

# Biofilm formation and persistence on abiotic surfaces in the context of food and medical environments

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**Abstract** The biofilm formation on abiotic surfaces in food and medical sectors constitutes a great public health concerns. In fact, biofilms present a persistent source for pathogens, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which lead to severe infections such as foodborne and nosocomial infections. Such biofilms are also a source of material deterioration and failure. The environmental conditions, commonly met in food and medical area, seem also to enhance the biofilm formation and their resistance to disinfectant agents. In this regard, this review highlights the effect of environmental conditions on bacterial adhesion and biofilm formation on abiotic surfaces in the context of food and medical environment. It also describes the current and emergent strategies used to study the biofilm formation and its eradication. The mechanisms of biofilm resistance to commercialized disinfectants are also discussed, since this phenomenon remains unclear to date.

**Keywords** Abiotic surfaces · Biofilm · Environmental conditions · Biofilm resistance · Disinfectants

## Introduction

The persistence of pathogenic bacteria, in food and health-care environments, presents a great risk for public health. In fact, infections with pathogens may lead to serious human diseases worldwide (Hota 2004; Newell et al. 2010). Food-associated infections usually arise when people consume food and drinking products contaminated with pathogens. These contaminations may occur at any stage of food processing via food handlers, contaminated equipment and food preparation surfaces (Verraes et al. 2013). The Center for Disease Control and prevention (CDC) stated that 48 million episodes of foodborne illness occur in the USA each year, leading to 128,000 hospitalizations and up to 3,000 deaths (CDC 2013). In the European Union, 5,609 foodborne outbreaks have been reported in 2007, involving about 39,727 human cases (11,283 in France), with 3,291 hospitalizations and 19 deaths (7 in France) (EFSA 2009). Otherwise, healthcare-associated infections (HAIs), also known as nosocomial infections, commonly occur via the hands of healthcare personnel, contaminated surfaces and devices (surgical instruments, catheters, breathing system, endoscopes, needles, etc.) (Weber et al. 2013). The National Hospital Discharge Survey (NHDS) estimated the number of HAIs occurring in US hospitals, between 1990 and 2002, to 1.7 million cases in which 98,987 cases were fatal (Klevens et al. 2007). In the Europe, the number of HAIs is estimated to be 3.2 million cases per year (Suetens et al. ). In France, the survey conducted on 1,938 healthcare facilities, between May and June 2012 (Réseau d'alerte, d'investigation et de surveillance des infections nosocomiales: Raisin), showed that 5.1 % of patients have at least one HAI, in which 7.8 % have surgical site infections and 10 % were exposed to invasive devices (vascular catheter, urinary catheter, tracheal intubation, etc.)

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(Raisin 2013). In addition to the human life losses, HAIs and food-associated infections cause substantial morbidity and economic losses. In fact, the aggregate cost of HAIs in the USA is about \$16.6 billion each year (Hassan et al. 2010). It has been reported that the resulting aggregated annual cost of foodborne illness in the USA is \$77.7 billion (Scharff 2012).

In natural and man-made ecosystems, bacteria have a tendency to live attached to surfaces and to form a complex structure, called a biofilm. Moreover, bacteria living under the biofilm state are phenotypically different from their planktonic counterparts (Lazazzera 2005) and present a high tolerance to antimicrobial agents (Donlan and Costerton 2002). Biofilm formation constitutes a critical issue for the surfaces and equipment of industries, which provide a favorable environment for their formation (Donlan and Costerton 2002; Simões et al. 2010). Moreover, numerous studies underlined that the HAIs and foodborne diseases are caused to a large extent by the biofilms formed on equipment surfaces of both food and medical fields (Donlan and Costerton 2002; Hall-Stoodley et al. 2004). Such biofilms constitute potential reservoirs for pathogens, which serve as a continuous source of infections and cross-contaminations. Thus, it is of importance to understand the mechanisms, and environmental conditions, that control the formation of biofilm in order to reduce the microbiological risk related to their formation.

In this regard, this review will focus on the effect of food and medical environments on bacterial adhesion and biofilm formation, in particular those of *Pseudomonas aeruginosa* and *Staphylococcus aureus*, on abiotic surfaces. In addition, the strategies of prevention and eradication of biofilms are highlighted. Moreover, the mechanisms of biofilms resistance to the major disinfectant agents are also discussed.

### ***Pseudomonas aeruginosa* and *Staphylococcus aureus***

*Pseudomonas aeruginosa* is a ubiquitous bacterium, commonly found in various inanimate and human environments. This gram-negative bacterium is widespread in the environment, often isolated from soil, water, plants, and other wet areas. The occurrence of *P. aeruginosa* in food-processing environments has been reported in many cases (Kim and Wei 2007). This opportunistic pathogen is also prevalent in hospital environments and continues to be one of the major causes of nosocomial infections despite the advances of health care (Mesaros et al. 2007). The National Healthcare Safety Network (NHSN) at CDC stated that *P. aeruginosa* was involved in 6,111 HAI cases (7.5 %) between 2009 and 2010 in US hospitals (Sievert et al. 2013). In the European Union (EU), about 901 HAIs were

linked to this bacterium between 2011 and 2012, representing about 8.9 % of total microorganisms isolated (10.2 % in France).

Otherwise, the gram-positive *S. aureus* is a versatile pathogen, which causes a wide range of diseases ranging from mild to severely life threatening (Cheesbrough 2006). This bacterium is considered as one of the major etiologic agents of food poisoning. In fact, the European Food Safety Authority (EFSA) reported that this bacterium caused about 1,945 cases of foodborne illness in 2007 (1,361 cases in France), 204 hospitalizations and 3 deaths (EFSA 2009). This bacterium also constitutes a major cause of HAIs. The NHSN at CDC reported that *S. aureus* was associated with 12,635 cases between 2009 and 2010, about 15.6 % of the total HAIs reported (Sievert et al. 2013). Between 2011 and 2012, *S. aureus* was associated with 1,243 HAIs, representing about 12.3 % of total microorganisms isolated in Europe (14.2 % in France) (Suetens et al. 2012).

### **Sectors affected by the biofilm formation**

The ability of bacteria to attach, and to form biofilms, on abiotic surfaces is a major concern of industries providing an appropriate environment for their formation (Donlan and Costerton 2002; Simões et al. 2010; Flemming et al. 2013). In fact, biofilms enhance the ability of bacteria to survive stresses and cause difficult problems in several sectors such as the food industries, medical facilities, and water systems.

### **Biofilms and food environments**

Food poisoning is a general term for diseases arising from eating foods, which are contaminated usually with bacteria, viruses, toxins, or parasites (EFSA 2009). People are entitled to expect wholesome and safe food to eat. However, several studies have reported the prevalence of pathogenic bacteria in a variety of food products. Wang et al. (2013), found that the retail raw chicken samples in china were contaminated by *S. aureus* and methicillin-resistant *S. aureus* (MRSA). Similarly, Manguiat et al. showed the high prevalence of *Escherichia coli* and *S. aureus* in ready-to-eat foods (Manguiat and Fang 2013). Further studies also showed that meat products (Gousia et al. 2011), dairy-based food products (Sasidharan et al. 2011), and seafood products (Zarei et al. 2012) were found to be contaminated with pathogenic bacteria such as *Escherichia coli* and *S. aureus*. The contamination sources in the food sector are water, crude foods, dust, equipment, animals, etc. In addition, food handlers are also recognized as an important source of contamination in the food sectors (Todd et al.

