on PC *(P < 0.05)* (Fig. 3c and d). Moreover, the adhesion rate of biofilm-detached cells was higher than that of planktonic cells *(P < 0.05)* whatever the studied conditions (Fig. 3a, b, c and d).

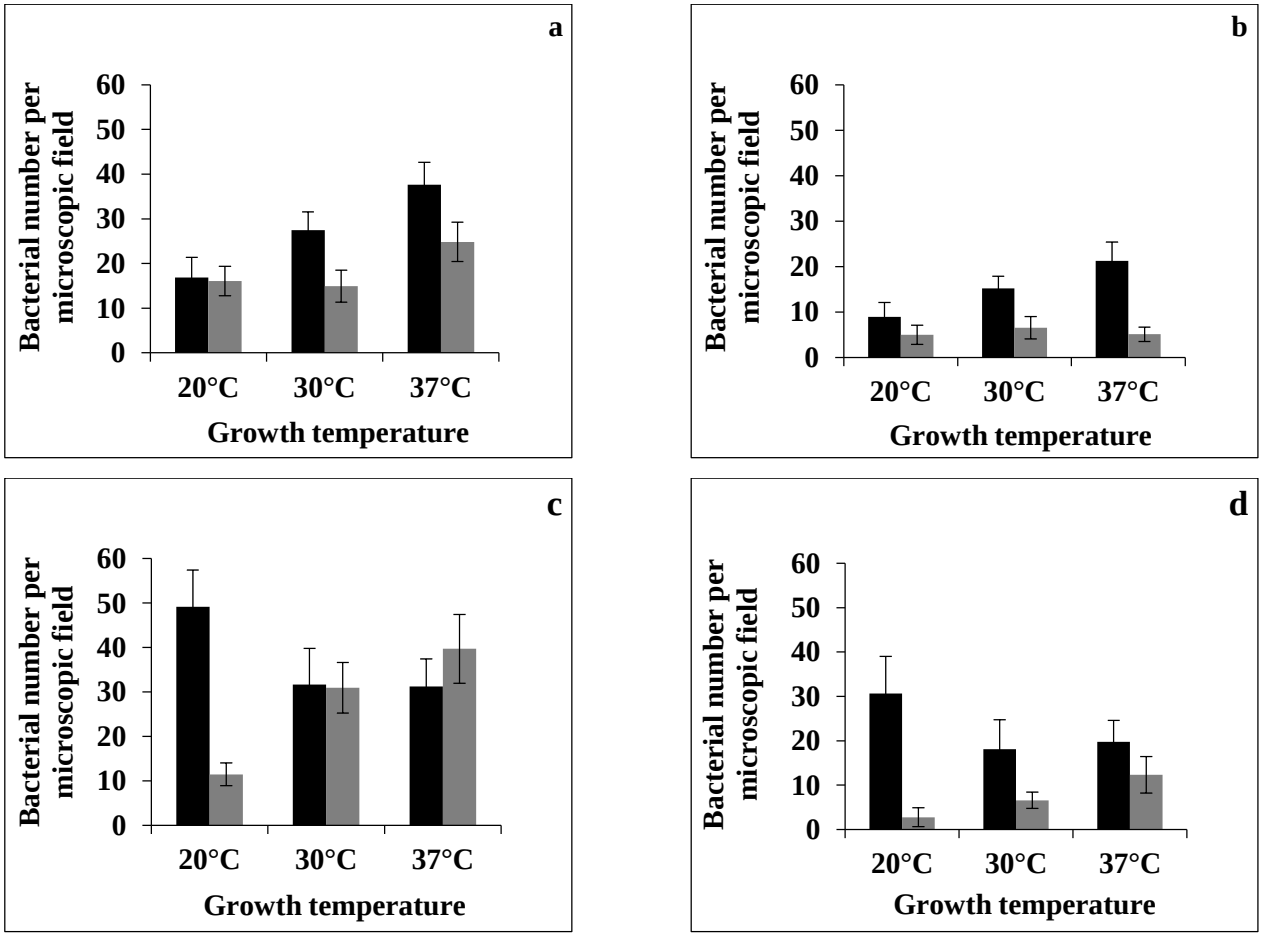


Figure 3 Adhesion of planktonic and biofilm-detached *Pseudomonas aeruginosa* cells on stainless steel and polycarbonate. Planktonic cells adhesion on stainless steel (a) and polycarbonate (b). Biofilm detached cells adhesion on stainless steel (c) and on polycarbonate (d). Cells grown for 24 (black column) and 48 h (grey column).

Effect of growth conditions on the proteases production by *P. aeruginosa*

The proteases activity was assessed in the supernatants recovered from planktonic and biofilm-cultures incubated at different growth conditions. The TSB has been used as a negative control and the results showed it had no proteolytic activity (Data not shown).

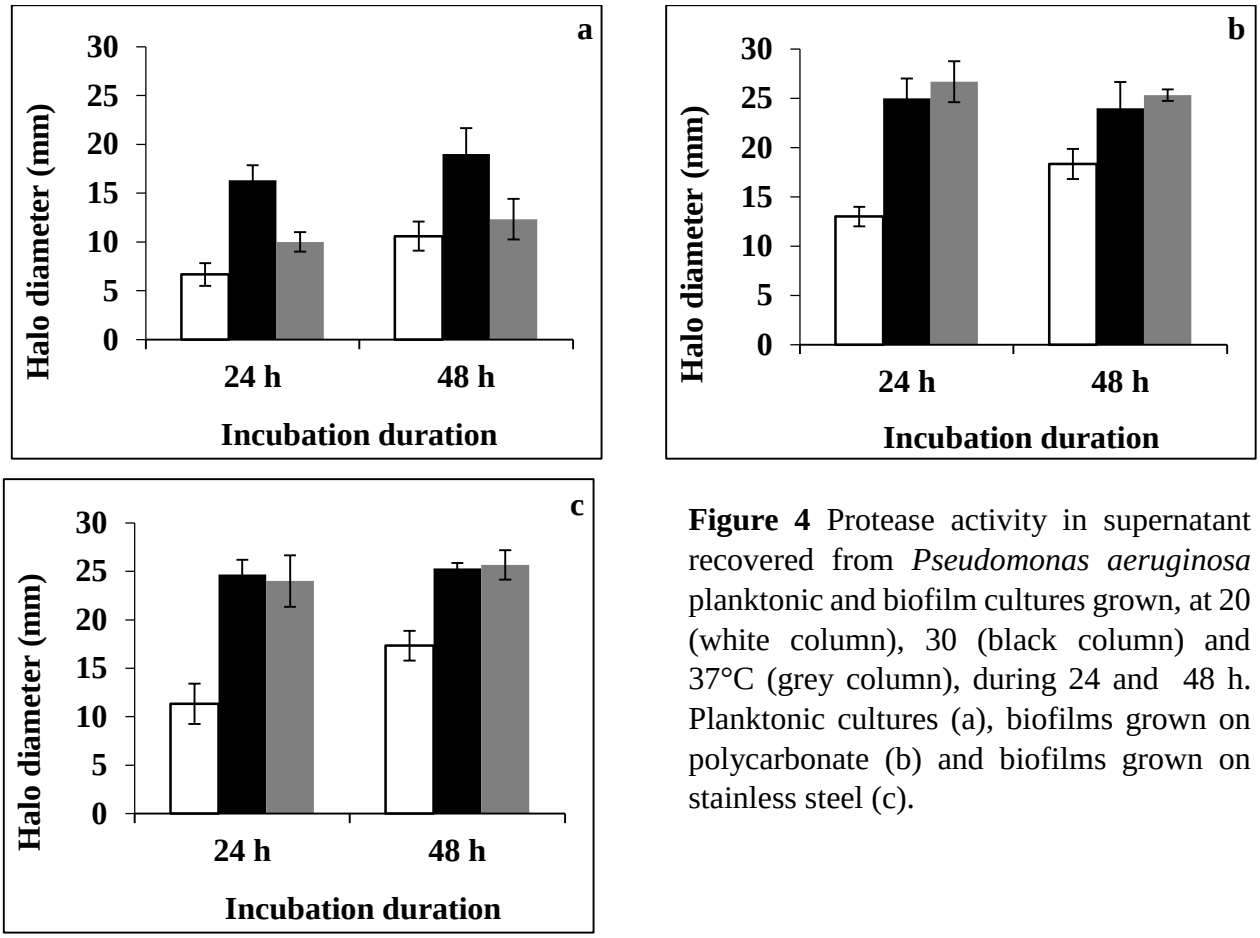


Figure 4 Protease activity in supernatant recovered from *Pseudomonas aeruginosa* planktonic and biofilm cultures grown, at 20 (white column), 30 (black column) and 37°C (grey column), during 24 and 48 h. Planktonic cultures (a), biofilms grown on polycarbonate (b) and biofilms grown on stainless steel (c).

The results showed that the proteolytic activity in the Planktonic-Culture-Supernatants (PCSs) seems to be dependent on the temperature and the incubation growth duration. The growth incubation temperature increase from 20 to 30°C, increased significantly ($P < 0.05$) the proteolytic activity of PCSs respectively by 2.4 and 1.8 fold after 24 and 48 h of incubation (Fig. 4a). When the temperature of incubation increased from 30 to 37°C, the proteolytic activity of *P. aeruginosa* PCSs decreased by *ca* 1.2-fold ($P < 0.05$) whatever the incubation duration (Fig. 4a). Our results also showed that the abiotic surface type had a significant effect on the proteolytic activity of sessile *P. aeruginosa* cells and this effect is dependent on the duration and the temperature of biofilm growth. When the growth incubation temperature increased from 20 to 37°C the proteolytic activity

of Biofilm-Culture-Supernatants (BCSs) increased by *ca* 2 and 1.4 fold ($P < 0.05$), respectively after an incubation of 24 and 48 h whatever the surface type (Fig. 4a and b). The proteolytic activity of BCSs was similar ($P > 0.05$) at 30 and 37°C whatever the studied conditions (Fig. 4a and b). Similar proteolytic activity was observed with culture supernatants recovered from biofilms grown on PC. Furthermore, the *P. aeruginosa* BCSs seem to have higher proteolytic activity than PCSs ($P < 0.05$) (Fig. 4a, b and c).

Incubation duration, growth temperature and surface type effect of on *P. aeruginosa* culture supernatant cytotoxicity

The cytotoxic effects of planktonic and biofilm culture supernatants against HeLa cells was investigated. The aim here is to assess the supernatant cytotoxicity of *P. aeruginosa* cells as a function of their growth conditions. Figure 5 showed the viability of HeLa cells, after an incubation of 3 h with the harvested supernatants. The results showed that TSB, used as a negative control, had no significant effect on the HeLa cells viability whatever the studied conditions (Fig. 5a, b and c). The results showed that the PCSs did not reduce the HeLa cell viability when cultures are incubated at 20 and 30°C (Fig. 5a). However, the 37°C PSCs reduced by 2-fold ($P < 0.05$) the HeLa cell viability whatever the incubation duration of planktonic cultures (Fig. 5a). Furthermore, after an incubation time of 24 h, the cytotoxicity of PC and the SS-BCSs significantly decreased the viability of HeLa cells respectively by 15 and 9.5 fold when the biofilm growth temperature increased from 20 to 37°C ($P < 0.05$) (Fig. 5b and c). The cytotoxicity of 48 h PC-BCSs significantly decreased the viability of HeLa cells to 6.8, 2.4 and 1.7 % respectively at 20, 30 and 37°C (Fig. 5b). At 20°C, the biofilm supernatants had not shown a significant cytotoxic effect against HeLa cells whatever the studied condition ($P > 0.05$) except for the 48 h PC-BCSs which reduced the HeLa cells viability to 6.8 % ($P < 0.05$) (Fig. 5b and c). Furthermore, the PC BCSs seem to be more cytotoxic than their SS and planktonic counterparts (Fig. 5a, b and c). After 24 h of incubation, the supernatants of 30 and 37°C PC-biofilms were respectively 7 and 5-fold more cytotoxic ($P < 0.05$) than that of their SS-biofilm (Fig. 5b and c). After 48 h, the cytotoxicity levels of the 20, 30 and 37°C PC-BCSs was respectively 12.5, 5.2 and 5.5-fold higher ($P < 0.05$) than those of 20, 30 and 37°C SS-BCSs (Fig. 5b and c). In addition, the cytotoxicity of the BCSs was significantly ($P < 0.05$) higher than that of PCSs (Fig. 5a, b and c).

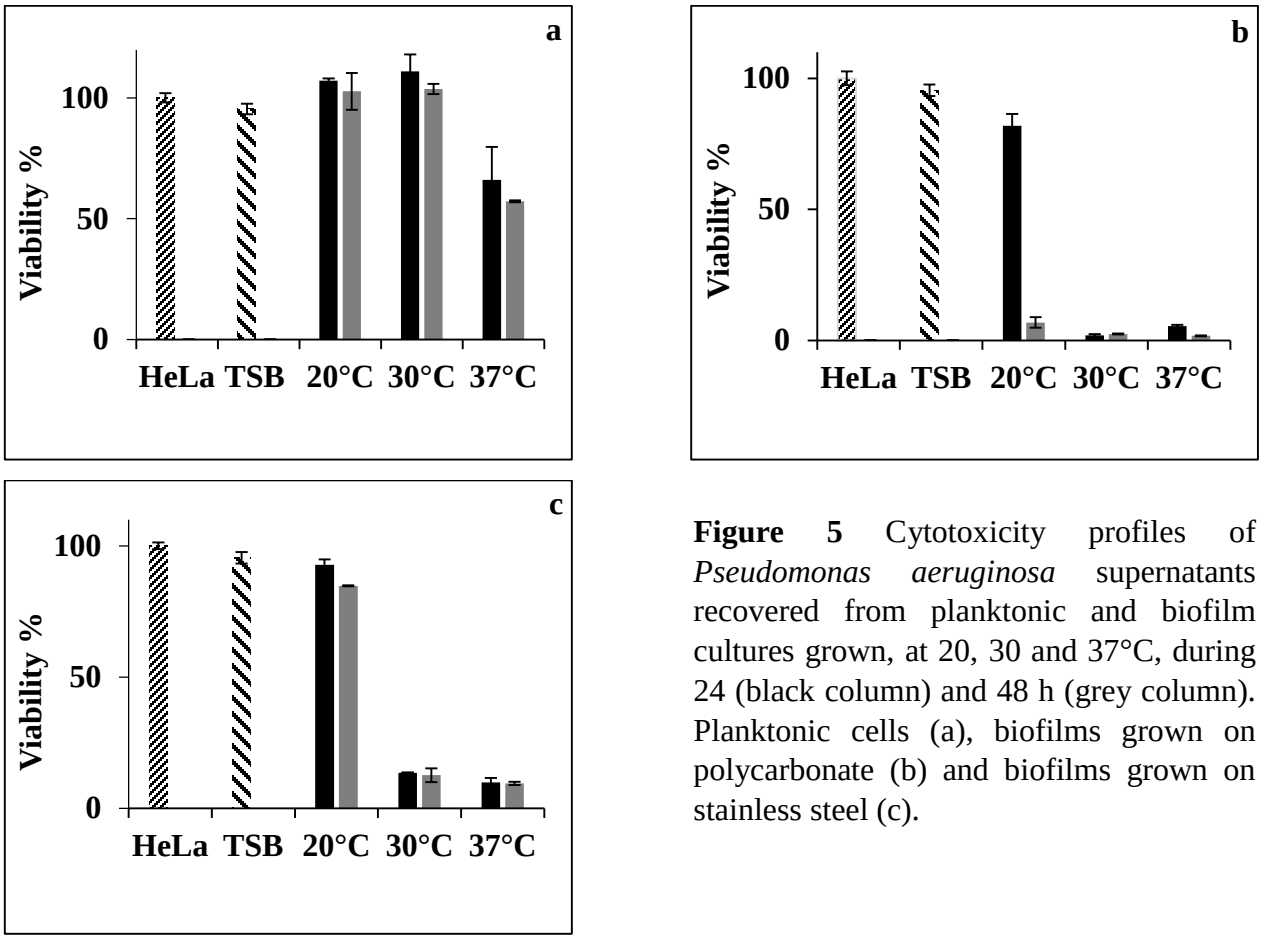


Figure 5 Cytotoxicity profiles of *Pseudomonas aeruginosa* supernatants recovered from planktonic and biofilm cultures grown, at 20, 30 and 37°C, during 24 (black column) and 48 h (grey column). Planktonic cells (a), biofilms grown on polycarbonate (b) and biofilms grown on stainless steel (c).

Effect of growth conditions on *P. aeruginosa* siderophore production

The results of Figure 6a showed that when the incubation temperature increased from 20 to 37°C, the siderophore units percentage of 24 and 48 h-PCSs increased respectively by 3.6 and 2-fold ($P < 0.05$). Moreover, the increase of the incubation duration from 24 to 48 h increased the siderophore units percentage by *ca* 3-fold at 20°C ($P < 0.05$) and by *ca 2* at both 30 and 37°C ($P < 0.05$) (Fig. 6a).