2009). Moreover, it is now established that when the contamination of food products occurs, the biofilms are the major source of contamination. In fact, the persistence of biofilm on food contact surfaces, and equipment, may constitute a continuous source of contamination. In addition, the environmental conditions encountered in this sector, such as temperature, nutrient availability, surface type, pH and humidity, were found to influence bacterial growth and biofilm formation. Moreover, several authors underlined the presence of biofilms on the food contact surfaces despite the use of disinfection procedures. Gutierrez et al. (2012) showed that the food contact surfaces in the dairy, meat and seafood industries were colonized by *S. aureus* biofilms. Similarly, Gounadaki et al. (2008) showed that the most of the food contact surfaces, in the small-scale-processing facilities, were contaminated by the biofilms of *Listeria monocytogenes*, *Salmonella* spp. and *S. aureus*. Likewise, Latorre et al. (2010) observed, using the scanning electron microscopy, the presence of biofilms on the equipment of the dairy farms. Sharma and Anand (2002) also highlighted the persistence of biofilms on different segments of pasteurization lines, in commercial plants, despite daily cleaning. These authors also stated the prevalence of a wide variety of pathogenic bacteria such as *S. aureus* and *E. coli*. Furthermore, the study conducted on the placement of stainless coupons near food contact surfaces, in a shrimp and a fish factory, also showed the formation of biofilms counting between  $10^4$  and  $10^6$  CFU/cm<sup>2</sup> (Guobjoernsdottir et al. 2005). Thus, the food sector provides a suitable environment for the biofilm formation, which compromises food safety and increases the public health risk. Moreover, it is of importance to improve hygienic conditions to control the emergence of biofilms in this sector.

### Biofilms and medical environments

Healthcare-associated infections (HAIs) referred to those contacted during the course of health care in hospitals or other healthcare facilities (CDC 2013). These HAIs are associated with a significant increase in morbidity, mortality, and economic losses, which are often preventable. The most significant hospital-acquired infections are those of surgical site infections and device-associated healthcare-associated infections (DA-HAIs): central line-associated bloodstream infections (CLABSIs), catheter-associated urinary tract infections (CAUTIs), and ventilator-associated pneumonia (VAP) (Rosenthal et al. 2012; Sievert et al. 2013). The survey of the International Nosocomial Infection Control Consortium (INICC), conducted on 422 intensive care units (ICUs) of 36 countries in Latin America, Asia, Africa, and Europe, showed that the number of patients suffering from CLABSIs, CAUTIs, and VAPs was

of 7,029, 6,595, and 12,145 cases, respectively, between January 2004 and December 2009 (Rosenthal et al. 2012). Moreover, there is a regular occurrence of infections, which are often linked to surfaces and devices colonized by biofilms (Donlan 2001; Donlan and Costerton 2002; Bryers 2008). In fact, Wang et al. (2010) showed, using transmission electron microscopy and scanning electron microscopy, that the urinary catheters were colonized by the mixed biofilm of *S. aureus*, *P. aeruginosa*, *E. coli* and *Klebsiella pneumoniae*. In addition, Vickery et al. (2012) reported that the sterile supply bucket, the opaque plastic door, the venetian blind cord, and the sink rubber in ICUs were colonized by biofilms and in certain cases by the methicillin-resistant *Staphylococcus aureus* (MRSA). Thus, the medical sectors constitute a favorable environment for the development of biofilms, and the fight against their formation represents a great challenge in order to ensure the safety of healthcare services and to reduce the microbiological risk associated with them.

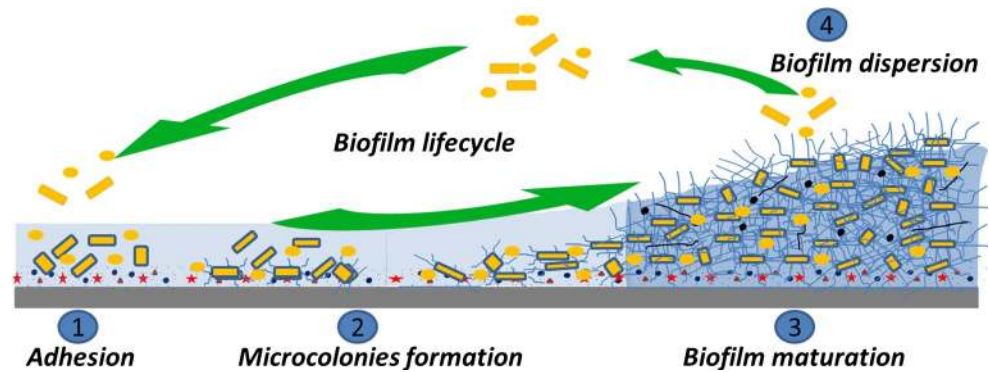
### Biofilms and other environments

The prevalence of pathogenic microorganisms in drinking water also presents a major public health concern (Sharma et al. 2003). In fact, several outbreaks were linked to the contamination of drinking water with pathogens (Brunkard et al. 2011). The most hazardous problem is the ability of these pathogens to form biofilms in the drinking water networks (Chaves Simoes and Simoes 2013). Such biofilms have detrimental effects on water quality, which may lead to waterborne infections, bio-corrosion, the reduction in the heat exchange efficacy, the cross-contamination of surfaces in both food and medicals fields, and increases in the maintenance costs of the distribution network (Anaissie et al. 2002; Chaves Simoes and Simoes 2013). Biofilms also cause severe problems in many different industries, such as paper production, petroleum, nuclear power plants, and marine industries (Bixler and Bhushan 2012; Flemming et al. 2013). In fact, such biofilms present a great impact on the deterioration and the failure of the materials of these industries.

### The biofilm formation

The definition of biofilm has evolved significantly since its discovery and researchers are still debating a common definition. However, the definition of Donlan and Costerton (2002) remains the most appreciated. These authors defined the biofilm as a structured community of microbial cells, enclosed in a self-produced polymeric matrix, and adherent to a surface, to interface, and to each other (Donlan and Costerton 2002).

**Fig. 1** Different steps of biofilm formation



Biofilm formation has four common stages. The first stage begins with the bacterial adhesion to surface (1), followed by the formation of microcolonies (2), and biofilm maturation (3). The final stage of the biofilm lifecycle is known as dispersion, in which the cells leave the biofilm structure in order to contaminate other surfaces (4). Bacterial adhesion to the surface (1) constitutes the first and essential step of the biofilm formation (Fig. 1) (Renner and Weibel 2011). This step is considered reversible and seems to be facilitated by many physical, chemical, and biological interactions (Bos et al. 1999; Renner and Weibel 2011). As the bacterial cell approaches a surface of interest, the entire cell will be exposed to nonspecific physiochemical forces such as Lifshitz-van der Waals, Lewis acid–base, and electrostatic interactions (Bos et al. 1999). The resultant force will allow a reversible bacterial adhesion to the surface. There is an increasing evidence that bacteria may sense the substratum, which allows them to conform to a biofilm condition. The bacterial appendages may constitute operative structures, which sense the abiotic surface and facilitate bacterial adhesion (Klausen et al. 2003; Weidenmaier and Peschel 2008). For example, it has been reported that motile bacteria sense the drag on its flagella motor caused by its interaction with the surface (Karatan and Watnick 2009). This phenomenon triggers a signal, which induces the expression of genes involved in biofilm formation, and repress flagellum synthesis (inhibition of motility) (Karatan and Watnick 2009). The adhesion to abiotic surfaces is also influenced by the environment surrounding the bacterial cells, such as temperature, organic matter, and pH. These factors may change bacterial and substrata surface properties and therefore the ability of bacteria to adhere to abiotic surfaces.