Our findings also showed that the surface type, the growth temperature and the incubation duration had a significant effect ($P < 0.05$) on siderophores production by sessile *P. aeruginosa* cells (Fig. 6b and c). The siderophore unit percentage of supernatant recovered from biofilms grown on the SS at 20°C was similar whatever the incubation duration (Fig. 6c). The increase of the incubation duration from 24 to 48 h increased the percentage of siderophore unit of SS-BCSs by *ca* 2-fold at 30 and 37°C (Fig. 6c). When the incubation temperature increased from 20 to 37°C, the siderophore units percentage of SS-BCSs increased from 10.5 to 44.2 % ($P < khelisssa0.05$) and from 11.8 to

76 % (*P < 0.05*) respectively after an incubation of 24 and 48 h (Fig. 6c). When the incubation temperature increased from 20 to 37°C the percentage of siderophore units of PC-BCSs, increased from 22.6 to 92 % (*P < 0.05*) and from 78.3 to 92.1 % (*P < 0.05*) respectively after incubation durations of 24 and 48 h (Fig. 6b). The percentage of siderophore units of PC-BCSs was similar at 30 and 37°C whatever the incubation duration. However, at 20°C, it increased from 22.6 % to 78.3 % (*P < 0.05*) when the incubation duration increased from 24 to 48 h (Fig. 6b). Furthermore, our results showed that the percentage of siderophore units of PC-BCSs was higher than those of SS-BCSs and PCSs (*P < 0.05*) whatever the condition studied (Fig. 6a, b and c).

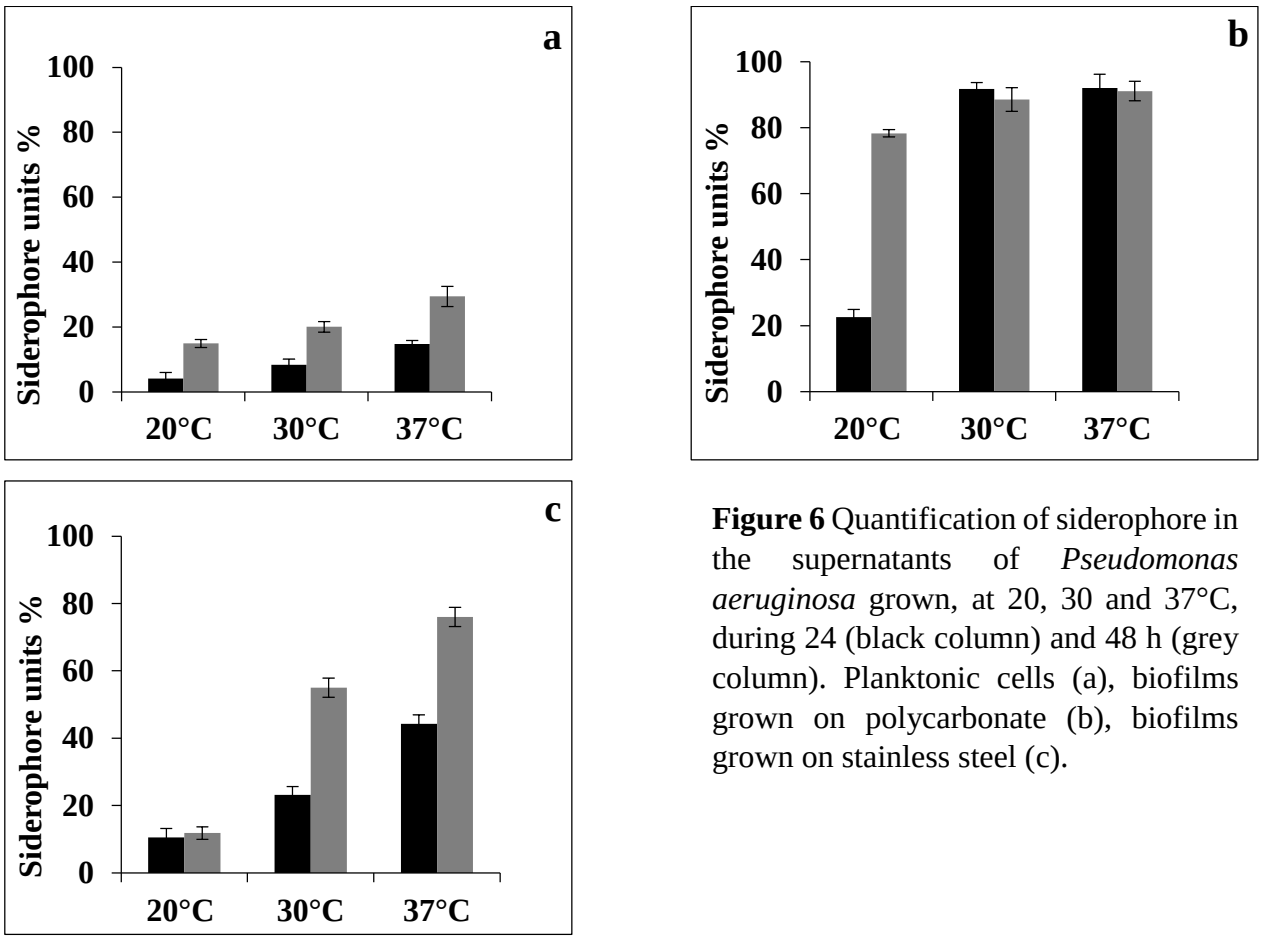


Figure 6 Quantification of siderophore in the supernatants of *Pseudomonas aeruginosa* grown, at 20, 30 and 37°C, during 24 (black column) and 48 h (grey column). Planktonic cells (a), biofilms grown on polycarbonate (b), biofilms grown on stainless steel (c).

Discussion

Our study investigated, in particular, the impact of growth conditions on the cell-surface physicochemical properties, the ability to adhere to the SS and PC, the production level of some virulence factors and cytotoxicity of *P. aeruginosa* populations grown under different physiological states. Our aim was to understand the impact of the studied growth conditions on the

physiology of *P. aeruginosa* biofilm-detached cells, and how they differ from those of planktonic cells, in order to assess the microbial risk related to their high persistence. Our results showed that the biofilm-detached *P. aeruginosa* cells adhered on abiotic surfaces more than their planktonic counterparts, these data extend this behavior to *P. aeruginosa*. Similar results underlined the greater ability of biofilm-detached *Halomonas venusta* and *Streptococcus mutans* cells to colonize new surfaces than their planktonic counterparts (Liu et al. 2013; Berlanga et al. 2015). In addition, our results underlined that the adhesion of all *P. aeruginosa* cell populations was greater on SS than on the PC and that the adhesion increased with the increase of the incubation temperature. Consistent with this observation, several studies highlighted a similar effect of the bacterial growth temperature and abiotic surface type on the adhesion of *P. aeruginosa* (Abdallah et al. 2014; Cappello and Guglielmino 2006). The significant difference between biofilm-detached and planktonic cells in terms of adhesion on SS and PC, suggests that these populations might have distinct phenotypic properties under our experimental conditions. Thus, we were interested in the surface physicochemical properties of the different bacterial populations, considering their involvement in the adhesion, as a function of their growth conditions. Our findings showed all *P. aeruginosa* cells were negatively charged probably due to the carboxylate and sulfate groups of the microbial surface structures such as proteins and fimbriae (Peel et al. 1988; Zita and Hermansson 1997). Furthermore, our results showed that biofilm-detached cells were less negatively charged than their planktonic counterparts which may result in a decrease of repulsive electrostatic forces between cells and negatively charged abiotic surfaces such as SS. Hence, these results may explain the higher adhesion rates of biofilm-detached cells on the SS as compared to that of planktonic cells. Nevertheless, the electrostatic interactions are considered to be negligible when a neutral polymeric surface, such as PC, is used (Gottenbos et al. 2000; Morra and Cassinelli 1997). Thus, our results suggest that electrostatic interactions could not always explain the bacterial adhesion to abiotic surfaces indicating the involvement of other physiochemical parameters. In this case, the acid-base interaction which involves the electric charge transfer between the bacterial wall and the abiotic surface may play an important role in the adhesion. In fact, according to the Lewis definition, bacteria and the substrate surfaces may show an acid or basic character related to the presence of chemical groups which behave as an electron acceptor or donor (Della Volpe and Siboni 2000; Briandet et al. 1999; Boonaert and Rouxhet 2000; Jones et al. 1996; Morra and Cassinelli 1996; Cunliffe et al. 1999). By contrast, our results underlined that the electron donor

characters of *P. aeruginosa* did not always describe the differences found in the bacterial adhesion on both surfaces. In addition, cell hydrophobicity is also a determinant parameter in the bacterial adhesion (Van Loosdrecht et al. 1990). Overall, our results showed that the hydrophobicity of *P. aeruginosa* biofilm-detached and planktonic cells increased when the growth temperature of this bacterium increased from 20 to 37°C. In general, the more hydrophobic cells adhere more strongly to hydrophobic surfaces, while hydrophilic cells strongly adhere to hydrophilic surfaces (Kochkodan et al. 2008; Giaouris et al. 2009). Therefore, the increase of the bacterial hydrophobicity with the increase of growth temperature may explain the increase of *P. aeruginosa* adhesion onto SS and PC. Considering our results, under our conditions, *P. aeruginosa* culture populations are relatively easily adhering to hydrophobic implants such as catheters, mechanical heart valves or pacemakers which are constructed from hydrophobic materials (silicon, teflon, etc.). However, and if we consider particularly the results related to the bacterial surface hydrophobicity, we could suggest that cell adhesion should be greater on hydrophobic supports such as the PC which is not the case under our experimental conditions. In fact, and in accordance with a previous study (Abdallah et al. 2014) our results showed a greater adhesion rate of *P. aeruginosa* cells on SS than on the PC. These results highlight that the attachment to surfaces depends not only on the hydrophobicity of cells and that other factors are involved in this process including the acid-base interactions (Bos et al. 1999).

Furthermore, it has been reported that intrinsic factors related to the cell envelope, such as adhesins, cell wall proteins, extracellular polymers, flagellar motility, pili are involved in bacterial attachment to abiotic surfaces (Hori and Matsumoto 2010). Compared with planktonic cells, the high ability of the biofilm-detached cells to attach to new surfaces may be explained by the upregulated of certain genes (Liu et al. 2013). These conclusions led us to believe that biofilm-detached cells represent a high risk for recolonization of surfaces: they adhere more easily to form biofilms than planktonic cells. Hence, this confirms the importance of considering attachment ability as a major bacterial virulence factor. Furthermore, an interesting investigation consisted in studying the virulence and cytotoxicity of the different *P. aeruginosa* populations, because the ability to develop biofilms is often associated with the expression of virulence (De Kievit et al. 2001). The proteasic activity of culture supernatants showed that the protease was produced with higher rates by sessile cells than by their planktonic ones. In addition, our results showed that the protease production increased with the increase of the growth temperature and the incubation

duration of planktonic and sessile cultures. Our results are in agreement with other investigations (Coenye et al. 2007; Mahoney et al. 2010) who underlined the greater virulence factors production by sessile bacteria than the planktonic cells. However, these results are in disagreement with Wang et al. (2011) who reported that the activity of virulence factors was greater in the supernatants of planktonic cultures when compared to that of the biofilm cultures. The high activity of the soluble protease in the biofilm supernatant was hypothesized to have a dispersal function by degrading the biofilm matrix that holds cells together (Silva and Benitez 2016). Furthermore, our results underlined a greater siderophore production in the supernatants of biofilm than those of planktonic cultures. *P. aeruginosa* cells living under biofilm state on PC seem to produce more siderophores than their SS counterparts. In *P. aeruginosa*, the production of siderophores under iron-limiting conditions contributes to virulence (Buckling et al. 2007). Kamerud et al. (2013) reported that iron and nickel particles may migrate from the SS to the culture media, the low availability of iron in the supernatant of biofilm grown on PC, which is a non-metallic surface, may result in an increase of the production of siderophores. In order to highlight the *P. aeruginosa* health risk, the cytotoxic effect of *P. aeruginosa* biofilm and planktonic-culture-supernatants on the HeLa cells was studied *in vitro*. Our results showed that the Biofilm-Culture-Supernatants (BCSs) had a higher cytotoxic effect on HeLa cells than the Planktonic-Culture-Supernatants (PCSs) under all the studied conditions. Moreover, our results showed that the cytotoxicity of BCSs and PCSs increased with the increase of the bacterial growth temperature and the incubation duration. Hence, this may explain the effect of these factors on the bacterial metabolism and production of virulence factors (Holler et al. 1998; Jozwiak et al. 2005; Secor et al. 2011; Khelissa et al. 2017b). Our results are in agreement with those of Secor et al. (2011) who reported that the different metabolic states in planktonic and biofilm cultures have a large pathogenic impact on the human cells. Our results showed that BCSs recovered from biofilms grown on the PC surface were more cytotoxic to HeLa cells than those of biofilm grown on SS.

Our findings showed that biofilm-detached-cells are phenotypically distinct from planktonically grown cells. In addition, this work underlined the hazardous characters of biofilm-detached-cells which appeared to have a better ability to adhere to abiotic surfaces than planktonic cells. Thus, to prevent the persistence and the spread of contamination it is of importance to fight against biofilm-detached cells. Our results underlined that physiochemical properties of bacterial cells cannot always fully explain the bacterial adhesion. An interesting perspective would consist in studying

the role of structural adhesins in the bacterial adhesion to abiotic surfaces. In addition, our results showed high growth temperature increase the virulence and the cytotoxicity of the *P. aeruginosa* cells. This effect is more important when cells are structured in biofilm at growth temperatures close to that of the human body. Moreover, biofilm formed on plastic surfaces, such as PC, showed higher pathogenic risk. Thus, the presence biofilm on indwelled medical devices which are mostly made of plastic material increases the risk of severe infections. Hence, the presence of biofilms in the hospital settings should be limited to prevent the emergence healthcare-associated infections. This work offers a novel knowledge about *P. aeruginosa* infectious potential depending on its physiological state, which suggests that a virulent strain may be able to increase its virulence by forming a biofilm and achieve persistent infection *in vivo*.

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Conclusion

In this chapter we studied the effect of bacterial growth temperature (20, 30 and 37°C), surface type (stainless steel and polycarbonate) and the incubation duration (24 and 48h) on the zeta potential, hydrophobicity and electron donor/acceptor character of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Two mode of growth were compared, the biofilm-detached and the planktonic one. This investigation aimed to unravel the relationship between the environmental conditions, and the bacterial growth mode, on the bacterial surface physicochemical properties and on bacterial adhesion behavior to abiotic surfaces. Our results showed that the bacterial growth conditions significantly influenced the physiology of both biofilm-detached and planktonic cells and at the same time the bacterial adhesion to the stainless steel and polycarbonate. In fact, our results showed that the increase of growth temperatures and incubation duration seemed to increase the bacterial adhesion to abiotic surfaces. The adhesion on the stainless steel was higher than that on the polycarbonate. Moreover, the adhesion rate of biofilm-detached cells was greater than that of planktonic ones. Furthermore, this work allowed to characterize the pathogenic character of the two studied cell populations as function of the growth conditions cited above. In fact, our data underlined that the supernatants recovered from biofilm cultures were more cytotoxic and presented greater virulence factor activity than those recovered from planktonic cultures. The increase of both temperature and duration of incubation enhanced the pathogenicity of the studied cells. This behavior was greater when biofilm were grow on polycarbonate.

Overall, our data highlighted the need to pay more attention to biofilm-detached cells, in the future researches, which present an important ability to contaminate abiotic surfaces and high cytotoxic potential. In addition, further experiments should focus on the quantification of bacterial adhesion forces using atomic force microscopy in order to extend the knowledge of the mechanisms mediating bacterial adhesion to abiotic surfaces and to reduce microbiological risk related to the biofilm formation in food and medical environments.