After attachment, the cells start to replicate into microcolonies (2). Reversible adhesion becomes irreversible mainly through the secretion of exopolymeric substances (EPS) that form the biofilm matrix. The extracellular matrix consists of a mixture of polymeric compounds such as polysaccharides, proteins, nucleic acids, and lipids (Flemming and Wingender 2010). These substances allow bacteria to

stick to surfaces and to each other (Ghafoor et al. 2011). At this stage, the process of biofilm maturation begins in order to create a mature biofilm in which the cells are encased in an extracellular matrix complete with a complex architecture with water channels. Such a matrix acts as a scaffold for the stabilization of the three-dimensional biofilm structure (Dunne 2002). It is now increasingly clear that the formation of a biofilm is under the control of several environmental signals such as temperature, nutrient availability. Bacteria may sense these environmental signals and trigger regulatory networks in order to modulate biofilm formation (Karatan and Watnick 2009). For example, *P. aeruginosa* sense the environmental signals by the sensor kinase/response regulators, such as the LadS, RetS, and GacS, which induce the expression of exopolysaccharides (Alginate, Psl, and Pel) (Harmsen et al. 2010). The intracellular signaling molecule, c-di-GMP, was also found to be a central regulator of genes controlling the biofilm state (Cotter and Stibitz 2007). Furthermore, *S. aureus* biofilm formation is linked to the expression of the polysaccharide intercellular adhesin (PIA), which is mediated by the intercellular adhesion (*ica*) locus. However, the *ica*-negative *S. aureus* strains retain the ability to form biofilm, indicating the involvement of another pathway such as the Bap-dependent one (Toledo-Arana et al. 2005). *S. aureus* cells also involve the SarA regulator factor, which enhances the *ica* operon transcription (for the *ica*-dependent pathway) and positively regulates the Bap-dependent pathway (Trotonda et al. 2005). The cell-to-cell communication mechanism or the quorum sensing (QS) also regulates the formation of biofilms. For example, *P. aeruginosa* has two acylated homoserine lactone (AHL)-based QS systems (Las and Rhl), which positively regulate the biosynthesis of exopolysaccharides (de Kievit 2009). However, the *S. aureus* QS system, the autoinducing peptide-based QS encoded by the (*agr*) locus, positively regulates the expression of several proteases promoting the dispersion of the *S. aureus* biofilm (Boles and Horswill 2008).

The last phase of the biofilm lifecycle is dispersion, which represents an option for the sessile cells to leave (4),



to contaminate other surfaces, and then to repeat the cycle. The biofilm dispersion is the result of several environmental events, such as alterations in nutrient availability, oxygen depletion, and other stress conditions, which promote the expression of genes involved in dispersion (McDougald et al. 2011). For example, it has been reported that the shift of a carbon source induced the expression of flagella, downregulated the pilus genes (twitching motility), and promoted the biofilm dispersion of *P. aeruginosa* (Sauer et al. 2004).

### The biofilm matrix

The EPS term corresponds to the different classes of exopolymeric substance such as polysaccharides, proteins, nucleic acids, and lipids (Czaczyk and Myszkowski 2007). The EPS constitutes approximately 50–90 % of biofilms organic matter. The composition of the biofilm matrix depends on numerous environmental conditions such as the nutriment availability, bacterial strains, and biofilm age (Czaczyk and Myszkowski 2007). Under the biofilm state, bacteria produce the extracellular polymeric substances in order to build up the matrix that holds the sessile cells together. *P. aeruginosa* cells were found to produce at least three secreted polysaccharides: the Alginate, the Psl (polysaccharide synthesis locus), and Pel (Pellicle). Alginate is a linear copolymer of 1,4-linked  $\beta$ -D-mannuronic acid (M) and its C-5 epimer  $\alpha$ -L-guluronic acid (G). The presence of anionic groups allows the association of divalent cations such as calcium, which increase the binding forces in a developed biofilm (Korstgens et al. 2001). The Psl polysaccharide consists of a repeating pentamer containing D-mannose, L-rhamnose, and D-glucose (Byrd et al. 2009). This polysaccharide was found to be involved in the first stage and the maturation of the *P. aeruginosa* biofilm (Ma et al. 2009; Zhao et al. 2013). Otherwise, the Pel structure, the third polysaccharide of *P. aeruginosa*, has not been revealed to date, but it seems to be a glucose-rich polysaccharide (Ma et al. 2007). Pel was found to increase the structural stability of microcolonies and plays a key role in the protection of *P. aeruginosa* biofilms against antimicrobial agents (Yang et al. 2011). In the gram-positive *S. aureus*, the main exopolysaccharide of the biofilm matrix, is the polymer of poly-N-acetyl- $\beta$ -(1-6)-glucosamine, also called PIA or poly-N-acetylglucosamine (PNAG). This partially de-N-acetylated polysaccharide is also produced by several bacteria such as *Staphylococcus epidermidis* and *E. coli* (Arciola et al. 2012). This cationic PNAG was found to mediate biofilm formation and resistance to antimicrobial agents (Ganeshnarayan et al. 2009). Recently, extracellular DNA (eDNAs) are recognized as one of the major components of biofilm matrix including those of *P.*

*aeruginosa* and *S. aureus* (Rice et al. 2007; Ghafoor et al. 2011). Moreover, eDNAs were found to improve the stability of biofilm structure and biofilm resistance toward antimicrobials (Mulcahy et al. 2008). For example, it has been reported that eDNA with the Pel increases the cell-to-cell interactions and compactness of the biofilm of *P. aeruginosa* in the absence of Psl (Ghafoor et al. 2011). The eDNAs of *S. aureus* biofilm matrix were found to enhance the compactness of the *S. aureus* biofilm, and its degradation promotes biofilm dispersion (Mann et al. 2009). Proteins, secreted or derived from cell lyses, are also abundant in the biofilm matrix. The matrix of *P. aeruginosa* biofilm harbors several proteins (CdrA, LapA, and lectins) and glycolipids also known as rhamnolipids (Mann and Wozniak 2012). Alternatively, *S. aureus* biofilms matrix contains several proteins, such as Bap, Aap and Spa, and teichoic acids (Arciola et al. 2012). Beside the role of these exopolymers in biofilm formation, they play an important role in the biofilm resistance to antimicrobial agents through the hindering of the biocides penetration inside biofilms.

### The mechanisms of bacterial adhesion to abiotic surfaces

Bacteria, in food and medical fields, are constantly exposed to different environmental conditions, which affect their adhesion to abiotic surfaces. Therefore, significant work has been done to understand the relationship between these factors and surface contamination. In fact, the environment surrounding bacterial cells, such as temperature, pH, and ionic strength, was found to influence bacterial adhesion to abiotic surfaces (Zita and Hermansson 1994; Zmantar et al. 2011). Moreover, the bacterial background may change the bacterial surface properties and therefore their adhesion rate to abiotic surfaces. In addition, bacterial growth under conditions relevant to food and medical environments, such as changes of growth temperature, pH, and culture medium, has been found to change the ability of bacteria to adhere to abiotic surfaces (Mafu et al. 2011; Gordesli and Abu-Lail 2012; Abdallah et al. 2014b). These factors may affect the structure of appendages of the cell walls, such as structural adhesins, cell wall proteins, extracellular polymers, flagellar motility, and pili, which are involved in bacterial attachment to abiotic surfaces (Hemery et al. 2007; Dehus et al. 2011). The initial adhesion of bacteria on non-living surfaces is a complex process and depends on three main components: the bacterial cells, the attachment surface, and the surrounding medium. The physicochemical properties of interacting surfaces (cells and substrata surfaces) may exert a strong influence on the bacterial adhesion to surfaces (Bos et al. 1999). However, the precise interaction, mediating the attachment of bacteria to abiotic surfaces, remains

unclear. It has been reported that the hydrophobic and the electrostatic interactions are the key forces modulating bacterial adhesion (Li and Logan 2004). In fact, it has been reported that the adhesion of *S. aureus* strains to hydrophobic surfaces was more important than to hydrophilic ones (Zmantar et al. 2011). However, *P. aeruginosa* cells seem to adhere more strongly to hydrophilic surfaces than to hydrophobic ones (Gomez-Suarez et al. 2002). On the other hand, Hamadi et al. (2005) found that the adhesion of *S. aureus* to different abiotic surfaces seems to be mediated by the acid–base interactions. In addition, the treatment of PVC with oxygen plasma yielded a hydrophilic surface and reduced the adhesion of *P. aeruginosa* by increasing the repulsive Lewis acid–base interactions (Triandafillu et al. 2003). Moreover, Carnazza et al. (2005) showed that the polar character of substrata may promote the secretion of exopolysaccharides and change the adhesion of *P. aeruginosa* to the polar surfaces. However, Mafu et al. (2011) have recently reported that the adhesion of *Aeromonas hydrophila*, *E. coli*, *Salmonella enteritidis*, and *S. aureus* was similar on both hydrophilic and hydrophobic surfaces. This finding seems to be consistent with the statement of Hui and Dykes (2012), which showed that the adhesion of *P. aeruginosa* and *S. aureus* to stainless steel and glass did not correlate with the bacterial and substrata surface properties. The bacterial adhesion on both hydrophilic and hydrophobic surfaces also seems to be dependent on the

bacterial genera and species. Indeed, Wang et al. (2011) have found that *E. coli* cells presented the highest adhesion rate to hydrophilic surfaces followed by *P. putida* and *P. aeruginosa*, in contrast to what has been observed on the hydrophobic ones. Otherwise, Bruinsma et al. (2001) found that the adhesion of the hydrophobic *P. aeruginosa* on both hydrophobic and hydrophilic lenses was more important than the adhesion of the hydrophilic *S. aureus*. It should be noted that there is no standard protocol to study bacterial adhesion. Thus, the variety of experimental procedures may result in contradicting outcomes and impede the comparison of results.

### The bacterial adhesion and the theoretical prediction

To control the surface contamination, several theories have been proposed to predict bacterial adhesion such as the thermodynamic and the extended Derjaguin, Landau, Verwey, and Overbeck (XDLVO) theories (Bos et al. 1999). The derived free energy of adhesion, according to the first one, is the summation of the Lifshitz-van der Waals ( $\Delta G_{\text{Adh}}^{\text{LW}}$ ) and the acid–base ( $\Delta G_{\text{Adh}}^{\text{AB}}$ ) forces at contact between bacterial and substratum surfaces (Table 1). The total derived free energy of adhesion does not account for distance dependence between the bacterial cells and solid surfaces. However, it is now established that the

**Table 1** The main theories used for the prediction of bacterial adhesion

Theory	Calculation	
Thermodynamic theory	$\Delta G_{\text{adh}}^{\text{tot}} = \Delta G_{\text{adh}}^{\text{LW}} + \Delta G_{\text{adh}}^{\text{AB}}$ $\Delta G_{\text{Adh}}^{\text{LW}} = -2 \left( \sqrt{\gamma_{\text{b}}^{\text{LW}}} - \sqrt{\gamma_{\text{l}}^{\text{LW}}} \right) \left( \sqrt{\gamma_{\text{sl}}^{\text{LW}}} - \sqrt{\gamma_{\text{l}}^{\text{LW}}} \right)$ $\Delta G_{\text{Adh}}^{\text{AB}} = 2 \left[ \left( \sqrt{\gamma_{\text{b}}^{+}} - \gamma_{\text{s}}^{+} \right) \left( \sqrt{\gamma_{\text{b}}^{-}} - \gamma_{\text{s}}^{-} \right) - \left( \sqrt{\gamma_{\text{b}}^{+}} - \gamma_{\text{l}}^{+} \right) \left( \sqrt{\gamma_{\text{b}}^{-}} - \gamma_{\text{l}}^{-} \right) \right]$	$\Delta G_{\text{adh}}^{\text{tot}}$ : interaction energy of adhesion $\Delta G_{\text{adh}}^{\text{LW}}$ : Lifshitz-van der Waals interaction energy $\Delta G_{\text{adh}}^{\text{AB}}$ : acid–base interaction energy $\gamma$ : surface free energy $\gamma^{\text{LW}}$ : Lifshitz-van der Waals component $\gamma^{\text{AB}}$ : Lewis acid–base component $\gamma^{+}$ : Lewis electron donor $\gamma^{-}$ : Lewis electron acceptor
DLVO	$\Delta G^{\text{tot}}(d) = \Delta G^{\text{LW}}(d) + \Delta G^{\text{AB}}(d)$ $\Delta G^{\text{LW}}(d) = -\frac{A}{6} \left\{ \frac{2r(d+r)}{d(1+2r)} - \ln \left( \frac{d+2r}{d} \right) \right\}$ $A = 12\pi d_0^2 \Delta G^{\text{LW}}(d_0)$ $\Delta G^{\text{AB}}(d) = 2\pi r \Delta G_{\text{Adh}}^{\text{AB}} \exp \left( \frac{d_0-d}{\lambda} \right)$	$\Delta G^{\text{tot}}(d)$ : Interaction energy of adhesion $d$ : the separation distance $d_0$ : the minimum separation (0.157 nm) $r$ : the radius of the bacterium $A$ : the Hamaker constant
Extended DLVO	$\Delta G^{\text{tot}}(d) = \Delta G^{\text{LW}}(d) + \Delta G^{\text{AB}}(d) + \Delta G^{\text{EL}}(d)$ $\Delta G^{\text{EL}}(d) = \pi \varepsilon \varepsilon_0 r \left( \xi_{\text{b}}^2 + \xi_{\text{s}}^2 \right) \left\{ \frac{2\xi_{\text{b}}\xi_{\text{s}}}{\xi_{\text{b}}^2 + \xi_{\text{s}}^2} \ln \left[ \frac{1+e^{(-\kappa d)}}{1-e^{(-\kappa d)}} \right] + \ln [1 + e^{(-2\kappa d)}] \right\}$	$\lambda$ : the correlation length of molecules in the liquid medium $\varepsilon \varepsilon_0$ : the dielectric permittivity $\xi_{\text{b}}$ and $\xi_{\text{s}}$ : the surface zeta potentials $\kappa$ : the reciprocal Debye length

DLVO Derjaguin, Landau, Verwey, and Overbeck theory

thermodynamic theory cannot fully predict bacterial adhesion and this is probably due to the inadequate description of electrostatic interactions (Sharma and Rao 2002). Therefore, the XDLVO theory (Table 1), which considers both repulsive and attractive forces acting in bacterial adhesion, has been used (Bos et al. 1999). According to this theory, the microbial adhesion is described as a balance between the Van der Waals, the electrostatic and the Lewis acid–base interactions (Table 1). The magnitude of these interactions is affected by the distance of the bacterium from the surface and the ionic strength of the surrounding environment. However, the validity of the XDLVO theory in the prediction of bacterial adhesion is still under investigation. Indeed, considerable studies have found that this theory is a useful tool to predict the bacterial adhesion to different abiotic surfaces (Bayouth et al. 2009; Hwang et al. 2010). However, further studies showed that this theory cannot predict the bacterial adhesion under all conditions (Chia et al. 2011; Nguyen et al. 2011). This discrepancy between theoretical predictions and empirical results could be related to the fact that this theory considers the complex living bacteria as ideal colloidal particles. In addition, the XDLVO may fail to take into account the appendages of the bacterial wall, which mediate bacterial adhesion (Houry et al. 2010). On the other hand, the calculation of interaction magnitudes is based on the contact angle measurement (CAM) outcomes. However, further studies stated that CAMs seem inadequate for the measurement of acid–base properties of bacterial surfaces (Nguyen et al. 2011). Although surface roughness is not included in the XDLVO theory, the failure of theoretical predictions may reflect the influence of surface topography on bacterial adhesion as previously reported (Singh et al. 2011). Moreover, Mitik-Dineva et al. (2009) indicated that the adhesion rate of *P. aeruginosa* and *S. aureus* appears to be inversely correlated with surface roughness. However, different studies underlined that the adhesion of bacteria, such as *E. coli*, *S. aureus*, and *S. epidermidis*, is not related to this factor (Prokopovich and Perni 2009). Thus, the microbial adhesion to abiotic surfaces seems to be a complicated process. The validity of the theoretical prediction and the involvement of surface roughness in bacterial adhesion are still a matter of debate, and more studies are required in order to understand this phenomenon.

### The effect of environmental conditions on biofilm formation

Bacteria in natural and industrial ecosystems are constantly exposed to various environmental conditions. Considerable studies have reported the effect of factors, such as temperature changes, nutrient availability, oxygen level, pH and

surface type, on the biofilm formation of pathogenic bacteria such as *S. aureus* and *P. aeruginosa*.