CHAPTER: III

ARTICLE IV AND V – RESULTS

Introduction

The biofilm formation on medical and food surfaces presents a continuous source of pathogenic bacteria which are generally involved in foodborne and nosocomial infections. Several strategies have been adopted to fight against the emergence of these infections. The use of disinfectant agents is one of the most frequently applied strategies to maintain an effectively clean and highly hygienic surfaces that are likely to be in contact with foods or with human body. Such strategy may be efficient to reduce the microbiological risk in the high affected areas. However, the high resistance of biofilm cells often leads to disinfection failure. In fact, most of disinfectant products are optimized against free floating cells which are up to 1,000 fold more sensitive to antimicrobial agents than their attached counterpart (Donlan and Costerton 2002). Thus, the biofilm is considered as a source of persistent contaminations which are difficult to eradicate and are involved in recurrent economic and health losses (Costerton et al. 1999). It has been reported that biofilm development process includes distinct stages (i) reversible and (ii) irreversible attachment, (iii) formation of micro-colonies, (iv) development of macro-colonies, and finally (v) cell detachment and dispersal (Donlan & Costerton, 2002; Sauer et al. 2002). It has been suggested that, the detachment step is due generally to nutrient and oxygen starvation of bacteria imbedded in biofilm. Bacterial detachment allows the spread of contamination by colonizing new surfaces (Allison et al. 1990; Sauer et al. 2002; Kaplan et al. 2003; Hunt et al. 2004). Nevertheless, few works have been focused on the detached cells to investigate their physiology and their susceptibility to disinfectants (Boles et al. 2004; Bester et al. 2005; Ymele-Leki & Ross, 2007). In fact, most of studies considered planktonic and biofilm structured cells and did not take into account the biofilm-detached population. Therefore, there is a need to elucidate the resistance behavior of biofilm-detached cells towards the disinfectant treatments. Such a study should help to assess the microbiological risk associated with the biofilm dissemination and to set up suitable and efficient disinfection protocols. Furthermore, it is also of great importance to understand the resistance mechanisms of biofilm cells. It has been reported that the biofilm resistance is thought to be linked to the involvement of the biofilm matrix which impedes the penetration of biocides inside the biofilm (Bridier et al. 2011). However, our previous study underlined that the biofilm matrix cannot always explain the biofilm resistance to disinfecting agents (Abdallah et al. 2014b). Thus the role of bacterial membrane fluidity and expression of certain resistance gene might be investigated (Campanac et

al. 2002). Such investigations should also consider environmental conditions commonly met in food and medical sectors, such as temperature changes, pH, aw, etc, (Donlan and Costerton 2002; Simões et al. 2010). Thus, the evaluation of disinfectants efficiency on biofilms-detached cells grown under different environmental conditions related food and medical sectors, should help to unravel the mechanisms of bacterial resistance to antimicrobial agents and also help to reduce issues associated to biofilms in these sectors.

Article IV

Biofilm-detached *Staphylococcus aureus* tolerance to benzalkonium chloride treatment and resistance mechanism on the cellular level

Simon Oussama KHELISSA[a, b], Marwan ABDALLAH[a], Charafeddine JAMA[a], Nour-Eddine CHIHIB[b] *

[a] CNRS, ENSCL, UMR 8207-UMET-PSI, Université de Lille 1, Avenue Dimitri Mendeleïev, 59652 Villeneuve d'Ascq, France

[b] CNRS, INRA, UMR 8207-UMET-PIHM, Université de Lille 1, 369 rue Jules Guesde, 59651 Villeneuve d'Ascq, France

*Corresponding author: email: nour-eddine.chihib@univ-lille1.fr, Tel: +33320435443

Abstract

Our study aimed to investigate the effect of the growth temperature on the resistance of *Staphylococcus aureus* to benzalkonium chloride (BAC) treatment. The antibacterial assay was performed against biofilm-detached and planktonic cells grown at 20 and 37°C, for 24 h. The increase of growth temperature increased the resistance of *Staphylococcus aureus* cells to BAC. The planktonic cells were found to be more susceptible to BAC than their biofilm-detached counterparts. The increase of growth temperature resulted in an increase of *S. aureus* cells membrane rigidity. Higher membrane fluidity was observed in the case of planktonic cells compared to the biofilm-detached ones. Overall, the resistance of biofilm-detached and planktonic *S. aureus* cells seems to depend on the growth temperature and involves the cell membrane fluidity. Our study represents an interesting report describing the impact of environment²al conditions on the susceptibility of the biofilm-detached cells to BAC. Membrane fluidity modulation is likely the key resistance mechanism of *S. aureus* to disinfectants at the cellular scale

Keywords: *Staphylococcus aureus*, biofilm-detached cells, environmental conditions, resistance to disinfectant, membrane fluidity

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) [1,2]. In order to fight against FBDs, disinfectants are constantly applied to maintain a high level of surface hygiene in food fields [3]. Such procedure is thought to be effective to decrease the microbiological risk in the highly affected areas. It is now established that most of the bacteria, live attached to surfaces and form a complex structure, called biofilm [4]. The biofilm-associated cells are different from those of free-floating planktonic cells [5–7]. Furthermore, biofilm cells have been shown to be significantly more resistant to disinfectants than their planktonic counterparts [5]. This high tolerance to biocides promotes pathogens persistence on abiotic surfaces. In the food sector, bacteria, are usually exposed to different environmental constraints such as temperature changes, shear forces, pH, etc. [5,8]. The exposure of biofilm to high shear forces can lead to the detachment of biofilm clumps, which may be enhanced when treated with sanitizers [9,10]. Thus, biofilm represents a bacterial reservoir which, once detached, serve as a continuous source of contamination resulting in severe FBDs. Although the detachment of cells from biofilms is of great importance to the dissemination of contamination and infection in public health settings [11].

The disinfection efficacy of commonly used biocides on biofilm-detached particles have not been deeply studied. Thus, the study of biofilm-detached S. aureus cells tolerance to disinfectants should help to understand the mechanisms of cell persistence, and to reduce issues associated to biofilms dispersal, in the food sector. In fact, several mechanisms have been proposed to explain the apparent resistance of biofilm cells to antimicrobial agents [12]. The biofilm resistance mechanisms might be observed at the macroscopic and the microscopic levels. At the macroscopic scale, the biofilm resistance is related to the production of an extracellular matrix composed of extracellular polymeric substances (EPS) which mainly include exopolysaccharides, proteins and nucleic acids. The multiple layers of cells and EPS may constitute a complex and compact structure within which biocides find it difficult to penetrate and reach internal layers, thus hampering their efficacy [12].

The biofilm resistance at the microscopic or cellular scale is thought to be linked to the modification of bacterial physiology. One important adaptive response of bacterial cells to non-optimal growth conditions is the modification observed in their membrane lipids [13]. The bacterial membrane, composed primarily of phospholipids and proteins, constitutes the first line of bacterial defence

against antimicrobial. The phospholipid fatty acyl chains, which are also influenced by the environmental conditions, determine the fluidity of bacterial membrane and may hinder the penetration of antimicrobial [14,15].

In this regard, the purpose of this work is to study the effect of growth temperature (20 and 37°C) on *S. aureus* cells detached from biofilm formed on stainless steel resistance to BAC, a quaternary ammonium compound (QAC) commonly used in food and health sectors. This study also aimed to study the membrane fluidity of biofilm-detached *S. aureus*, in order to characterize it as a mechanism of cellular resistance to BAC treatment.

Materials and methods

Bacterial culture conditions and suspension preparation

Staphylococcus aureus CIP 4.83 strain used in this study was stored frozen (- 80°C) in Tryptic Soy Broth (TSB; Biokar Diagnostics, Pantin, France) containing 40% (v/v) of glycerol. Precultures were prepared by the addition of 100 µl from frozen stock to 5 ml of TSB in sterile glass test tube and then incubated at 20 (for 48 h) or 37°C (for 24 h). The cultures were prepared by inoculating 10^4 CFU/l from the preculture tubes in 50 ml of TSB and incubation under shaking (160 rpm) at 20 or 37°C. Cultures were stopped at the late exponential phase. *S. aureus* cells from cultures were harvested by centrifugation (5000 *g* for 5 min at 20°C). Bacteria were washed twice with Potassium Phosphate Buffer (PPB; 100 mM, pH 7) and finally, the bacterial suspensions were adjusted to 10^8 CFU/ml by adjusting the optical density to OD_{620nm} = 0.110±0.005 using a Jenway 6320D UV/visible light spectrophotometer, in PPB.

Slide preparation

The SS (304L, Equinox, France) slides were first immersed in acetone (Fluka, Sigma-Aldrich, France) for 1 h then rinsed under tap water, followed by three washes with distilled water. The slides were then soaked in 500 ml of DDM ECO detergent (1 %) for 15 min at 20°C (ANIOS, France), rinsed 3 times in distilled water and three times in ultrapure water (Milli-Q® Academic, Millipore, France) at 20°C. The SS slides were dried and sterilized by autoclaving at 121°C for 20 min.

Development of biofilms

The upper face of each coupon (90 x 90 mm) was covered by 12 ml of the corresponding cell suspension (20 or 37°C) adjusted to 10^7 CFU/ml and incubated at 20°C for 1 h to allow the bacterial adhesion. Afterwards, coupons rinsed twice by immersing in PPB using sterile forceps to remove loosely attached cells. The upper face of each coupon was covered by 12 ml of TSB and the biofilm formation was started by incubating coupons statically, at the same temperature of bacterial-cell-cultures (20 or 37°C), for an incubation duration of 24 h. Following the 24 h incubation, each coupon was rinsed twice by pipetting with 12 ml of PPB in order to remove loosely attached cells. The strongly attached bacteria were recovered into 10 ml of PPB by surface scraping, harvested by centrifugation at 5000 *g* for 5 min at 20°C and then washed once with 20 ml of PPB. In order to remove the biofilm matrix residue, attached cells were sonicated at 37 kHz for 5 min at 20°C. The bacterial suspensions were adjusted to a cell concentration of 10^8 CFU/ml for the anti-bacterial assays.

BAC minimum inhibitory concentration (MIC) determination

The MIC of BAC was determined by micro-dilution growth inhibition assays using a Bioscreen C (Labsystems, Helsinki, Finland), which measures kinetically, the development of turbidity by vertical photometry. *S. aureus* cells were cultured, as previously, in Mueller Hinton Broth (MHB) (Bio-Rad, France). Two-fold dilutions of BAC ranging from 25 to 0 mg/l in MHB (Bio-Rad, France), were made in micro-plates and *S. aureus* suspension was added to give a bacterial density of approximately 10^6 CFU/ml. For each test plate, 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 then incubated in the Bioscreen C at 37°C under continuous agitation and $OD_{600\ nm}$ was measured every 2 h for 48 h. The MIC was defined as the lowest concentration of the antibiotic that prevented growth, as measured by optical density. Micro-dilution plate growth inhibition assays were repeated in triplicate and mean log $OD_{600\ nm}$ values were plotted versus time.

Disinfection of bacteria detached form biofilms formed on SS

For the disinfection assay, 1 ml of bacterial suspension, adjusted to 10^8 CFU/ml was introduced to 1 ml of 6 mg/l BAC solution. After 5 min contact time at 20°C, 1 ml of this mixture was transferred into 9 ml of neutralizing solution [16] to stop the antibacterial action. After the disinfection

treatment, surviving culturable cells were enumerated by Tryptic Soy Agar (TSA) plating after 10-fold serial dilutions in PPB. Plates were incubated at 37°C for 24h and the number of viable and culturable cells was expressed in log CFU/ml. The results represent the mean of three independent experiments. For the control assays, the disinfectant solution was replaced by TSB.

BAC-induced potassium (K^+) leakage assessment

Biofilm-detached and planktonic *S. aureus* cells grown at 20 and 37°C were concentrated to 10^{10} CFU/ml. 5 ml of the concentrated bacterial suspensions were introduced into silicone cap glass reaction vessels (100-ml wide-necked flasks) containing 45 ml of BAC solution (final concentration of 3 mg/l in 50 ml final volume) or HEPES buffer (negative control). Before the introduction of cells to the biocide, a 10-fold dilution of the stock inoculum in HEPES buffer was made, and 5 ml of that suspension was passed through a 0.2 µm filter (Sartorius™ Minisart™ NML Syringe Filters, France) into a 15-ml glass Bijou bottle. This filtrate represented the level of K^+ leakage from *S. aureus* cells at time zero. This procedure was performed before the inoculation of each reaction vessel. After the addition of bacterial cells to the reaction vessel, 4-ml samples were removed and filtrated at 5, 10, 15, 20, 30, 60 and 90 min. Each sample was removed using a 10-ml sterile plastic syringe attached to a sterile needle to enable easy access to the reaction mixture suspension through the silicon cap. All filtrates were stored at -80°C until analysis. The results represent the average of three independent experiments and each experiment was done in duplicate.

K^+ analysis

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). Before calibration and measurement of samples, the instrument was autozeroed with HEPES buffer. The instrument was calibrated using K^+ standards (analytical grade; Sigma-Aldrich, Poole, United Kingdom) of 0, 0.5, 1, 2.5, and 5 mg/l (final concentration) prepared in HEPES buffer. The filtrate samples were diluted in HEPES buffer to give K^+ levels that could be detected at the midpoint of the calibration graph. The K^+ standards were re-measured periodically during the experiment to verify instrument accuracy. The results represent the average of three independent experiments.

Cellular fatty acids extraction and analysis

Biofilm-detached and planktonic *S. aureus* cells were harvested as above, either by scrapping cells embedded in biofilm from the rinsed coupons or by centrifuging planktonic culture then resuspended in 10 ml of PPB. Then the cells were sonicated (37 kHz, 5 min), and vortexed for 30 s. The cells were pelleted by centrifugation (10 000 *g*, 10 min at 4°C), and the pellets, containing 10^9 CFU/ml were washed twice with cold distilled water. The washed pellet was mixed with 1 ml of the saponification solution [13] and transferred to extraction tube. Subsequently, cells were submitted to the saponification and methylation. Fatty acid methyl esters extraction was realized as described previously by [13]. Methyl esters analysis were performed on a GC-2014 gas chromatograph (Shimadzu, Japan) equipped with a Zebron ZB-FFAP (30 m×0.25 mm) capillary column (Phenomenex, Australia), and connected to Thermo-Finnigan Trace DSQ mass spectrometer (Thermo Fisher Scientific, USA) according to Abdallah et al. [16]. The results represent the average of three independent experiments and each experiment is done in duplicate.

Scanning electron microscope (SEM) observation

The morphology of *S. aureus* cells upon BAC treatment was assessed by SEM. 1 ml of the BAC or TSB treated then neutralized planktonic and 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, critical point dried and coated with carbon thin film before examination in the scanning electron microscope. Microscopy was performed with a Hitachi S4700 microscope at 3 kV.

Statistics

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) to determine the significance of differences. Results were considered significant at a *P* value of < 0.05.

Results

Determination of the BAC minimum inhibitory concentration by microplate growth inhibition assays

Microplate growth inhibition assays were performed on *S. aureus* in order to analyse their growth in presence of two-fold serial dilution of BAC final concentrations ranging from 25 to 0 mg/l (Figure 1).

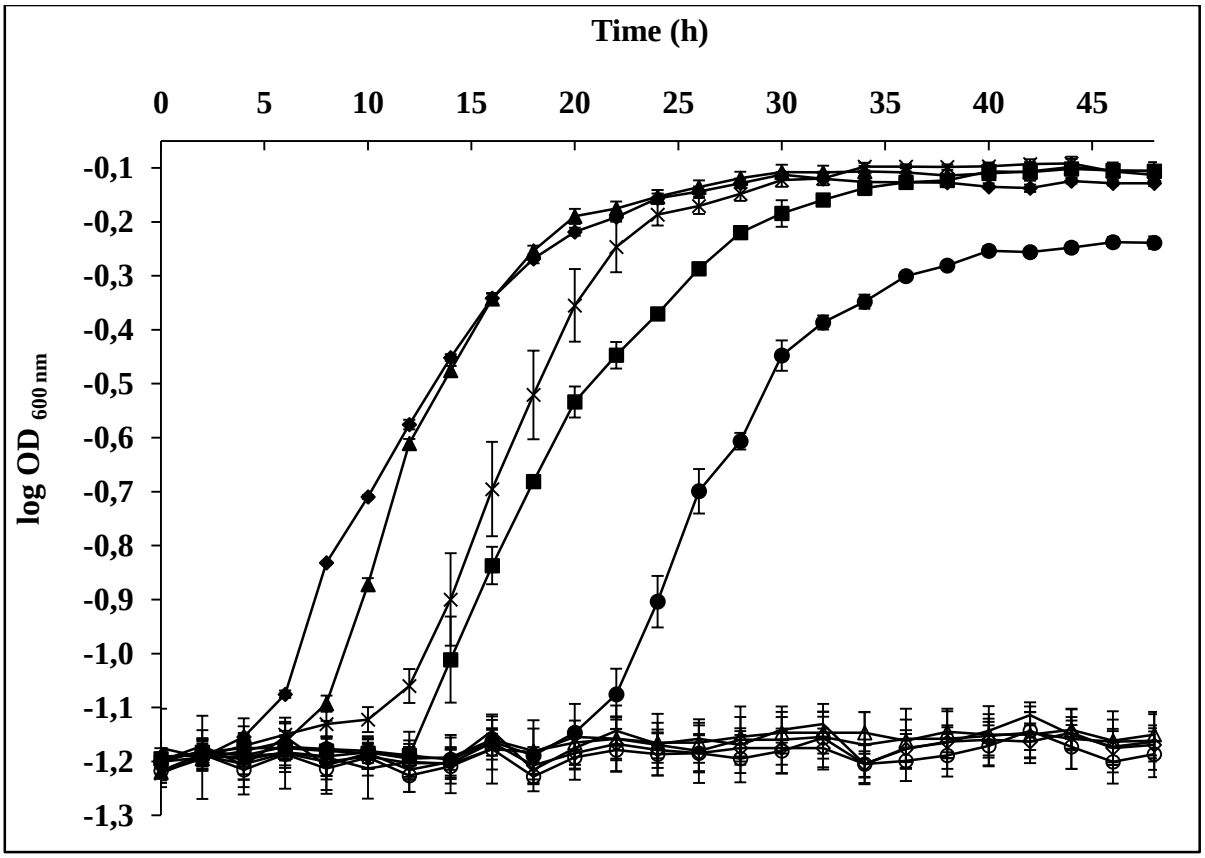

Figure 1 Growth curves of Staphylococcus aureus inoculums in presence of benzalkonium chloride concentrations (BAC) of 25 (−), 12.5 (○), 6.25 (◊), 3.12 (Δ), 1.56 (●), 0.8 (■), 0.4 (x), 0.2 (▲) and 0 mg/L (♦). The concentration at which there was no visible growth was considered the MIC. The bacterial growth in MH broth without bacteria and BAC was measured to ensure the sterility of the growth medium (sterile control) (+).

Figure 1 showed that at a concentration of 0.2 mg/l, cells have the same growth behaviour ($P < 0.05$) as the control (cells without BAC). At of 0.4, 0.8 and 1.5 mg/l of BAC, the growth profile was different from the control ($P < 0.05$). At BAC concentration of ≥ 3 mg/l, the bacterial growth was totally inhibited (Figure 1). According to those data, the MIC of BAC was considered at 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 values of *S. aureus* cultures were 0.066 and

0.046 h^{-1} when the BAC concentrations were ranging respectively from 0 to 0.2 and from 0.4 to 1.5 mg/l. Furthermore, the lag time consistently extended with increased BAC concentrations (Figure 1). When the BAC concentrations increased from 0 to 1.5 mg/l the λ increased from 8 to 20 h.

Effect of growth temperature on the resistance of biofilm-detached and planktonic *S. aureus* to BAC

The resistance of *S. aureus* biofilm-detached and planktonic cells, grown at 20 and 37°C, to BAC treatment was studied. The results showed that the TSB treatments, used as the negative control, had no significant effect ($P < 0.05$) on the initial population of *S. aureus*, whatever the studied conditions (Figure 2). The average of viable and culturable counts of cells, after TSB treatment for 5 min, was of *ca* 7.3 log CFU/ml (Figure 2).

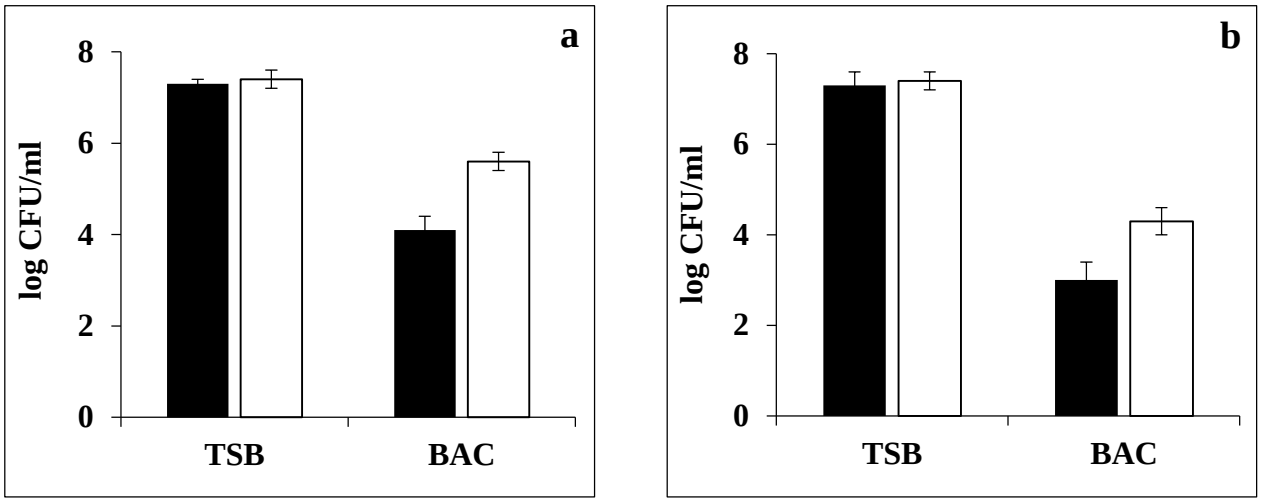

Figure 2 Effect of benzalkonium chloride (BAC) treatment on biofilm-detached (a) and planktonic (b) Staphylococcus aureus grown during 24 h at 20°C (black column) and 37°C (white column). The bacterial density is presented in log CFU/ml. The ± SEM for three replicates are illustrated after treatments with TSB (control) and BAC.

When *S. aureus* biofilm-detached cells were grown at 20 and 37°C, the 5 min BAC treatment at 3 mg/l, resulted in a significant reduction of the initial viable and culturable count ($P < 0.05$) by 3.2 and 1.8 log CFU/ml respectively (Figure 2a). However, when *S. aureus* planktonic cells were grown at 20 and 37°C the BAC treatment at 3 mg/l led respectively to a significant reduction of the initial viable and culturable count by 4.3 and 3.1 log CFU/ml ($P < 0.05$) (Figure 2b). Furthermore, the results also showed that the remained viable and culturable count of biofilm-

detached cells after BAC treatment was *ca.* 1.3 log higher than that of planktonic cells ($P < 0.05$) whatever the cells growth temperature (Figure 2).

Effect of BAC treatment on the cell membrane integrity

Measurements of K^+ efflux from the bacterial cells were realized to assess the biofilm-detached cells resistance to BAC treatment when grown at 20 and 37°C. This was carried out by monitoring the extracellular K^+ concentration in *S. aureus* biofilm-detached and planktonic cells grown at 20 and 37 °C for 24 h (Figure 3).

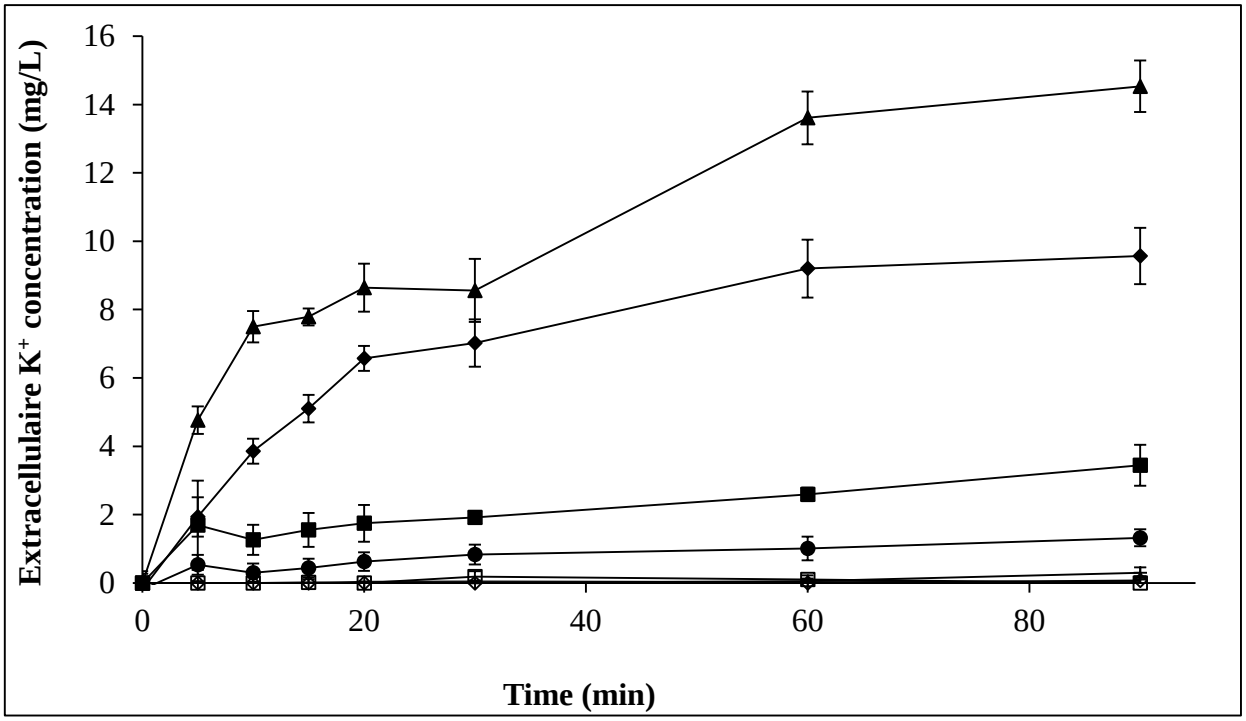

Figure 3 Kinetics of potassium (K^+) efflux in the suspension medium filtrates of biofilm-detached and planktonic *Staphylococcus aureus* cells upon Benzalkonium Chloride (BAC) treatment: BAC-treated biofilm-detached cells grown at 20 (♦) and 37°C (●); BAC-treated planktonic cells grown at 20 (▲) and 37°C (■); HEPES-treated biofilm-detached cells grown at 20 (+) and 37°C (◊); HEPES-treated planktonic cells grown at 20 (-) and 37°C (□). The K^+ concentrations are presented in mg/l. The ± SEM for three replicates are illustrated after treatments with HEPES buffer (control) and BAC

The addition of BAC at a final concentration of 3 mg/l resulted in an immediate increase of extracellular K^+. Figure 3 shows that K^+ efflux was higher in planktonic than biofilm-detached cells whatever the temperature conditions ($P < 0.05$). The addition of BAC resulted at 5 min in an increase of extracellular K^+ concentration to 4.8 ($P < 0.05$) and 1.7 mg/l ($P < 0.05$) in the planktonic

cell suspensions grown respectively at 20 and 37°C (Figure 3). Under the same condition, the extracellular K^+ concentration in the biofilm-detached cell suspensions grown at 20 and 37°C increased respectively to 1.9 mg/l ($P < 0.05$) and 0.5 mg/l ($P < 0.05$) (Figure 3).

The results also showed a significant effect of growth temperature on K^+ efflux when *S. aureus* was grown at 20°C (Figure 3). After BAC addition, the extracellular K^+ concentration increased from 0 to a mean value of 16 mg/l ($P < 0.05$) and from 0 to a mean value of 14 mg/l ($P < 0.05$) respectively in the planktonic and biofilm-detached cells suspension of *S. aureus* grown at 20°C (Figure 3). However, the K^+ extracellular concentration of planktonic and biofilm-detached *S. aureus* cells grown at 37°C increased respectively, to 5 ($P < 0.05$) and 2.5 mg/l ($P < 0.05$) (Figure 3). Our results also showed that the HEPES buffer addition had no effect on K^+ efflux which remained stable whatever the studied condition.

Morphological changes and observations

To investigate structural modifications of *S. aureus* biofilm-detached and planktonic cells after the exposition to BAC treatment, bacterial samples were analyzed by SEM. As shown in (Figure 4a, c, e and g), the untreated (control samples) *S. aureus* biofilm-detached and planktonic cells cultivated at 20 and 37°C looked round and exhibited an undamaged normal smooth lining. However, when *S. aureus* biofilm-detached and planktonic cells were exposed for 5 min to BAC 3 mg/l (CMI concentration) significant morphological changes were observed in both biofilm-detached and planktonic cells (Figure 4b, d, f and h). *S. aureus* planktonic cells grown at 20°C showed holes in their cell wall. In addition, cell lysis was also observed resulting from BAC treatment a result of deep, irreversible membrane damage (Figure 4b). S. aureus biofilm-detached cells cultivated at 20°C showed multiple dents on their surface (Figure 4d). However, the morphological changes of *S. aureus* biofilm-detached and planktonic cells grown at 37°C seemed less pronounced than those of their 20°C grown counterparts (Figure 4f and h). Hence, the BAC treated planktonic cells grown at 37°C were less bulky and their membrane seemed to be rougher, wrinkled and deformed compared with untreated cells (Figure 4f). The treated *S. aureus* biofilm-detached cells grown at 37°C became generally distorted in shape and had few craters in their cell walls (Figure 4h).

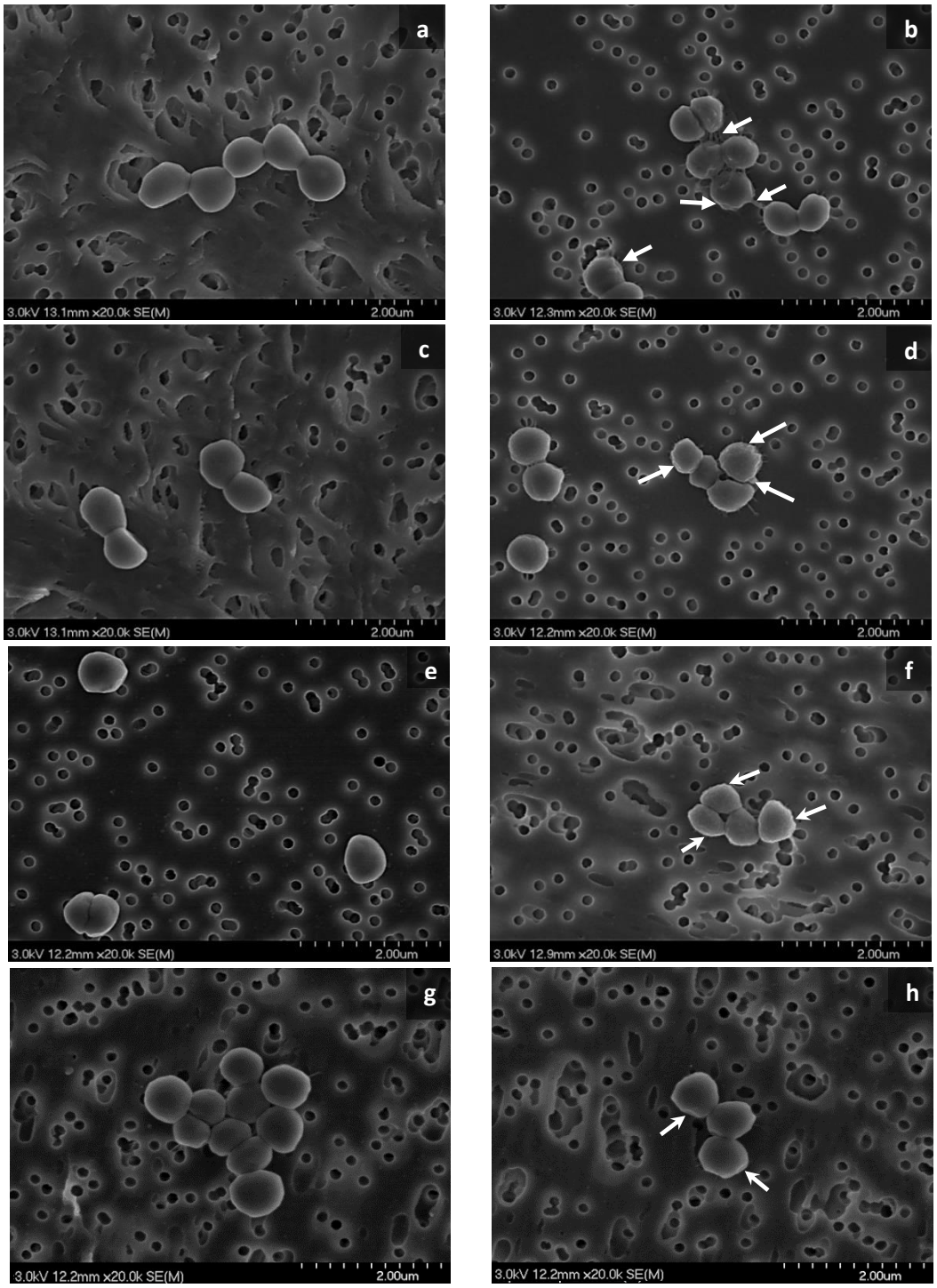

Figure 4 SEM images of untreated *Staphylococcus aureus* cells. Untreated planktonic grown at 20°C (a) untreated biofilm-detached gown at 20°C (c), untreated planktonic grown at 37°C (e) and untreated biofilm-detached gown at 37°C (g) showing a smooth cell membrane of a normal shaped cells; treated *S. aureus* cells with benzalkonium chloride for 5 min: planktonic grown at 20°C (b), biofilm-detached gown at 20°C (d), planktonic grown at 37°C (f) and biofilm-detached gown at 37°C (h), cell morphologies changed, distortion and even collapse in cell morphology are observed.

Effect of incubation time, growth temperature and surface type on membrane fatty acids of planktonic and sessile *S. aureus*

Membrane fatty acid (FA) profiles of *S. aureus* cells detached from biofilms and their planktonic counterparts grown for 24 h at 20 and 37°C was analyzed (Figure 5). This investigation was performed to study the effect of growth temperature on the membrane fatty acid composition that controls the membrane fluidity. Figure 5 showed that the amounts of anteiso C15 (aC15) were maintained in a stable level whatever the growth temperature ($P > 0.05$). The results also showed that the aC15 amount in the biofilm-detached cell membranes was significantly lower than that of planktonic cells ($P < 0.05$). Moreover, the total long-chain FA amounts of biofilm-detached cells were 1.4 and 1.2-fold ($P < 0.05$) higher than their planktonic counterparts respectively at 20 and 37°C (Figure 5). The increase of long-chain FA amounts was tightly related to the aC19, C18 and C20 FAs amounts which increased significantly ($P < 0.05$) with the increase of growth temperature from 20 to 37°C (Figure 5). In addition, biofilm-detached cells displayed greater ($P < 0.05$) levels of these three fatty acids (aC19, C18 and C20) than the planktonic ones whatever the studied temperature (Figure 5).