### Effect of temperature changes and nutrient availability

Several studies have showed that the temperature changes, which take place in both food and medical environments, affect biofilm formation (Cerca and Jefferson 2008; Nilsson et al. 2011). The biomass of the *P. aeruginosa* biofilm seems to increase with increases in incubation temperature (Hostacka et al. 2010). However, the effect of temperature changes remains unclear on the biofilm formation of *S. aureus*. In fact, Choi et al. (2013) and Vazquez-Sanchez et al. (2013) have found that the biomass of *S. aureus* biofilms grown at 37 °C was more important than those grown at 25 °C on polystyrene. However, Pagedar et al. (2010) reported a higher cell count of the *S. aureus* biofilm at 25 °C in contrast to that obtained at 37 °C on stainless steel (Pagedar et al. 2010). Otherwise, Da Silva Meira et al. (2012) showed that there is no clear effect of the incubation temperature (7 and 28 °C) on the biofilm formation of *S. aureus*. This discrepancy may reflect the difference in experimental conditions, or the synergistic effect of the growth temperature and other environmental factors such as nutrient availability and surface type on biofilm formation. In fact, Oulahal et al. (2008) found that the effect of the growth temperature on the formation of *S. aureus* biofilm is dependent on the type of the nutrient, the surface type, and the incubation time. When the pasteurized skim milk was used as a culture medium, these authors have found that the biomass of biofilms grown at 25 °C was lower than those formed at 12 °C after an incubation time of 8 days. However, when they used the raw milk, no significant difference was found in the biofilm biomasses with the variation in growth temperature. Likewise, Rode et al. (2007) also found that the effect of temperature on biofilm formation was dependent on the presence of glucose and NaCl (Rode et al. 2007).

Bacteria, in the medical and food processing environments, are usually exposed to divergent levels of nutrients which may influence biofilm formation. It has been reported that nutrient-rich growth media may enhance biofilm formation (Herrera et al. 2007). The presence of glucose and NaCl also appears to influence the biofilm formation of pathogenic bacteria such as *S. aureus* and *P. aeruginosa* (Rode et al. 2007; Huang et al. 2009). The presence of iron has been also found to increase the biofilm biomass of *P. aeruginosa* and *S. aureus* (Banin et al. 2005; Lin et al. 2012). In biological systems,  $\text{Ca}^{2+}$ , mainly recognized as intracellular secondary messenger molecule, is involved in the formation and structure stability of biofilm structure (Shukla and Rao 2013). In fact, the divalent cations seem to

maintain the biofilm structure by bridging and cross-linking polymers of the biofilm matrix. Other factors such as the non-lethal concentration of antimicrobial residues were found to enhance biofilm formation (Knobloch et al. 2001).

### Effect of substrata surface properties and topography

Food and biomedical equipment are often subject to bacterial contamination and biofilm formation. Moreover, several reports have already shown the ability of bacteria to form biofilms on materials commonly encountered in these fields, such as the stainless steel, glass, rubber, polycarbonate, polyurethane, polystyrene, polypropylene, titanium, aluminum, and ceramic. (Donlan 2001; Simões et al. 2010). It has been reported that the biofilm formation does not correlate with the initial adhesion rate (Cerca et al. 2005). Therefore, the substratum surface properties are thought to be more involved in the late stage of biofilm formation rather than the first one. Considerable studies have found that the biofilm formation on hydrophobic substrata occurred to a greater extent than that on hydrophilic ones (Cerca et al. 2005; Pagedar et al. 2010). However, the superhydrophobic surfaces were found to inhibit the biofilm formation (Loo et al. 2012). Otherwise, Chavant et al. (2002) have found that *L. monocytogenes* formed biofilms more rapidly on hydrophilic surfaces than on hydrophobic ones. Recently, Da Silva Meira et al. (2012) have stated that stainless steel (hydrophilic) and polystyrene (hydrophobic) have no significant effect on the biofilm formation of *S. aureus*. Moreover, the surface roughness has been found as an essential factor affecting biofilm formation including those of *P. aeruginosa* and *S. aureus* (Arnold and Bailey 2000; Tang et al. 2011). However, further studies have stated that the correlation between surface roughness and biofilm formation was poor (Rodriguez et al. 2008). The role of surface properties on biofilm formation may depend on bacterial genera, species, and strains used in each study. For example, Litzler et al. (2007) reported that the attachment of *P. aeruginosa* and *S. epidermidis* to pyrolytic carbon may correlate with surface roughness, while *S. aureus* attachment appeared to be independent of this factor. The conditioning of substratum also affects the bacterial attachment and biofilm formation. The substratum surfaces could be coated, in both medical and food fields, by a film of organic matter, such as proteins from milk, blood, meat, and even the EPS produced by bacteria, which may influence the bacterial adhesion and biofilm formation (Herrera et al. 2007; Hwang et al. 2013). Those findings showed that environmental factors have a strong effect on biofilm formation. However, this discrepancy found on their involvement in bacterial adhesion, and biofilm formation may reflect the difference in the method of biofilm formation

which may give conflicting results. Moreover, the lack of a reference method for biofilm formation makes it often difficult to obtain consistent results.

### The systems used to study biofilm formation

To increase knowledge about bacterial sessile life, a variety of systems, commercially available or standardized in the laboratory, are used to study biofilm formation. The concept of each one is designed to simulate and to represent the application domains such as food and medical ones. In this review, these systems are classified into two major groups: the static and the flow systems. These different systems have progressively contributed to the current knowledge of biofilm formation and regulation, to the study of preventive or curative strategies to control biofilm-related problems.

Static biofilm systems may be preferable to continuous flow methods for a number of reasons. The main advantages of these systems are the simplicity of experimental procedures, the adaptation to a variety of conditions, and a high screening capacity. Microtiter plate (MTP)-based systems are among the most common systems used for the study of biofilm formation and resistance toward disinfectants (Theraud et al. 2004). In the MTP-based systems, the biofilm is either grown on the bottom or on the walls of wells. The advantage of this method is the high number of conditions that can be analyzed in one experiment and the low cost of experiments (small volumes of culture medium). The MTP-based system was upgraded thereafter to as we know now the “Calgary Biofilm Device,” commercialized as the MBEC assay (“minimal biofilm eradication concentration” assay) (Ceri et al. 1999). In this system, biofilm grow on 96 pegs, fixed to the lid of the microplate, was immersed in the culture medium. The agar plate system is also among the systems proposed for the study of biofilm formation under static conditions. This method is based on the deposition of bacterial suspension into sterile filter posed on the solid agar medium (Hammond et al. 2011). Then, the biofilm can be studied directly on the filter or on the sterile test slide placed on the inoculated filter. Another method is the Biofilm Ring Test, developed by Chavant et al. (2007), which uses the microplate. This system is based on the concept of the immobilization of magnetic beads by sessile bacteria. These colonized beads have enough strength to overcome the magnetic attraction forces applied on them in each well. No colonized beads are attracted to the bottom of each well, and the colonized beads are quantified using a spectrophotometer. Otherwise, there are a variety of systems based on the immersion of test slides in culture medium in different ways.



Despite the advantages of the above-described systems, the static biofilm systems are limited by nutrient availability. In fact, continuous flow systems were developed in order to overcome nutrient limitation by a continuous or semi-continuous medium flow. In contrast to static systems, the flow systems allow a biofilm growth under (semi-) continuous flow of culture medium. Several flow systems have been developed to study biofilm formation under dynamic conditions, such as the modified Robbins device (MRD), the Centre of Disease Control reactor (CDC), drip flow biofilm reactors, and the rotating disk reactor.

The MRD is developed by Jim Robbins at the University of Calgary. The MRD is composed of a rectangular channel in which a press-fit plug, holding a disc of biofilm formation, is inserted in individual ports (Coenye et al. 2008). The CDC is composed of several coupon holders, which are suspended from the lid of the glass vessel. The mixing is provided by a sterile magnetic bar placed in the bottom of vessel (Buckingham-Meyer et al. 2007). The rotating disk reactor consists of a disk designed to hold slides in the bottom of a vessel. The bottom of the rotating disk contains a bar magnet to allow disk rotation in the liquid growth media. Drip flow biofilm reactors consist of different isolated rectangular channels, holding one standard coupon (Buckingham-Meyer et al. 2007). In this system, the biofilms are formed under low-shear forces (low flow of culture medium). The flow cell consists of a base made of plastic or a square glass tube through which microorganisms and nutrient are pumped for biofilm formation. This device can be placed on the stage of a microscope and allows real-time non-destructive microscopic analyses of biofilms (Haagensen et al. 2007).

### The strategies to control biofilm formation

The harsh consequences of biofilms in the industrial fields generated extensive research to avoid biofouling. In this regard, several strategies have been proposed to control biofilm formation. These strategies can be classed into two major groups. The aim of the first one is to prevent bacterial adhesion and biofilm formation with either surface property modification or antimicrobial surface coating. The second one aims to eradicate/disrupt formed biofilms using antimicrobial agents, physical forces, enzymes, phages, etc.

### Prevention of biofilm formation

The strategies involving the modification of surface properties have emerged as an option to prevent biofilm formation. For example, the design of superhydrophobic surfaces has been found to be effective in the prevention of cell

attachment and the biofilm formation of several bacteria such as *P. aeruginosa* and *S. aureus* (Lin et al. 2011; Loo et al. 2012). In addition, the modifications of surface topography have also been found as promoting tool to prevent the bacterial adhesion of pathogenic bacteria (Verran and Whitehead 2005). Recently, different approaches have been proposed, which consist of antimicrobial-coated surfaces in order to reduce biofouling and associated infections. Moreover, various synthetic technologies have been extensively explored to immobilize the active agents such as antimicrobial peptides, anti-quorum sensing, essential oils, enzymes, and quaternary ammonium (QA), on abiotic surfaces (Glinel et al. 2012; Karam et al. 2013). The antimicrobial-coated surfaces have been found suitable for the inhibition of biofilm formation by either killing bacteria or preventing their adhesion. However, some antimicrobial coating can be toxic for humans, limiting the implementation of these methods in the food and medical fields.

### Eradication of biofilms with disinfectant agents

Ideally, the prevention of the biofilm formation would be safer than eradicating it. However, there is no perfect technique to prevent the formation of biofilms. In fact, the modified surfaces may only reduce and not completely prevent bacterial adhesion. Furthermore, bacteria may use different mechanisms of attachment in response to these surface modifications. The overuse of antimicrobial-coated surfaces may allow bacteria to develop resistance against the antimicrobial of interest and increase microbiological risks. Thus, there is a demand for curative agents across industries, such as food processing and healthcare, in order to maintain a high level of hygiene and to fight against biofilm formation in these fields.

It is now established that regular cleaning and disinfection represent the main strategy to prevent biofilm formation (Donlan 2001; Simões et al. 2010). In health facilities and food sector, biocides are widely used to decontaminate surfaces, instruments, and equipment that come into contact with the human body or with the food product. In addition, there are a variety of commercialized disinfectants, commonly used within these fields, such as alcohol-based products, hypochloric solutions including sodium hypochlorite, aldehydes, peracetic acid, hydrogen peroxide, ozone, chlorhexidine digluconate, polyhexamethylene biguanides (PHMB), and QA compounds (Grobe et al. 2002; Maillard 2005; Buckingham-Meyer et al. 2007; Harrison et al. 2008; Belessi et al. 2011). The same agent can be used by different sectors with the main difference being the concentration at which it is employed (Maillard 2005). Bacteria vary in their susceptibility to biocides, with bacterial spores being the most resistant, followed by

mycobacteria, gram-negative, gram-positive, and fungal microorganisms (Maillard 2005). However, it is not possible to predict which microorganisms will be present on surfaces. Thus, disinfectant products must be adapted whatever the lifestyle and the kind of harmful microorganisms.

Unlike antibiotics, which affect a specific physiological process, the disinfectant molecules in general have more than one target site. Biocides, such as QA, phenols, biguanides, and alcohols, have the cytoplasmic membrane as a main target (McDonnell and Russell 1999). These active agents may promote the *precipitation* of cellular material (McDonnell and Russell 1999). It has been reported that the QAs interact with the bacterial membrane causing its disruption and the leakage of intracellular components (Ioannou et al. 2007). The QA contains one quaternary nitrogen, which is associated with at least one major hydrophobic substituent and an anion such as Cl or Br. The mode of action of these cationic biocides seems to involve both hydrophilic and hydrophobic moieties (Palermo et al. 2011). The hydrophilic moiety of QAs is thought to adsorb to the relatively anionic bacterial cell walls, while the hydrophobic tail integrates into the lipid bilayer causing its disruption (Gilbert and Moore 2005). However, the PHMB, which are poly-cationic disinfectants, bind to the negatively charged phosphate head groups of phospholipids and do not integrate into the bilayer (Gilbert and Moore 2005). The PHMB bridges between pairs of adjacent phospholipid head groups causing its aggregation and disruption in the cell walls. The antimicrobial action of alcohols, which are also dehydrating agents, is related to the denaturation of surface and intracellular proteins. Disinfectants based on alkylating agents, hydrogen peroxide, ozone, peracetic acid penetrate inside cells and interact with the cells constituents, such as proteins, ribosome, nucleic acid, and enzymes, causing the cells death (McDonnell and Russell 1999).

### Factors affecting the disinfectant efficacy against the biofilm

The activity of biocides against biofilms depends upon a number of factors. The principal physical and chemical factors that influence the efficiency of these agents are the concentration, contact time, temperature, and pH of treatment. The efficacy of disinfectants against biofilms usually increases with the increase in both biocide concentration and time of treatment (Mafu et al. 1990; Grobe et al. 2002; Surdeau et al. 2006; Belessi et al. 2011). However, there needs to be a balance between efficacy and toxicity. The increase in temperature and pH of the treatment may increase the efficiency of disinfectant agents (Mafu et al. 1990; Chavant et al. 2004). In addition to the effect

of the treatment conditions, other factors related to the environmental conditions of biofilm growth may affect the efficiency of disinfectant products. For example, it has been reported that the increase in the biofilm growth temperature increases biofilm resistance to disinfectant agents (Belessi et al. 2011; Nguyen and Yuk 2013). Otherwise, the increase of the duration in incubation time of biofilms often results in an increase in biofilm resistance to antimicrobial agents (Stopforth et al. 2002; Nilsson et al. 2011). The surface type may also influence the efficacy of biocides in killing and removing biofilms from the abiotic surfaces. The involvement of the surface type could be related to the effect of surface properties on the biofilm shield and architecture (Singh et al. 2011), or on the effectiveness of cleaning and sanitizing (Chaturongkasumrit et al. 2011; Schlisselberg and Yaron 2013). In fact, the surface defects/roughness often makes the cleaning process more difficult.

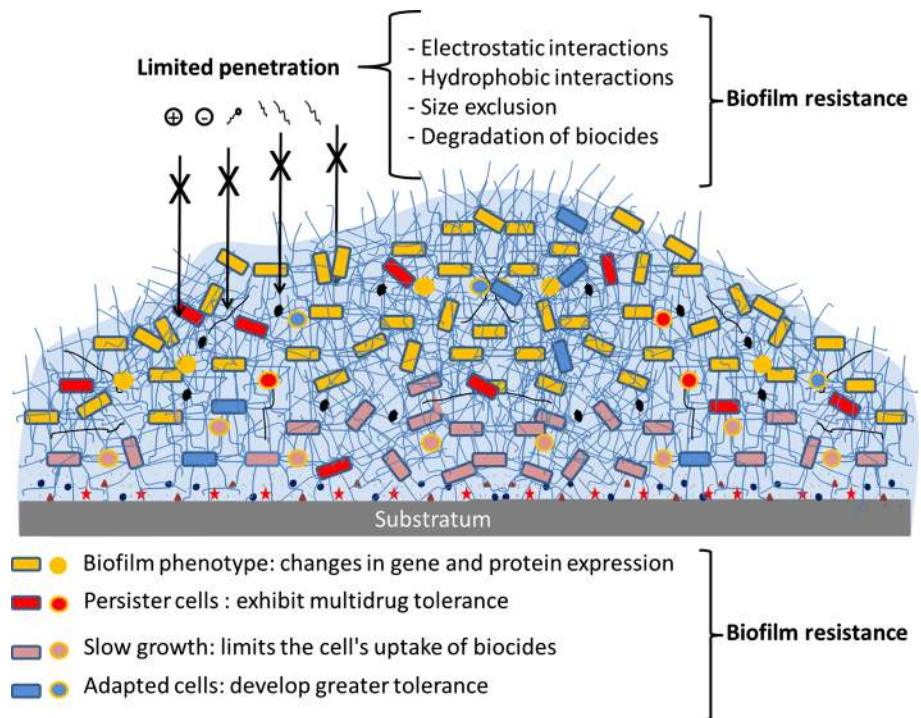
### The biofilm resistance to disinfectant agents