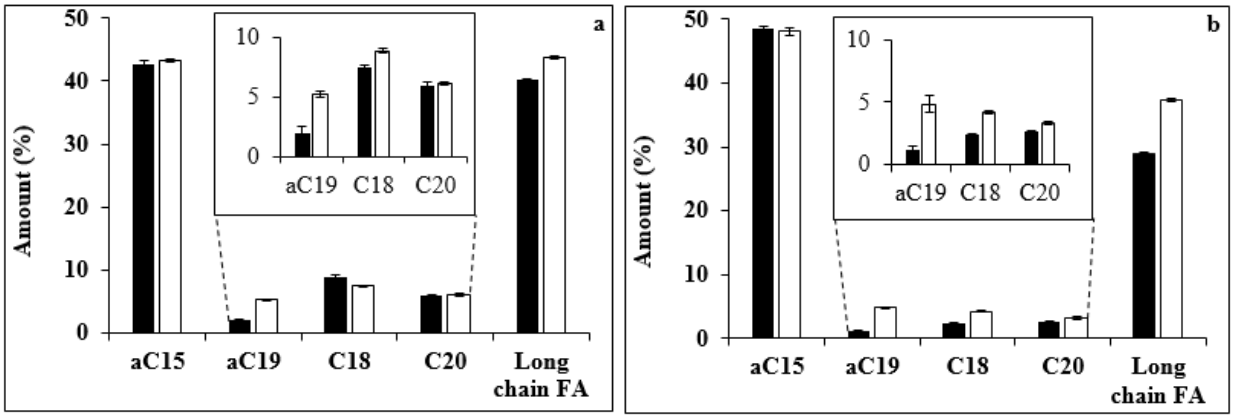

Figure 5 Fatty acid (FA) composition of *Staphylococcus aureus* biofilm-detached (a) and planktonic cells (b) cultivated at 20°C (black column) and 37°C (white column) for 24 h. a for anteiso

Discussion

Biofilm-detached cells constitute a major source of bacterial dissemination and contamination of food contact surfaces [11]. Thus, it is of importance to carry out research on biofilm-detached cells to further assess the microbiological risk associated with these cells and to optimize appropriate

disinfection procedure. *S. aureus* is a pathogenic bacterium, associated with serious FBDs, able to adhere and form biofilms on food contact surfaces [2,3,16]. Our findings showed that biofilm-detached cells phenotype is highly different from the planktonic one. Cells grown under biofilm state are known to be more resistant to antimicrobial agents than those grown under floating state [6]. This resistance is often associated with the extracellular matrix, a compact structure, which may prevent disinfectants from penetrating and reaching the bacterial cells [17,18]. The goal in the present work is to investigate the resistance at the cellular level when they are in a biofilm or when they are detached from a biofilm.

Our results underline that the increase of growth temperature from 20 to 37°C increased the resistance of *S. aureus* biofilm-detached and planktonic cells to BAC treatment. Moreover, the resistance of biofilm-detached cells to BAC was significantly higher than that of planktonic ones whatever the growth temperature conditions. However, Rollet et al., (2009) reported that the sessile, biofilm-detached and planktonic *Pseudomonas aeruginosa* showed the same antibiotic susceptibility profile [11]. According to our results, biofilm-detached cells in food processing industry represent a serious public health problem. In fact, after being released from biofilm, the cells represent a real threat as they acquire resistance and require an effective antimicrobial treatment adapted to this physiological state. It has been reported that Quaternary Ammonium Compounds (QACs) have several cell targets such as the perturbation of bacterial membranes [17,20]. In this context, our results showed that when biofilm-detached and planktonic *S. aureus* cells were exposed to 3 $mg.l^{-1}$ of BAC, an immediate K^+ leakage was measured. The K^+ efflux rate decreased with the increase of the incubation temperature from 20 to 37°C. These results also showed that at a given incubation temperature (20 or 37°C) K^+ leakage was higher in Planktonic than in biofilm-detached cells. Thus, the BAC bactericidal activity depends both on the physiological state and the incubation temperature. These data highlighted that the resistance at a cellular level when bacteria are embedded or when they are detached from a biofilm, in addition, is due to other mechanisms in addition to the well-studied protective effect of the matrix. These findings were comforted by structural modifications observed on *S. aureus* cells when exposed to BAC and analysed by SEM. Cells treated with BAC 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, it is expected that *S. aureus* resistance to BAC may be developed through the modification of the cellular membrane fatty acid composition which controls the membrane fluidity. In the present study, *S. aureus* biofilm-detached cells displayed a significantly higher SFAs amount compared to planktonic cells, due to the high increase of long-chain SFAs, such as 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. However, 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 temperature. In fact, the aC15 has been reported to be a major determinant of membrane fluidity for many Gram-positive bacteria regarding its low melting point [21]. In addition, shortening fatty acid chain length results in lower-melting-point fatty acids [22]. 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 [23].

Thus, the membrane fatty acids profiles of the studied *S. aureus* cells would have probably resulted in a lower fluidity of the biofilm-detached cells membranes compared to those of planktonic cells. This could explain the greater resistance of the biofilm-detached cells to BAC treatment. The phase transition temperatures of the phosphatidylcholine containing aC19:0 (36.7°C), C18 (26°C) are significantly higher than those of phosphatidylcholine containing aC15 (-13.9°C) [24,25]. Thus, 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 temperatures. Wang et al. (2016) recently showed that the increase of growth temperature decreased the fluidity of *S. aureus* membrane in response to electroporation [22]. Thus, growth temperature increase results in *S. aureus* cells membrane more impermeable to BAC. Taken together our findings, may explain the increase of planktonic and biofilm-detached cells resistance to BAC treatment with the increase of incubation 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

Conclusion:

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. *S. aureus* is extremely susceptible to BAC which had high antimicrobial leading to membrane collapse and irreversible loss of membrane integrity with consequent leakage of intracellular K^+. The resistance behaviour correlated with the membrane fluidity. Therefore, it is suggested that modification of membrane fatty acids composition occurred as a direct resistance mechanism at the cellular scale by *S. aureus* cells.

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Article V

A study of biofilm-detached *Pseudomonas aeruginosa* susceptibility to benzalkonium chloride and associated bacterial resistance strategy

Simon Oussama KHELISSA [a, b], Marwan ABDALLAH [a], Charafeddine JAMA [a], Nour-Eddine CHIHIB [b] *

[a] CNRS, ENSCL, UMR 8207-UMET-PSI, Université de Lille 1, Avenue Dimitri Mendeleïev, 59652 Villeneuve d'Ascq, France

[b] CNRS, INRA, UMR 8207-UMET-PIHM, Université de Lille 1, 369 rue Jules Guesde, 59651 Villeneuve d'Ascq, France

* Corresponding author: Dr. Nour-Eddine CHIHIB.

Tel: +33 320435443

Email: nour-eddine.chihib@univ-lille1.fr

Abstract

This study aimed to study *P. aeruginosa* cells detached from biofilm formed on stainless steel and to compare them with their planktonic counterparts as a function of growth temperature (20 and 37°C). First, we tested their susceptibility to benzalkonium chloride (BAC). Then we investigated the effect of BAC on their membrane integrity and we studied the role of the membrane fluidity in the cell-scale-resistance mechanism. Our results showed that biofilm-detached cells were more susceptible to BAC treatment than the planktonic ones. The leakage of intracellular potassium after BAC treatment was more important in the case of biofilm-detached cells, which mirrors their membrane vulnerability. Interestingly, the increase of the growth temperature from 20 to 37°C increased the membrane rigidity of planktonic cells as compared to their biofilm-detached counterparts. Our data highlighted that when are released from the biofilm structure they lose rapidly their biofilm phenotype. Thus under our experimental conditions, the phenotype of *P. aeruginosa* biofilm-detached cells are distinguishable from those of planktonic and biofilms cells. The modification of membrane fatty acid profiles seems to be an effective resistance strategy at a cellular scale, for *P. aeruginosa* to survive BAC treatment.

Keywords: *Pseudomonas aeruginosa*, biofilm-detached cell, planktonic cell, benzalkonium chloride, disinfectant resistance, membrane fluidity

Introduction

In natural and human-made environments, bacteria live mostly attached to surfaces and form a complex and protective structure called biofilm, while the freely floating cells form (planktonic cells) seems to be a transitory growth mode (Davey and O'toole 2000; Jefferson 2004). *Pseudomonas aeruginosa* is Gram-negative bacterium able to form a biofilm on abiotic and biotic surfaces (Dunne 2002). This ubiquitous bacterium is naturally present in the soil, on vegetation, and in water. It is known as an opportunistic human pathogen which resists to antibiotics and antiseptics, this makes it the main causes of nosocomial infections affecting immune-compromised patients (Elsen *et al.* 2014). In fact, *P. aeruginosa* is one of the main colonizers of clinical environments.

Planktonic and biofilms cells are physiologically different, particularly in their resistance towards biocides and antibiotics treatments (Davies 2003). During biofilm formation cycle bacteria can detach from biofilm and colonize new surfaces (Allison *et al.* 1990; Sauer *et al.* 2002; Smith and Hunter 2008; Kaplan 2010). This step is critical in case of contaminated closed surfaces. However, few works have been carried to assess the microbiological risk associated with biofilm-detached cells in term of resistance to sanitizing agents. Quaternary Ammonium Compounds (QACs) are commonly used as disinfectants in both medical and food sectors. QACs affect the membrane permeability of bacteria causing leakage of cytoplasmic material (Russell, Suller and Maillard 1999). Benzalkonium chloride is one of the most a widely used QAC for the disinfection of medical and food-processing environments (Mustapha and Liewen 1989).

Therefore, we aimed to study and compare the resistance of biofilm-detached and planktonic *P. aeruginosa* cells grown at 20 and 37°C to BAC. This will allow understanding the resistance strategy adapted by *P. aeruginosa* when grown under these two lifestyles. We completed our work with an original comparison of the effect of BAC treatment on the leakage of the intracellular potassium, used as a direct membrane damage indicator, to investigate the ability of both cell populations to maintain their membrane integrity after disinfectant treatment. The membrane fatty acid profile of biofilm-detached and planktonic cells was investigated in order to study the involvement of membrane fluidity as a mechanism of their resistance to BAC.

Materials and methods

Culture conditions and cell suspension preparation

Precultures were prepared by inoculating 5 ml of TSB with 100 µl of *P. aeruginosa* CIP 103467 strain stored at -80°C in Tryptic Soy Broth (TSB; Biokar Diagnostics, Pantin, France) in stock tubes containing 40% (v/v) of glycerol. Precultures were incubated for 24 h at 37°C, or for 48 h at 20°C. The cultures were prepared by inoculating 10^4 CFU.ml^{-1} from the preculture tubes in 500-ml sterile flasks containing 50 ml of TSB, then incubated under shaking (160 rpm) at 20 or 37°C. The cultures were stopped at the late exponential phase and cells were pelleted by centrifugation at 5000 g for 5 min at 20°C and washed twice with 20 ml of 100 mM Potassium Phosphate Buffer (PPB; pH 7). The bacterial suspensions were sonicated at 37 kHz (Elmasonic S60H, Elma®) for 5 min at 20°C to disperse cells before being adjusted to 10^7 CFU.ml^{-1}.

Stainless steel coupons preparation

The SS (304L, Equinox, France) coupons used for this study were soaked in ethanol 95° (Fluka, Sigma-Aldrich, France) overnight after which they were rinsed with distilled water before being soaked in 1 % DDM ECO detergent (ANIOS, France), for 15 min, at 20°C. Coupons were then vigorously rinsed in distilled water and then in ultrapure water (Milli-Q® Academic, Millipore, France) at 20°C in order to remove detergent residue. Coupons were air-dried before being sterilized by autoclaving at 121°C for 20 min.

Biofilm formation assays

Sterile coupons were placed in cell culture dishes. 12 ml of the cell suspension (10^7 CFU.ml^{-1}) were deposited on the SS coupons, then left to stand for one hour at 20°C to allow the bacterial adhesion. After one hour, the deposited volume was removed and coupons were gently rinsed twice with 12 ml of PPB. The coupons were covered by 12 ml of TSB then incubated at the same temperature of bacterial-cell-cultures (20 or 37°C), for 24 h. The biofilms covering the SS coupons surfaces were rinsed twice with 12 ml of PPB in order to remove loosely attached cells. Then attached cells were recovered into 10 ml of PPB by surface scraping and pelleted by centrifugation at 5000 g for 5 min at 20°C. The pelt was washed once with 20 ml of PPB. In order to remove residual biofilm matrix, attached cells were resuspended in 20 ml of PPB then sonicated at 37 kHz for 5 min at 20°C. Finally, the harvested bacteria were recovered in 20 ml of PPB, after being centrifuged for 5 min

at 5000 *g*. The bacterial suspensions were adjusted to a cell concentration of 10^8 CFU.ml^{-1} to perform the anti-bacterial assays.

BAC minimum inhibitory concentration (MIC) determination

The MIC of BAC was determined by micro-dilution growth inhibition assays using a Bioscreen C (Labsystems, Helsinki, Finland), which measures kinetically, the development of turbidity by vertical photometry. *P. aeruginosa* cells were cultured like above in Mueller Hinton broth (MHB) (Bio-Rad, France). Two-fold serial dilutions of BAC (ranging from 50 to 0 mg.l^{-1} in MHB), were distributed to each well of the Bioscreen micro-dilution plates (100 µl). Then, 100 µl of *S. aureus* test suspension (10^6 CFU.ml^{-1}) were added. For each test plate, two BAC-free controls were maintained, one with 200 µl of the MHB alone (sterility control) and the other with 100 µl of MHB plus 100 µl of inoculum suspension (growth control). The micro-dilution plates were then incubated in the Bioscreen C at 37°C under continuous agitation and $OD_{600\ nm}$ was measured every 2 h for 48 h. The MIC was defined as the lowest concentration of the BAC that prevented growth, as measured by optical density. Micro-dilution plate growth inhibition assays were repeated in triplicate and mean log $OD_{600\ nm}$ values were plotted versus time.

BAC antibacterial assays

For the antibacterial assays, planktonic cells were harvested by centrifugation for 5 min at 5000 *g* at 20°C and supernatants were discarded. Biofilm cells were scraped from SS coupons as described previously. 1 ml of bacterial suspension, adjusted to 10^8 CFU.ml^{-1} (as confirmed by colony counts on Tryptic Soy Agar broth (TSA; Biokar Diagnostics, France) plates) was added to 1 ml of 25 mg.l^{-1} BAC solution. 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. In order to count the surviving culturable cells, serial dilutions were realized in PPB. Samples of 100 µl were spread on TSA plates and incubated at 37°C for 24 h. The number of viable and culturable cells is expressed in log CFU.ml^{-1}. The results represent the mean of three independent experiments. For the control assays, the TSB was used instead of BAC.

BAC-induced K^+ leakage assessment

Harvested biofilm-detached and planktonic *P. aeruginosa* cells grown at 20 and 37°C were concentrated to 10^{10} CFU.ml^{-1} in HEPES buffer. 5 ml of the concentrated bacterial suspensions

were introduced into silicone cap glass reaction vessels containing 45 ml of BAC prepared in HEPES buffer (final concentration of 12.5 $mg.l^{-1}$ in 50 ml final volume) or HEPES buffer without BAC (negative control). Before the introduction of cells to the biocide, a 10-fold dilution of the stock inoculum in HEPES buffer was made, and 5 ml of that suspension was passed through a 0.2 µm filter (Sartorius™ Minisart™ NML Syringe Filters, France) into a 15-ml glass Bijou bottle. This filtrate represented the level of K^+ leakage from *P. aeruginosa* cells at time zero. This procedure was performed before the inoculation of each reaction vessel.

After the addition of bacterial cells to the reaction vessel, 4-ml samples were removed and filtrated at 5, 10, 15, 20, 30, 60 and 90 min. Each sample was removed using a 10-ml sterile plastic syringe attached to a sterile needle to enable easy access to the reaction mixture suspension through the silicon cap. All filtrates were stored at -80°C until analysis. The results represent the average of three independent experiments and each experiment was done in duplicate.

Potassium analysis

The potassium ion 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). Before calibration and measurement of samples, the instrument was autozeroed with HEPES buffer, and this was repeated periodically throughout the analysis. The instrument was calibrated using potassium standards (analytical grade; Sigma-Aldrich, Poole, United Kingdom) of 0, 0.5, 1, 2.5, and 5 $mg.l^{-1}$ (final concentration) prepared in HEPES buffer. There was a linear relationship between emission and potassium concentration. The filtrate samples were diluted in HEPES buffer to give potassium levels that could be detected at the midpoint of the calibration graph. The potassium standards were re-measured periodically during the experiment to verify instrument accuracy. The results represent the average of three independent experiments.