The most clinical guidelines for the use of biocides have been developed for planktonic microorganisms (Cerf et al. 2010). However, most of the microorganisms live as surface-adherent communities. In addition, considerable works have already shown that the cells living under a biofilm state can be up to 1,000-fold more resistant to disinfectant products than their planktonic counterparts (Campanac et al. 2002; Grobe et al. 2002; Belessi et al. 2011; Bonez et al. 2013). Thus, the commercialized disinfectants may have a confirmed efficiency on the planktonic cells and often be unable to eradicate biofilm cells. This high tolerance of sessile cells to biocides may increase the risk of further disinfection failure leading to severe health problems and economic losses. Therein, the current researches are focused on the mechanisms of the biofilm resistance to disinfectant agents in order to understand them and to improve biofilm control strategies. In fact, several mechanisms have been proposed to explain the apparent increased resistance of biofilm cells (Fig. 2). The biofilm resistance is thought to be linked to the: (1) restricted penetration of biocides into the biofilm, (2) the biofilm phenotype and adaptation of cells to the biofilm environment, and (3) presence of disinfectant-adapted and persister cells.

### The restricted penetration of biocides inside the biofilm

The cells living under the biofilm state are embedded in self-produced exopolysaccharides, DNA, proteins, and lipids. Thus, antimicrobial agents should encounter this physical barrier, which prevents them from reaching their targets in the deeper layers of the biofilm. This hypothesis

**Fig. 2** Mechanisms of biofilm resistance to antimicrobial agents



is supported by several experimental studies indicating that the biofilm matrix may hinder the penetration of numerous disinfectant molecules into biofilms (Stewart and Raquepas 1995). The involvement of the extracellular matrix in the resistance to QA was underlined by Campanac et al. (2002), who showed that the dispersion of *P. aeruginosa* biofilm increased the sensitivity of sessile cells to the biocide treatment. Moreover, it has been reported that the penetration of chlorine dioxide ( $\text{ClO}_2$ ) was delayed in the mixed biofilm of undefined bacteria from unpasteurized whole milk (Jang et al. 2006). These authors showed that the  $\text{ClO}_2$  failed to reach more than 100  $\mu\text{m}$  into the biofilm with a thickness varying from 150 to 200  $\mu\text{m}$ . In addition, Stewart et al. (2001) reported that penetration of the hypochlorite is delayed in the mixed biofilms of *P. aeruginosa* and *Klebsiella pneumoniae*. Using time-lapse confocal laser imaging, Davison et al. (2010) have found that the penetration of biocides inside the biofilm of *S. epidermidis* was retarded by the factor of 600 and 60, respectively, for chlorine and QA. Using the same technique, Bridier et al. (2011) also observed that diffusion–reaction limitations are involved in the resistance of *P. aeruginosa* biofilms to benzalkonium chloride.

Those findings showed that biofilm resistance seems to be related to the involvement of the biofilm matrix in the retention of biocides. Furthermore, several hypotheses have been proposed for the mechanisms of antimicrobial interactions with the biofilm matrix. In fact, the biofilm matrix was found to limit the diffusion of antimicrobial in biofilms either by size exclusion or by electrostatic interactions

(Zhang et al. 2011). When they used different probes to study the diffusion inside of *Streptococcus mutans* biofilms, Zhang et al. (2011) found that the relative diffusion coefficients decreased with the increasing of the probe size and the negative charge. The involvement of electrostatic interactions was also underlined by Tseng et al. (2013), who showed that the positively charged biocides is sequestered to the biofilm periphery, while the neutral ones readily penetrated. The same observation was stated by Mulcahy et al. (2008), who found that the matrix of *P. aeruginosa* sequesters the cationic antimicrobial and increased biofilm resistance. Ganeshnarayan et al. (2009) also underlined that the cetylpyridinium chloride (CPC), a QA, binds reversibly to matrix of *Actinobacillus pleuropneumoniae* and *S. epidermidis*, and this was probably due to the contribution of electrostatic interactions. In fact, these authors showed that the CPC could be eluted from the biofilms using 1M of NaCl solution. Since both CPC and PNAG are cationic, it is possible that CPC binds to the PNAG by the mean of its hydrophobic tail. On the other hand, Sandt et al. (2007) suggested that the interactions between the QA groups and the biofilm matrix are not the prime contribution to strong CPC binding, while the length of the hydrophobic tail plays a role in this association through hydrophobic interactions. Interestingly, Epstein et al. (2011) underlined that *Bacillus subtilis* biofilm surface remained non-wetting against up to 80 % ethanol as well as other organic solvents and commercial biocides. Thus, the biofilm matrix may involve both electrostatic and hydrophobic interactions in order to hinder the penetration of antimicrobial agents into the

deeper layers. However, this property seems to be dependent on the nature of the biocides used. Bridier et al. (2011) reported that the matrix of *P. aeruginosa* delayed the penetration of benzalkonium chloride, while peracetic acid was not so affected. The deactivation of biocide is also among the mechanisms of biofilm resistance proposed. The biofilm matrix may accumulate degradative enzymes, such as the catalase that prevent the full penetration of hydrogen peroxide into biofilms (Stewart et al. 2000).

### The phenotype of biofilm cells

Different approaches have been proposed to explain the biofilm resistance to biocides, since the inhibition of diffusion inside biofilms cannot always explain the resistance of sessile cells to an antimicrobial compound. Corbin et al. (2011), using green fluorescent dye, observed that the time for the loss of the green color after the biofilm treatments with ethanol, sodium lauryl sulfate, triclosan, chlorhexidine digluconate, CPC, and nisin was much longer than the time of diffusion predicted for each agent. These findings suggested that other factors such as decreased growth rate, membrane permeability changes and the adaptation of cells to biofilm environments could be involved in the resistance of sessile cells to biocide agents.

The reduced growth rate of bacterial cells under the biofilm state is also among the hypothesis proposed for the biofilm resistance to antimicrobials (Schulte et al. 2005). It is now established that the bacteria grown in the stationary phase present an enhanced resistance to disinfectant agents than those in the exponential phase (Luppens et al. 2002; Cherchi and Gu 2011). Moreover, cells grown under a biofilm state were found to resemble stationary phase rather than the planktonic stage. Thus, it is easy to imagine that bacterial growth in the deeper layer of biofilm is slowed or arrested, owing to substrate and oxygen limitation, and may diminish the uptake of antimicrobials.

The sessile cells have been found to be phenotypically different from their planktonic counterparts. This phenotype, also called “biofilm phenotype,” was proposed to explain the resistance of sessile cells to antimicrobial agents. The upregulation of exopolysaccharide production is considered a phenotypic characteristic of surface-attached bacteria. In fact, the transition from floating to sessile state increased the expression of genes involved in the biosynthesis of EPS as previously reported for *P. aeruginosa* and *S. aureus* (Friedman and Kolter 2004; Resch et al. 2006). The transition to the sessile phenotype can induce changes in the membrane fatty acids profile, which maintain the membrane fluidity of bacterial cells. For example, the transition from planktonic to sessile state was found to decrease the membrane fluidity of *Listeria monocytogenes*

(Gianotti et al. 2008) and *P. aeruginosa* sessile cells (Benamara et al. 2011). Such an increase in membrane rigidity may hinder the penetration of biocide into the lipid bilayer and enhance the resistance of biofilm cells to disinfectant agents at the cellular level (Bisbiroulas et al. 2011; Abdallah et al. 2014a). The transition to the sessile phenotype was found to induce changes in the expression of membrane and cytosolic proteins. Sauer et al. (2002) found that the transition of *P. aeruginosa* cells from planktonic to sessile state varied the expression of more than 800 proteins. Further study showed that *E. coli* sessile cells induced the expression of 35 proteins and downregulated about 59 proteins when compared to stationary-phase cells (Perrot et al. 2000). *Salmonella enterica* serovar Enteritidis PT4 growing under the biofilm state also changed the expression of 61 proteins (Giaouris et al. 2013). Moreover, this study showed that the sessile cells differed from planktonic ones by the expression of a group of proteins involved in the stress response, nutrient transport, and DNA metabolism (Giaouris et al. 2013). Thus, the protein expression changes may also be a part of biofilm resistance to biocides since several upregulated proteins have been associated with the resistance to disinfectant agents (Tabata et al. 2003).