Extraction and analysis of bacterial membrane fatty acids

Biofilm-detached and planktonic *P. aeruginosa* cells were harvested as stated above then sonicated at 37 kHz during 5 min then vortexed for 30 s. The cells were pelleted by centrifugation (10 000 *g*, 15 min at 4°C). The supernatant was discarded and the pellet containing 10^{10} $CFU.ml^{-1}$ was washed twice with cold distilled water. The pellet was mixed with the saponification solution (Chihib *et al.* 2003). Subsequently, cells were submitted to the saponification and methylation. Fatty acid methyl

esters extraction was realized as described by Chihib *et al.* (2003). Methyl esters analysis were performed on a GC-2014 gas chromatograph (Shimadzu, Japan) equipped with a Zebron ZB-FFAP) capillary column (Phenomenex, Australia), and connected to Thermo-Finnigan Trace DSQ mass spectrometer (Thermo Fisher Scientific, USA) according to Abdallah *et al.* (2015). The MS spectra and retention index were compared both with those available in libraries to identify the compounds. The results represent the average of three independent experiments and each experiment is done in duplicate.

Scanning electron microscope (SEM) observation

The cell morphology of *P. aeruginosa* cells grown for 24 h at 20 and 37°C after 5 min of BAC treatment was assessed by SEM. 1 ml of the BAC or TSB treated then neutralized planktonic and 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, critical point dried and coated with carbon thin film before examination in the scanning electron microscope. Microscopy was performed with a Hitachi S4700 microscope at 3 kV.

Statistics

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

Results

Determination of the BAC MIC

Microplate growth inhibition assays were performed on *P. aeruginosa* in order to determine the MIC of BAC (Fig. 1). The bacterial growth was assessed in presence of twofold serial dilution of BAC final concentrations ranging from 25 to 0 mg. l^{-1} (Fig. 1). Figure 1 showed that when *P. aeruginosa* was exposed to BAC concentrations (from 0.2 to 6.25 mg.l^{-1}), bacteria were able to grow. Figure 1 showed also that the BAC MIC concentrations is 12.5 mg.l^{-1} as at this concentration

the bacterial growth was totally inhibited. The growth rate (μ), and lag time (λ) of *P. aeruginosa* were dependent on the BAC concentration (Fig. 1). In fact, our results showed that the μ and the λ values of *P. aeruginosa* cultures were respectively 0.07 h^{-1} and 4 h when the BAC concentrations were ranging respectively from 0 to 3.2 $mg.l^{-1}$. When the BAC concentration was at 6.25 $mg.l^{-1}$ the μ of *P. aeruginosa* culture decreased significantly to 0.02 h^{-1} and the lag time significantly was extended to 12 h *(P < 0.05)* (Fig. 1).

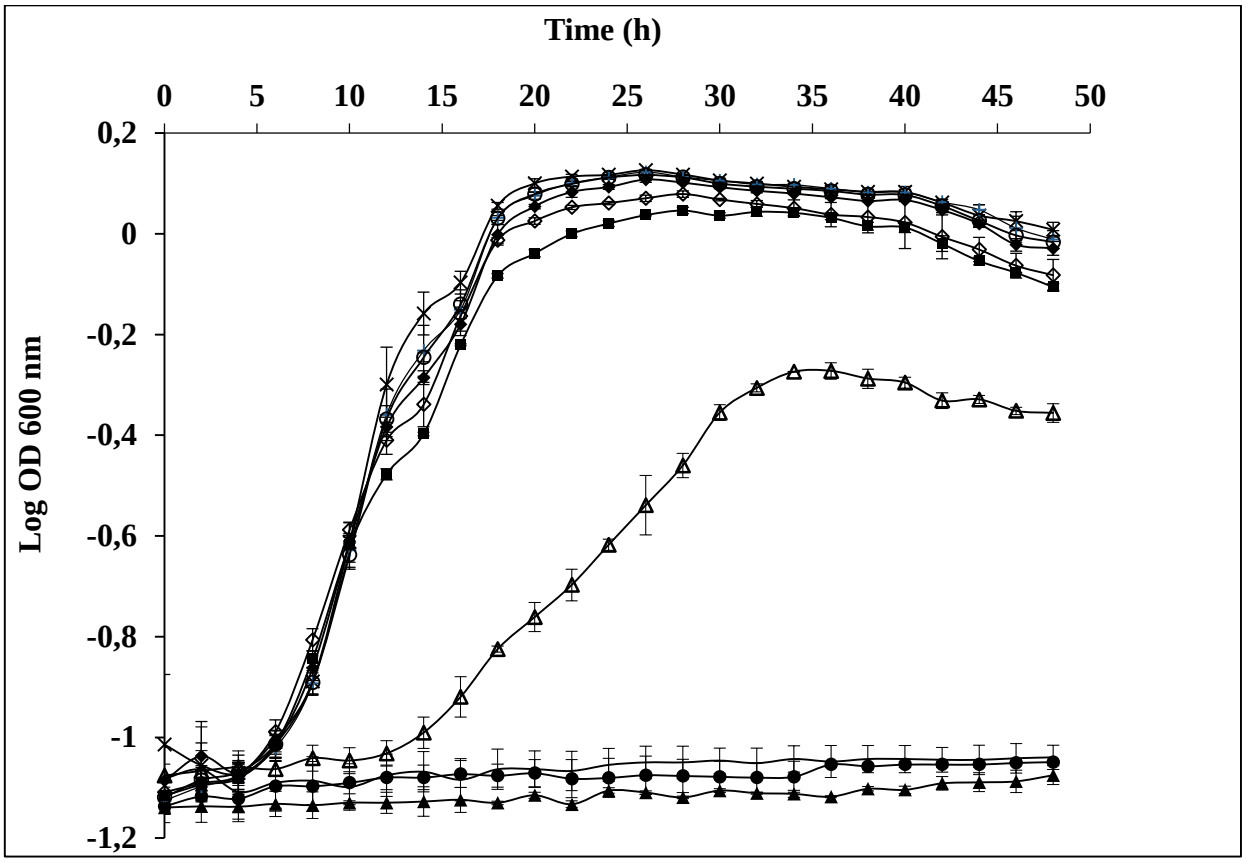


Figure 1 Growth curves of *Pseudomonas aeruginosa* in presence of BAC concentrations of 25 (▲), 12.5 (−), 6.25 (Δ), 3.12 (■), 1.56 (◊), 0.8 (♦), 0.4 (○), 0.2 (+) and 0 $mg.l^{-1}$ (x). The concentration at which there was linear growth inhibition was considered the MIC. The bacterial growth in Mueller Hinton Broth without bacteria and benzalkonium chloride was measured to ensure the sterility of the growth medium (sterile control) (●). The ± SEM for three replicates are illustrated.

Effect of growth temperature on the resistance of *P. aeruginosa* cells to BAC

The resistance of biofilm-detached cells grown on SS and that of planktonic *P. aeruginosa* to BAC treatment was studied as a function of the bacterium growth temperature (20 and 37°C) (Fig. 2).

The increase of the growth temperature, from 20 to 37°C, resulted in a significant decrease of biofilm-detached and planktonic cells sensitivity to the BAC *(P < 0.05)* (Fig. 2a and b). The treatment of *P. aeruginosa* biofilm-detached cells, with BAC at 12.5 mg.l^{-1} for 5 min, resulted in a significant reduction of the initial viable and culturable count *(P < 0.05)* by 2.4 and 3.5 log CFU.ml^{-1} respectively when cells were grown at 20 and 37°C (Fig. 2a). However, when *P. aeruginosa* planktonic cells were grown at 20 and 37°C the BAC treatment at 12.5 mg.l^{-1} reduced significantly the initial viable and culturable count by 1.3 and 3.2 CFU.ml^{-1}, respectively *(P < 0.05)* (Fig. 2b). Furthermore, the results also showed that the remained viable and culturable count of planktonic cells after BAC treatment was significantly higher than that of biofilm-detached cells, with an average value of 1 *(P < 0.05)* and 0.4 log *(P < 0.05)* when the cells were grown respectively at 20 and 37°C (Fig. 2a and b).

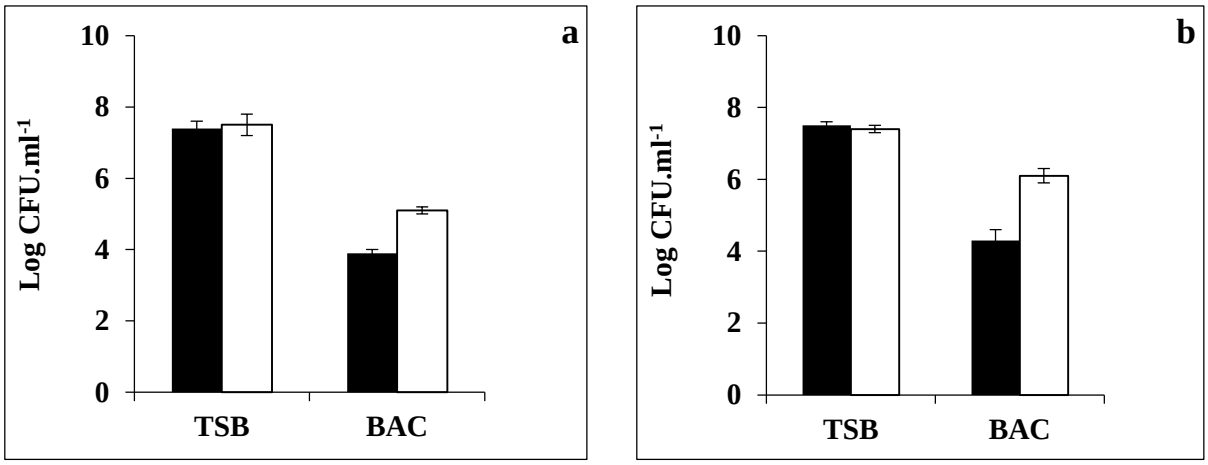


Figure 2 Effect of benzalkonium chloride (12.5 mg.l^{-1}) treatment on *Pseudomonas aeruginosa* detached-biofilm (a) and planktonic cells (b) grown during 24 h at 20°C (black column) and 37°C (white column). The biofilms were grown on stainless steel. The bacterial density is presented in log CFU.ml^{-1} ± SEM after treatments with tryptone soy broth (TSB) and benzalkonium chloride.

Effect of BAC treatment on the cell membrane integrity

The leakage of intracellular K^+ was monitored after BAC **(**12.5 mg.l^{-1}) treatment of the planktonic and biofilm-detached *P. aeruginosa* cells grown at 20 and 37 °C for 24 h. Our results showed that the HEPES buffer had no effect on the K^+ efflux which remained stable whatever the studied conditions (Fig. 3). The contact of *P. aeruginosa* with of BAC at a final concentration of 12.5 mg.l^{-}

[1] resulted in an instantaneous and a significant increase of the extracellular K^+ concentrations (Fig. 3). The BAC addition led after 5 min in an increase of extracellular K^+ concentration to 5.6 *(P < 0.05)*, 1.4 mg.l^{-1} *(P < 0.05)*, 0.8 mg.l^{-1} *(P < 0.05)* and 0.4 mg.l^{-1} *(P < 0.05)* respectively in the biofilm-detached cell suspension and planktonic cell suspensions grown 20, biofilm-detached cell suspension and planktonic cell suspension grown at 37°C (Fig. 3). Figure 3 also showed a significant effect of growth temperature on K^+ efflux when *P. aeruginosa* was grown at 20°C. 90 min after the BAC addition, the extracellular K^+ concentration increased from 0 to 13 mg.l^{-1} (*P < 0.05)* and from 0 to a mean value of 8 mg.l^{-1} (*P < 0.05)* respectively in the biofilm-detached and planktonic and cells suspension of *P. aeruginosa* grown at 20°C (Fig. 3). However, the extracellular K^+ concentration of biofilm detached and planktonic *P. aeruginosa* cells grown at 37°C increased respectively, to 7 *(P < 0.05)* and 3 mg.l^{-1} (*P < 0.05*), 90 min after the BAC addition (Fig.3).

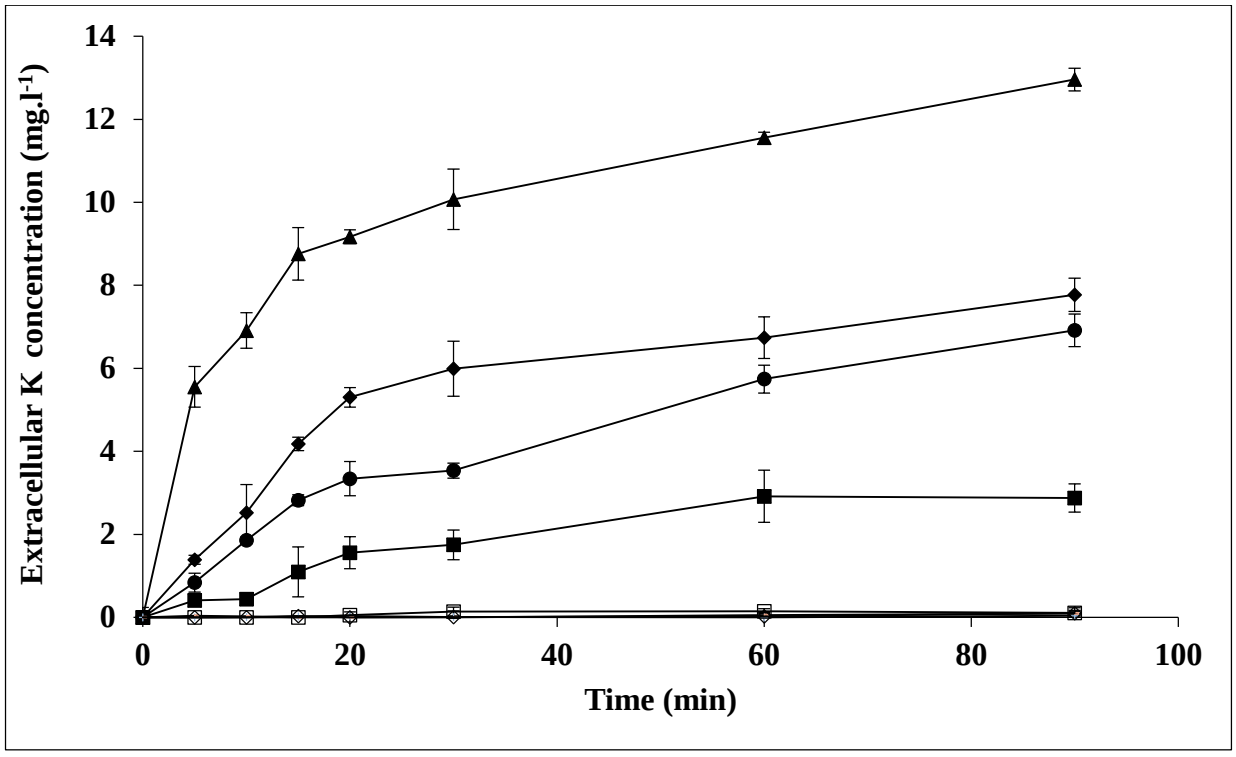
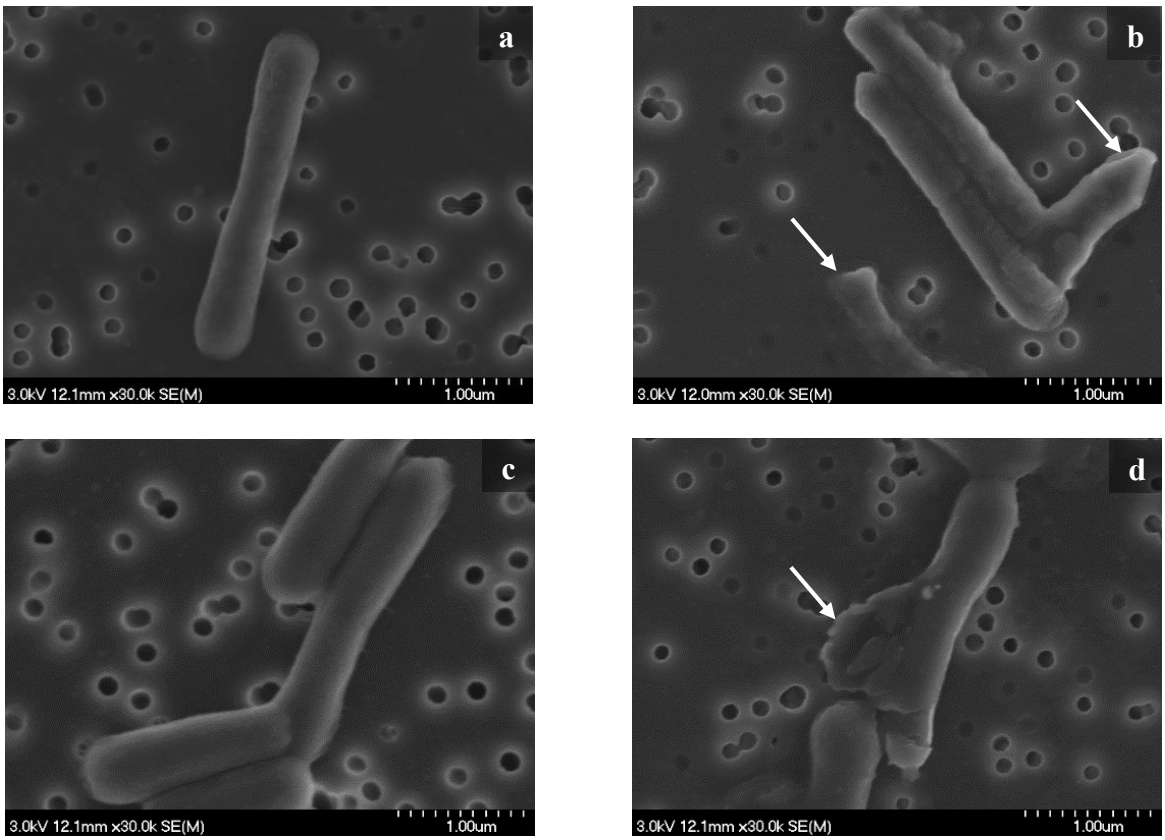


Figure 3 Kinetics of potassium efflux in the medium filtrates of planktonic and biofilm-detached *Pseudomonas aeruginosa* suspensions upon treatment with benzalkonium chloride (BAC) at 12.5 mg.l^{-1}. BAC-treated biofilm-detached cells grown at 20 (▲) and 37°C (●); BAC-treated planktonic cells grown at 20 (♦) and 37°C (■); HEPES-treated biofilm-detached cells grown at 20 (+) and 37°C (◊); HEPES-treated planktonic cells grown at 20 (-) and 37°C (□). The K+ concentrations are presented in mg.l^{-1}. The ± SEM for three replicates are illustrated after treatments with HEPES buffer (negative control) and BAC

Morphological changes after the BAC addition

Scanning electron microscopy was used to check out the morphological changes in *P. aeruginosa* cells, grown at 20 and 37°C, after exposure to BAC at a concentration of 12.5 mg.l^{-1} (Fig. 4). The untreated *P. aeruginosa* cells showed a normal shape and a smooth surface whatever the growth temperature (Fig. 4a, c, e and g). However, when the treated cells were grown at 20°C, their membranes were deeply damaged. Figure 4b showed that the planktonic cells lost their normal form and had holes on their surfaces. Whereas, the biofilm-detached cells were completely lysed and seemed to have their membrane collapsed under the effect of BAC (Fig. 4d). Furthermore, the exposure of *P. aeruginosa* cells grown at 37°C cells to BAC MIC for 5 min, resulted in pronounced modification of their shape and aspect whatever the mode of growth without disorganizing the membrane structure. Figures (4.f, h) showed that, in the presence of BAC, *P. aeruginosa* cells appeared misshapen and had undulating and rougher than those of control cells.



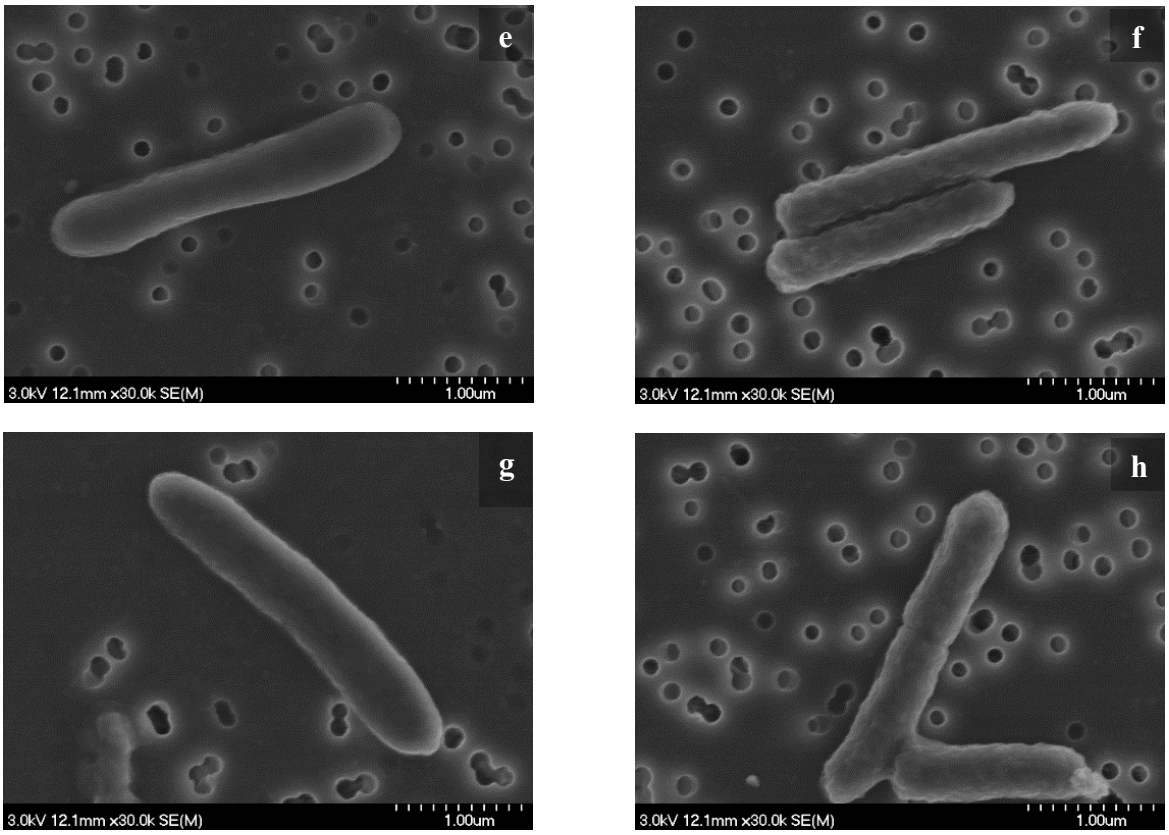


Figure 4 SEM images of untreated *Pseudomonas aeruginosa* cells: Planktonic grown at 20°C (a) Biofilm-detached gown at 20°C (c), Planktonic grown at 37°C (e) and Biofilm-detached gown at 37 °C (g), showing a smooth cell membrane of a normal shaped cells; *P. aeruginosa* cells treated with benzalkonium chloride for 5 min: Planktonic grown at 20°C (b) Biofilm-detached gown at 20°C(d), Planktonic grown at 37°C (f) and Biofilm-detached gown at 37 °C (h), cell surfaces are rough, distorted and even collapsed

Effect of growth temperature on membrane fatty acids of *Pseudomonas aeruginosa* cells

The modifications of the membrane fatty acid profiles were investigated, as a function of growth temperature, for the planktonic and biofilm-detached cells grown on SS (Fig. 5). This investigation was performed to study the relationship between the membrane fluidity of *Pseudomonas aeruginosa* cells and their resistance to BAC treatment used in this work. Our results indicated that the biofilm-detached and planktonic cells increased respectively their Saturated Fatty Acids (SFA) from 25 to 38% *($P < 0.05$)* and from 25 to 41% *($P < 0.05$)* and decreased respectively their Unsaturated Fatty Acids (UFA) from 62 to 47% and from 57 to 31%, in response to the increase of growth temperature from 20 to 37°C (Fig. 5a, b).

Our results also showed a significant rise of Cyclic Fatty Acids (CFA) amounts from 0.8 to 5% and from 3 to 6% ($P < 0.05$) with the increase of growth temperature (20 to 37°C), respectively for biofilm detached and planktonic cells (Fig. 5a, b).

Our findings also showed that the UFA/SFA ratios decreased significantly with the increase ($P < 0.05$) of the growth temperature whatever the studied conditions (Fig. 5a, b). In addition, Figure 4 showed that the UFA/SFA ratios of biofilm-detached cells were higher than those of planktonic cells whatever the studied condition.

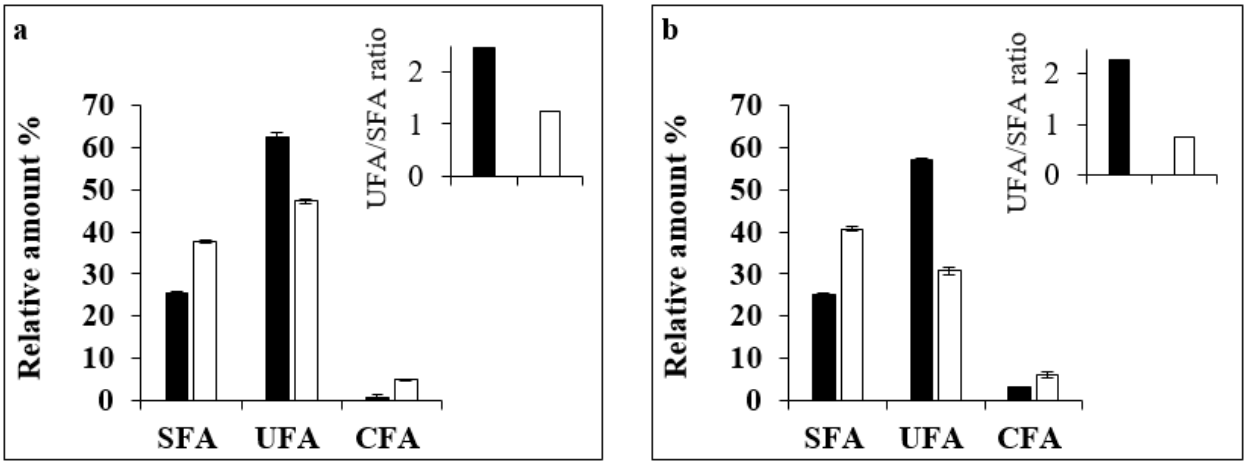


Figure 5 Membrane fatty acids profiles of biofilm-detached (a) and planktonic (b) *Pseudomonas aeruginosa* cells grown, at 20 (black column) and 37°C (white column). Biofilms were grown on stainless steel. Values present the relative amount ± SEM. SFA: saturated fatty acids; UFA: unsaturated fatty acids; CFA: cyclic fatty acids.

Discussion

Our study was conducted to assess the microbiological risk related to *P. aeruginosa* cells detached from biofilm formed on SS. Subsequently, the resistance of these cells to BAC treatment was studied as a function of the growth temperature (20 and 37°C). The cell homeostasis after BAC treatment and the membrane fluidity of biofilm-detached and planktonic cells were characterized to check out the effect of the environmental conditions and the physiological state on the mechanisms of bacterial cell resistance to BAC.

The results of the antibacterial assays showed that the growth temperature significantly affected the resistance of *P. aeruginosa* cells to BAC. The rise of the growth temperature from 20 to 37°C, increased the resistance to BAC whatever the studies cell population. These findings are in

agreement with what has been reported on the effect of growth temperature on the *P. aeruginosa* biofilm resistance to disinfectants (Abdallah *et al.* 2014). Moreover, our findings showed that biofilm-detached cell phenotype is highly different from the planktonic one. Our results underline that the resistance of biofilm-detached cells to BAC at a given incubation temperature (20 or 37°C) was significantly lower than that of planktonic cells. Our results are consistent with those of Bester *et al.* (2005) who reported that *Pseudomonas* sp. strain CT07 biofilm-detached cells were more susceptible to a biocide mix consisting of glutaraldehyde (12%) and isothiazolones (4%), than their planktonic counterparts. It has been shown that biofilm cells are more resistant to treatment with antimicrobial agents than the planktonic ones (Brooun, Liu and Lewis 2000). However, according to our finding, it seems that the recently biofilm-detached cells do not maintain their biofilm character and lose their greater resistance compared to planktonic cells. Yet, *P. aeruginosa* biofilm-detached cells grown at 37°C are still more resistant than their planktonic counterparts grown at 20°C. Thus, biofilm-detached cells in food processing industry, such as in dairy industry where the processing temperature are higher than 20°C, represent a real threat to consumer safety as they acquire resistance that needs an efficient and adapted antimicrobial treatment. In order to highlight the resistance mechanism of biofilm-detached and planktonic *P. aeruginosa* cells to BAC treatment, we investigated the bacterial membrane integrity by monitoring the leakage of K^+ after exposure to BAC MIC (12.5 $mg.l^{-1}$) concentration. Our results showed an immediate K^+ leakage after the BAC addition to *P. aeruginosa* cells. 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 incubation temperature (20 or 37°C) K^+ leakage was higher in planktonic than in biofilm detached cell suspensions. Hence, the bacterial response to the BAC treatment seems to be related to the bacterial physiological state and the growth temperature. The K^+ leakage kinetics of *P. aeruginosa* due to BAC treatment were in accordance with the obtained results of the cultivability reduction studies. Hence we assume that the potassium leakage monitoring would be a reliable indicator to study the susceptibility of *P. aeruginosa* cells to QACs. Our data also highlighted, that *P. aeruginosa* at the cellular level, the bacterium exhibited different membrane properties to modulate its resistance to BAC. It has been reported that QACs compromises the integrity of the cytoplasmic membrane by forming holes or gaps inducing the leakage of cell contents and death (Maillard 2002; Johnston *et al.* 2003). In order to observe the effect of BAC treatment on *P. aeruginosa* membrane structure, bacterial samples were analysed by SEM. The *P. aeruginosa* control cells (TSB treated) had a

normal appearance and smooth surface whatever the studied conditions. Whereas, treatment for 5 min with BAC had an obvious effect on the shape of biofilm-detached and planktonic cells grown at 37°C which appeared rough and corrugated. Although when treated cells were grown at 20°C they seemed to have their membrane disintegrated and damaged. Thus, the leakage of cell contents demonstrated by the K^+ efflux through gaps in the plasma membrane after BAC treatment is supported by these electron microscopy investigations. In fact, QACs are membrane-active agents interacting with the cytoplasmic membrane of bacteria (Gerba 2015). Several studies have mentioned that positively charged QACs disturb the bacterial membranes structure by interacting with the negatively charged acidic phospholipids (Gilbert and Moore 2005; Abdallah *et al.* 2014). It has been shown that resistance to BAC in *Listeria monocytogenes* is related to changes in the cell membrane fatty acid composition (To *et al.* 2002). Therefore, our work showed that in *P. aeruginosa* cells the membrane fatty acids composition, controlling the membrane fluidity, could be involved in the resistance of biofilms-detached and planktonic cells to BAC.

The membrane fatty acid composition was strongly influenced by the increase of growth temperature and the physiological state of the cells. The membrane fatty acids in both biofilm-detached and planktonic *P. aeruginosa* cell cultures showed an increase in the relative amount of both SFA and CFA and a corresponding decrease in UFA with the increase of growth temperature from 20 to 37°C. Higher SFA and CFA and lower UFA amounts of were observed in planktonic cells compared to biofilm detached ones respectively at 37 and 20°C. In fact, SFAs increase membrane packaging and rigidity by rising the phase transition of the membrane bilayers (Zhang and Rock 2008). A higher amount of the straight-chain saturated fatty acids makes the space between the phospholipid molecules became smaller due to the optimized steric arrangement of fatty acid chains. Thus, the membrane becomes more dense, rigid and impermeable, simultaneously, to BAC penetration and K^+ leakage. Furthermore, an increase of CFA amounts, with the increase of growth temperature, was reported to increase the structural stability of bacterial membranes (Brown *et al.* 1997). Thus, our results indicated that when studied at the same incubation temperature (20 or 37°C) the membrane fluidity of planktonic *P. aeruginosa* cells was lower than that of biofilm-detached cells.

In conclusion, our results showed that the resistance of biofilm-detached and planktonic cells to BAC treatment was dependent on the physiological growth mode in addition to the growth

temperature. In addition, our work showed that the membrane fluidity of *P. aeruginosa* cells may explain their resistance to BAC which targets the bacterial cell membrane.

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Conclusion

Pseudomonas aeruginosa, and *Staphylococcus aureus*, biofilms are a significant cause of infections worldwide such as nosocomial and foodborne infections. In this context, we studied the effect of growth temperature commonly met in these sectors on the resistance of *P. aeruginosa*, and *S. aureus* biofilm-detached and planktonic cells to benzalkonium chloride (BAC) treatments.

Our results showed that the increase of growth temperature resulted in a significant increase of the biofilm-detached and planktonic cell resistance to BAC treatment. Such results highlight the fact that equipment constantly exposed to temperature close to 37°C should be constantly disinfected in order to avoid the biofilm formation under such temperatures and therefore to reduce the microbiological risk associated with. In addition, our results suggest that the assessment of antibiofilm products should take into consideration different environmental conditions in order to set up efficient disinfecting products. Furthermore, our results underlined that the membrane fluidity of both biofilm-detached and planktonic cells seems to be involved in the bacterial resistance to BAC treatment and there is a necessity to consider it when developing new disinfectants.