The sessile cells are subject to several stresses such as starvation, osmotic, and oxidative stress (Stewart and Franklin 2008). It is known that these cells, in response to stress conditions, induce an adaptive stress response such as the expression of the stress sigma factor RpoS ( $\sigma^S$ ). The  $\sigma^S$  is the master regulator of the general stress response and was found to be upregulated in the gram-negative biofilm cells such as *P. aeruginosa* and *E. coli* (Waite et al. 2005). Moreover, this factor ( $\sigma^S$ ) was found to positively control the expression of more than 240 genes encoding stress management proteins, metabolic enzymes, membrane proteins, and regulatory proteins (Weber et al. 2005). The alternative sigma factor SigB ( $\sigma^B$ ), controlling the cellular stress responses of gram-positive bacteria, has been found to be upregulated under the biofilm state (Rachid et al. 2000). Thus, it can be expected that these factors may affect biocide resistance, by the regulation of biofilm formation and the regulation of genes involved in the resistance to biocides. Moreover, the deletion of sigma factor has been found to increase the sensitivity of both planktonic and sessile *L. monocytogenes* cells to the benzalkonium chloride and peracetic acid (van der Veen and Abee 2010), the *P. aeruginosa* sessile cells to hydrogen peroxide (Cochran et al. 2000), and the sessile *S. aureus* to different house cleaners (Davis et al. 2005). Furthermore, it has been reported that the increased antioxidative capacities in the biofilm, in response to oxidative stresses, may also increase the resistance of sessile cells to oxidative agents such as sodium hypochlorite and hydrogen peroxide (Leung et al. 2012). Otherwise, the biofilm formation is under the control of



several factors which in turn are regulated by the QS molecules. Although the role of QS in resistance of sessile cells to disinfectant is not yet clear, the deletion of *lasI* and *rhlI* of *P. aeruginosa* was found to increase its sensitivity to disinfectant agents (Hassett et al. 1999).

### The presence of disinfectant-adapted and persister cells

The increased use of disinfectants at lower concentrations than that recommended by the manufacturer has raised some concerns about their overall efficacy, but also about the emergence of microbial resistance to biocides. In fact, the bacterial adaptation to disinfectant products has been reported for several bacteria (Langsrud et al. 2004; Condell et al. 2012). In addition, the food and the medical environments constitute a reservoir of bacteria presenting high tolerance to disinfectant products, which is due to misuse of these agents (Romao et al. 2005; Marino et al. 2011). Moreover, disinfectant-adapted bacteria may exhibit cross-resistance to other disinfectant agents (Langsrud et al. 2004). For example, the benzalkonium chloride adapted *P. aeruginosa* presented a cross-resistance to other membrane-active disinfectants such as CPC and cetrimide (Loughlin et al. 2002). Thus, food and medical equipment is constantly confronted with the formation of biofilms harboring already disinfectant-resistant bacteria, which increase the chance of biofilm cells survival in the biocide treatment. Furthermore, the exposure of bacteria to a sublethal biocide concentration engendered adapted phenotype changes, and this was predominantly due to the contribution of efflux pump activity (Mc Cay et al. 2010). The efflux proteins, also known as multidrug resistance (MDR), remove toxic substances, including antimicrobial agents from the cells (Morita et al. 2003). However, the involvement of these structures in the resistance of the biofilm is still not fully understood, since their expression it is not induced under the biofilm state (Folsom et al. 2010).

Recently, the involvement of persister cells in biofilm has been proposed as a hypothesis of the biofilm resistance to biocides. The persister cells describe a bacterial phenotype, which is highly tolerant to antimicrobial treatments (Simoes et al. 2011). This population has been estimated to reach about 0.1–10 % of total biofilm cells. However, the exact cause leading to the formation of this protected subpopulation, “persister cells,” remains not fully understood (Lewis 2010).

### Mechanical and enzymatic treatments

The disinfection processes previously described have some disadvantages such as the reaction with material and the

toxicity of some disinfectant agents. In an effort to stem the increase in biofilm resistance to the conventional control strategies with chemical-based disinfectants, new approaches have been introduced in order to overcome and to control the biofilm-related problems such as the use of mechanical forces, green strategies, and the phage.

Mechanical cleaning is one of the most effective ways to fight against biofilms (Donlan and Costerton 2002). The high shear forces affect the mechanical stability of biofilms and facilitate its removal from abiotic surfaces or the accessibility of antimicrobial compounds. For example, the combination of high flow rates with detergents showed an important efficiency of biofilm removal in the endoscopes (Vickery et al. 2009). The use of ultrasonication also seems to be a useful option for improving disinfectant efficiency and biofilm removal (Shen et al. 2010). It is now established that the exopolymeric matrix is a part of biofilm resistance to disinfectant products. In addition, even if disinfectant agents can reduce completely the viable count of sessile cells, most biocides leave the matrix undisturbed (Tote et al. 2010). Thus, the dispersion of the biofilm matrix is among the approaches proposed to remove biofilms and to disrupt their structure (Xavier et al. 2005). The uses of enzymes, promoting the degradation of exopolymers such as proteases, amylase proteases, and DNAase, have been found as a suitable option to facilitate the breakdown of the biofilm matrix. This approach has the advantage of reducing the excessive use of toxic antimicrobial agents. In addition, the combination of enzymatic treatment and other strategies such as surfactants, chelating agents, and ultrasonic may improve the enzymatic activity and biofilm removal (Oulahal et al. 2007; Lequette et al. 2010). However, the application of enzymes in the control of biofilms is still limited due to the low price of chemical agents in comparison with enzymes.

### The treatment of biofilms with bacteriophages

Bacteriophage treatment is another emerging method of the biofilm control and removal. For example, the phage K has showed successful effect in the removal and prevention of *S. aureus* biofilms (Kelly et al. 2012). Similarly, T7-like lytic phages isolated from river water dispersed the biofilm of multidrug-resistant strains of *P. aeruginosa* (Donlan 2009). The engineered enzymatic bacteriophage, producing depolymerases that hydrolyze biofilm extracellular polymers, has been found to be a promising tool of biofilm control (Donlan 2009). Moreover, biofilm removal by enzymatic bacteriophage has been found to be more efficient than the classical enzymatic treatment (Lu and Collins 2007). The combination of phages with other antibacterial agents also showed interesting outcomes (Zhang and Hu 2013).

## Conclusion

The food and medical sectors have the prevention/eradication of biofilms as their main objective. Both sectors may provide a suitable environment for biofilm development, which threatens public health and increases economic loss. The environmental conditions, encountered in both sectors, may enhance the bacterial adhesion and biofilm formation on abiotic surfaces. It is therefore of interest to be conscious of the bacterial ecological background and the environmental conditions of biofilm formation such as temperature, surface type, and biofilm age, which influence the biofilm resistance. Moreover, a clearer understanding of the mechanisms of bacterial adhesion is needed in order to reduce the surface contamination and therefore biofilm formation. Furthermore, the biofilm resistance to disinfectant agents seems to be multifactorial and involves several parameters. The biofilm matrix may constitute a physiological barrier hindering the penetration of biocides inside biofilms. Moreover, the adaptation of bacterial cells to biofilm environments may contribute to the resistance of sessile cells to disinfectant agents. In order to control the issues related to biofilms, further experiments should focus on the relationship between the environmental condition of biofilm formation and biofilm resistance to disinfectant agents. Such studies will be useful to understand the biofilm resistance and therefore to improve the biofilm treatments.

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