GENERAL CONCLUSION AND PERSPECTIVES

General conclusion

Biofilm formation is considered as serious issue with dramatic consequences in food processing and healthcare environments (Brooks and Flint 2008; Donlan and Costerton 2002). Despite the cleaning and disinfection procedures, pathogenic bacteria may persist on abiotic surfaces and form biofilm. In fact, biofilm provides reservoir for pathogenic microorganisms which are at the origin of fatal human infections such as nosocomial and foodborne ones. In addition to human life losses, biofilms are also an important cause of material deterioration in industry, such as the marine, paper production, oil drilling, etc. (Donlan and Costerton 2002; Simões et al. 2010). In such industries, disinfecting agents are constantly applied in order to ensure the cleanness of equipment surfaces and therefore to prevent biofilm establishment and their related issues. Unfortunately, the biofilm enables bacteria to survive chemical and physical stresses. Hence, one of the major advantages for bacteria growing under biofilm state is the high tolerance to antimicrobial agents and ability to survive environmental stresses. It has been reported that, bacteria growing within biofilm state may be up to 1,000-fold more resistant to disinfecting agents than their planktonic counterparts (Bridier et al. 2011). Furthermore, the resistance of bacteria living under biofilm state to disinfectant agents is known to be influenced by the environmental conditions under which bacterial cells are exposed such as temperature, humidity, pH, etc. Thus, it's of importance to understand the mechanisms of the biofilm formation and resistance to disinfectants agents as a function of bacterial growth under environmental conditions commonly met in food and medical sectors.

Biofilm formation is a complex process, characterized by a succession of different steps. The bacterial adsorption or the reversible adhesion to a substrata is the first step of the biofilm development. This phenomenon seems to be triggered via non-covalent forces, such as van der Waals, acid-base and electrostatic interactions (Bos et al. 1999). Thereafter, bacteria will subsequently attach to surfaces in an irreversible manner. The colonization of the surface is achieved through the multiplication of microorganisms which will form microcolonies and macrocolonies while secreting the extracellular matrix to form a mature biofilm (Costerton et al. 1999). At this stage, some bacteria will detach from the biofilm structure and colonize other surfaces (Stoodley et al. 1999). The bacterial detachment from the biofilm structure may be initiated by external forces such as fluid shear and the use of disinfectants or by the bacteria themselves as consequence of nutrient and oxygen starvation (Kaplan 2010). Interestingly, the vast

majority of biofilm studies is associated to planktonic or biofilm-structured cells and very few investigations have been conducted on biofilm-detached cells in order to elucidate their behavior, in term of their ability to contaminate abiotic surfaces.

In this regard two bacterial models were selected for this study: *Staphylococcus aureus* and *Pseudomonas aeruginosa* regarding their involvement in both nosocomial and foodborne infections (EFSA 2009; Rosenthal et al. 2012). The impact of growth conditions, of biofilm-detached and planktonic cells on their physiology, microbial risk related to the production of virulence factors, adhesion to surfaces and resistance to benzalkonium chloride (BAC) were investigated. The polycarbonate (PC) and the stainless steel (SS), two surfaces commonly encountered in both food and medical equipment, were selected as substrata of bacterial adhesion and biofilm formation assays. In addition, the involvement of membrane fluidity of biofilm-detached and planktonic cells in the resistant against BAC was also studied.

In the first part of this work, we studied the effect of bacterial growth temperature (20, 30 and 37°C), surface type (SS and PC) and incubation duration (24 and 48h), of *P. aeruginosa* CIP 103467 and *S. aureus* CIP 4.83, on their bacterial surface properties (zeta potential, donor/acceptor character, hydrophobicity), adhesion to stainless steel and polycarbonate, production of virulence factors (DNases, proteases and siderophores) and cytotoxicity against human cells.

Our results underlined that the bacterial growth conditions has a significant effect on the bacterial surface properties and this effect seems to influence the bacterial cell adhesion to abiotic surfaces. In fact, *P. aeruginosa* and *S. aureus*, cells grown under at 37°C were the most adherent to stainless steel and polycarbonate, followed by those grown at 30°C and 20°C. Moreover the adhesion rate of bacterial cells was higher on SS than on the PC whatever the studied conditions. Interestingly, our data highlighted that the biofilm-detached cells presented an adhesion rate, on both SS and PC surfaces, higher than that of their planktonic counterparts. Thus, our investigations underlined the importance to have a closer look at the cell detachment stage in the process of biofilm development. These results should contribute to more effective management of disinfection strategies, especially by ensuring a rapid removal and killing of cells detached from contaminated surfaces in order to prevent the persistence and the spread of contaminations.

Furthermore, our results showed that the increase of both temperature (from 20 to 37°C) and duration (24 to 48h) of incubation enhanced the production of virulence factors by biofilm and planktonic cells. In addition, the type of substrata of biofilm growth also play an important role in

virulence factors production and cytotoxic molecules by attached bacteria. In fact, the virulence factor activity and the cytotoxicity towards HeLa cells, in biofilm supernatants were found to be greater than those of their planktonic supernatants. In addition, when biofilms were grown on plastic abiotic surface, such as PC, they exhibited greater DNase, protease and siderophore activities whatever the studied condition. Hence, we showed that sessile cells produce higher amounts of different virulence factors which represent a serious threat. Thus, the biofilm formation of *S. aureus* and *P. aeruginosa* under growth temperatures close to that of the human body increases virulence potential of bacterial cells and therefore the cytotoxicity against human cells. In addition, our results highlight the fact that the biofilm colonization of plastic indwelling medical devices, such as catheters, may increase the infection severity. Therefore, disinfectant procedures of such equipment must be optimized in food and medical sectors in order to ensure their sterility and avoid the biofilm establishment.

In order to control the persistence and the spread of biofilm contamination on food and medical contact surfaces, the second part this work consists of the study of *S. aureus* and *P. aeruginosa* biofilm-detached cells resistance to BAC. This investigation aimed to bring and improve our knowledge regarding the mechanisms of the bacterial resistance/tolerance to disinfectant molecules such as BAC. BAC is a quaternary ammonium compound (QAC) that is described as one of the most widely used disinfectants in the food industry and health care facilities (Ceragioli et al. 2010). Our results showed that the resistance of biofilm-detached and planktonic cells to disinfectants was dependent on several environmental factors commonly found in food and healthcare sector. In fact, antibacterial assays highlighted the significant effect of the growth temperature on the resistance of *S. aureus* and *P. aeruginosa* to disinfectant treatments. The increase of growth temperature from 20 to 37°C resulted in a significant increase of *S. aureus* biofilm-detached cells resistance to BAC than their planktonic counterparts whatever the studied conditions. However, the *P. aeruginosa* biofilm-detached cells were more sensitive than their planktonic counterparts whatever the studied growth temperature. These data highlight the fact that it is therefore important to be conscious of environmental conditions, such as growth temperature and bacterial mode of growth, when testing the antibiofilm efficacy of disinfectants products. Our results underlined the necessity to consider different environmental conditions of biofilm formation when testing the antibiofilm efficacy of disinfectant products. Such investigation may allow the development of efficient disinfecting

products and decrease the microbiological risk related to biofilms in food and medical environments.

The action mechanism of a disinfectant may be defined according to the bacterial structure with which it interacts. Thus, three targets may be described: outer cellular components, the cytoplasmic membrane and cytoplasmic constituents. However, it is possible that disinfectant acts simultaneously at all three levels with the bacterial cells to produce its antimicrobial activity (Maillard 2002). Commonly, the term "membrane active agents" is used for antimicrobials such as BAC, a quaternary ammonium compound QAC, which target the cytoplasmic membrane (van der Veen and abee 2011). The bacterial cytoplasmic membrane damage is often indicated by the leakage of intracellular components, such as the K^+ ions (Lambert and Hammond 1973; Maillard 2002). The bacterial permeability and the rate of leakage depend on several parameter, including the micro-organisms, the membrane active agent type, the concentration of the biocide and environmental temperature. In fact, the rate of leakage as effect of QAC treatment, is generally higher for Gram-positive than for Gram-negative bacteria. In addition, the concentration of the applied disinfectant is a major factor in biocidal activity (Russell and McDonnell 2000). Disinfectant are usually used at high concentrations to ensure the elimination of contaminant on surface. However, minimal inhibitory concentrations have also been used to evaluate the emergence of biocide resistance in bacteria (Russell and McDonnell 2000). In order to study the effect of BAC on *S. aureus* and *P. aeruginosa* membranes, we investigated the relationship between the growth temperature (20 and 37°C) and the rate of K^+ leakage for biofilm-detached and planktonic *S. aureus* and *P. aeruginosa* cells treated with BAC (at the MIC). This investigation aimed to confirm antimicrobial results of BAC treatments. Our results showed that the bacterial membrane damage was dependent the growth temperature and lifestyle of treated cells. In fact, our findings showed that the BAC treatment resulted in an immediate K^+ leakage which seemed to be higher when the bacterial cells were grown at 20 °C rather than at 37°C. In addition, our data showed that the K^+ leakage correlated perfectly with the resistance profile of both *P. aeruginosa* and *S. aureus* cells to BAC. In order to explain the observed results, we have assessed indirectly the membrane fluidity of biofilm-detached and planktonic cells. In this regard, the analysis of the membrane fluidity of biofilm-detached and planktonic cells was investigated trough the study of membrane fatty acids of *S. aureus* and *P. aeruginosa* cells. Our results showed that the increase of growth temperature increased the membrane rigidity of both, biofilm-detached and planktonic,

S. aureus and *P. aeruginosa* cells. In fact, the increase of growth temperature of biofilm-detached cells increased the relative amount of branched aC19 and long chain saturated fatty acids (C18 and C20) for *S. aureus* cells and decreased the SFA/UFA ratio for *P. aeruginosa* cells. Furthermore, the increase of membrane rigidity of studied bacteria with the rise of growth temperature (from 20 to 37°C) may explain to the increase of the resistance biofilm-detached cells to BAC. Furthermore, our data also underlined that the switch from planktonic to attached growth mode for *P. aeruginosa* and *S. aureus* promoted respectively an increase and a decrease of the cell membrane fluidity whatever the studied conditions. These results also correlate with the results of disinfectant sceptibility where the biofilm-detached *P. aeruginosa* cells were more sensitive to BAC treatments than their planktonic counterparts. In the case of *S. aureus*, our results showed that the decrease of membrane cell rigidity, with the switch from planktonic to detached form, may also explain the observed increase in the resistance to BAC treatments. Thus, our results underlined that the growth temperature presents an effect on the membrane structure and composition, which control its permeability and consequently the susceptibility to disinfectants. Our results seem to correlate with the results of Abdallah et al. (2014b) who showed that the membrane fluidity of attached cells may plan an important role in the biofilm resistance to disinfectant agents. Overall, the first part of this study showed that the environmental conditions and bacterial growth mode had a significant effect on *S. aureus* and *P. aeruginosa* surface physicochemical properties, adhesion to SS and PC and pathogenic potential. Furthermore, our work showed that the biofilm-detached and planktonic cell resistance to BAC was dependent on the bacterial type and the growth conditions. Our data underlined that the membrane fluidity could explain the resistance profile of biofilm-detached and planktonic cells to BAC. In addition, the physiological state of biofilm-detached cells seems to be an important element of the biofilm resistance to disinfectant and should be considered when disinfecting agents are developed.

Perspectives

To enlarge our understanding to *S. aureus* and *P. aeruginosa* biofilm-detached cells behavior and physiology, it would be interesting to complete the results of bacterial adhesion to studied surfaces. In fact, our findings underlined that the modification of bacterial physicochemical properties cannot always fully explain their adhesion. The purification and quantification of structural adhesins of bacteria grown under the different environmental conditions may help to understand the results of adhesion to SS and PC. An interesting perspective would consist in focusing on the quantification of bacterial adhesion forces using atomic force microscopy in order to extend the knowledge of the mechanisms mediating bacterial adhesion to abiotic surfaces and to develop new strategies for the prevention of the biofilm formation.

In addition, the effect of other growth conditions such as incubation durations and the type of abiotic surfaces on the bacterial resistance and on the membrane fatty acid profiles controlling the membrane fluidity should be elucidated. The instantaneous membrane fluidity variations in *S. aureus* and *P. aeruginosa* biofilm-detached and planktonic may be assessed by fluorescence anisotropy measurements.

Moreover, the expression of gene encoding for efflux pumps, another mechanism of resistance to antimicrobials, would be interesting to study.

It should be noted that all experiments conducted on biofilm-detached cells were done, immediately after being harvested and washed to remove the residual biofilm matrix. Thus, it can be envisaged to do the same experiments on biofilm-detached cells within time after being harvested to highlight whether they would recover the planktonic phenotype.

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The listed references below concern only introduction of thesis and chapters, as well as the final conclusion of the thesis

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Abstract

The contamination of abiotic surfaces by pathogenic microorganisms in health-care and food-processing sectors leads to the establishment of biofilm. Bacterial biofilms are considered as a main cause of serious human infections, such as foodborne and nosocomial deseases. The threat of biofilm-cells comes from their higher resistance to disinfectants, when compared to their planktonic counterparts. After biofilm maturation, bacteria may detach from biofilm and colonize new surfaces. Yet, few studies have been conducted on biofilm-detached bacteria to assess their microbial risk. In this regard, the goal of the present work was to conduct a comparative study of growth conditions effect on some physiological properties of biofilm-detached and planktonic *Staphylococcus aureus* and *Pseudomonas aeruginosa* cells. The surface physicochemical properties which control the bacterial adhesion to stainless steel (SS) and polycarbonate (PC) were investigated. Moreover, the pathogenic potential of both cell populations was studied. The results showed that the bacterial growth conditions and lifestyle influenced their surface properties and therefore their adhesion on SS and PC. The growth temperature (20, 30 and 37°C), surface type (SS and PC) and incubation duration (24 and 48h) affected significantly the virulence factors production and the cytotoxicity in the supernatants recovered from biofilm and planktonic cultures. Thereafter, the effect of growth temperature (20 and 37°C) on the resistance of biofilm-detached and planktonic cells to Benzalkonium chloride (BAC) was investigated. The results showed that, in addition to the growth temperature and the lifestyle, the resistance to BAC treatment depended on the studied strain. In order to understand the mechanisms of resistance to BAC, investigations were carried out at a cellular level. In fact, the damage of bacterial membranes associated to BAC was monitored by the efflux of the intracellular potassium. In addition, the membrane fluidity of biofilm-detached and planktonic cells was investigated through the study of membrane fatty acid profiles. The results showed that biofilm-detached and planktonic bacteria were phenotypically different. Their pathogenicity and resistance response to BAC treatment depended on several parameters. The results also showed that BAC targeted and damaged the bacterial membrane. Finally, our study highlights that the modulation of bacterial membrane fluidity may be an effective strategy adopted by bacteria in response to BAC treatment.

Keywords: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, biofilm-detached cells, planktonic cells, physiology, nosocomial infections, foodborne diseases, pathogenicity, resistance to benzalkonium chloride.

Résumé

La contamination des surfaces abiotiques par des micro-organismes pathogènes dans les secteurs hospitalier et alimentaire conduit à la formation de biofilm. Le biofilm est considéré comme une cause principale d'infections nosocomiales et alimentaires. Le risque biologique élevé des bactéries structurées en biofilm provient de leur plus haute résistance aux désinfectants, comparée aux cellules planctoniques. Après la maturation de biofilm, des bactéries peuvent se détacher et coloniser de nouvelles surfaces. A ce jour, peu d'études ont été menées sur les bactéries détachées de biofilm pour évaluer le risque microbiologique associé à ce type de bactérie. À cet égard, l'objectif de ce travail était de mener une étude comparative de l'effet des conditions de croissance sur les propriétés physicochimiques de surface de *Staphylococcus aureus* et *Pseudomonas aeruginosa* sous leurs formes détachées de biofilm et planctoniques. Ceci a permis d'élucider l'impact des propriétés de surface sur l'adhésion bactérienne sur l'acier inoxydable (SS) et le polycarbonate (PC). Le pouvoir pathogène des deux populations bactériennes a également été étudié. Les résultats ont montré que les conditions et le mode de croissance bactérienne influencent les propriétés de surface et par conséquent l'adhésion de *S. aureus* et *P. aeruginosa* sur le SS et le PC. De plus, la température de croissance (20, 30 et 37°C), le type de surface (SS et PC) et l'âge physiologique (24 et 48h) influencent significativement la production des facteurs de virulence et la cytotoxicité des surnageants récupérés de biofilm et de cultures planctoniques. Par la suite, l'effet de température de croissance (20 et 37°C) sur la résistance des cellules détachées de biofilm et planctoniques au chlorure de benzalkonium (BAC) a été évalué. Les résultats ont montré que, en plus de la température et du mode de croissance, la résistance au traitement BAC dépend de la souche étudiée. Les mécanismes de résistance, ont été étudiés au niveau cellulaire. En effet, les lésions des membranes bactériennes associées au BAC ont été suivies par l'efflux des ions K+ intracellulaires. En outre, la fluidité membranaire de deux populations bactériennes a été caractérisée à travers l'étude de profils d'acides gras membranaires. Les résultats ont montré que les bactéries détachées de biofilm et celles à l'état planctonique sont phénotypiquement différentes. Leur pouvoir pathogène et leur résistance au BAC dépendent de plusieurs paramètres. Les résultats ont également montré que le BAC endommage la membrane bactérienne. Enfin, notre étude a mis en évidence que la modulation de la fluidité de la membrane bactérienne peut être une stratégie efficace adoptée pour résister au traitement antibactérien.

Mots clés : *Staphylococcus aureus*, *Pseudomonas aeruginosa*, cellules détachées de biofilm, cellules planctoniques, physiologie, infection nosocomiale, intoxication alimentaire, pathogénicité, résistance au chlorure de benzalkonium.