



Biofilm-Based Nosocomial Infections

Edited by

Gianfranco Donelli

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Gianfranco Donelli (Ed.)

Biofilm-Based Nosocomial Infections



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About the Guest Editor

Gianfranco Donelli is Director of the Microbial Biofilm Laboratory at the Fondazione Santa Lucia Research Hospital in Rome. His primary research interests are the biofilm-based healthcare-associated infections, as well as the possible strategies to prevent and counteract the development of microbial biofilms. FEMS Delegate as representative of the Italian Society of Microbiology since 2002 and Vice-Chairperson of the Executive Committee of ESCMID Study Group for Biofilms from 2009 to 2013, he has been a Guest Editor for “Biofilms I” and “Biofilms II”, the two Special Issues published by FEMS Immunology and Medical Microbiology in 2010 and 2012, respectively. Author of over 200 full-length papers, he is on the list of Top Italian Scientists (H-index = 41). Currently, he is also the editor of the new Springer Series, “Advances in Microbiology, Infectious Diseases and Public Health”.

Preface

The well-known persistence in the nosocomial environment of multidrug resistant bacterial and fungal species, today responsible for a wide variety of healthcare-associated infections, is believed to be greatly promoted by the ability of most of them to adhere and to grow in sessile mode on mucosal and soft tissues of hospitalized patients, as well as on the inner and outer surfaces of indwelling medical devices, including intravenous catheters, orthopaedic, cardiac valves, intrauterine devices, and contact lenses.

In this regard, a large number of these microorganisms, such as *Acinetobacter baumannii*, *Candida albicans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, give rise to highly organized, sessile community defined biofilms, in which microbes grow encased in a hydrated matrix of extracellular polymeric substances produced by themselves and are well protected from the attack of antimicrobial molecules and from the host immune response, by resisting phagocytosis and other body's defense systems. The great influence of the sessile growth on the effectiveness of the antibiotic therapies is due to both the structure and function of these microbial communities, making these also 1000 times more tolerant to antibiotics and disinfectants. Thus, alternative approaches to the common antibiotic treatments are emerging for preventing and treating both the mono-species and the most frequent multi-species biofilms, including enzymes able to disrupt mature biofilms and new biomaterials for the coating of medical devices to counteract microbial adhesion and biofilm formation.

The aim of this Special Issue is to report on the state-of-art of the basic and applied research in the field of biofilm-based nosocomial infections that can be acquired by patients in both general hospitals and long-term care settings. Particularly, the involvement of microbial biofilms in medical device-related infections and other healthcare-associated infections, so far underestimated and/or scarcely investigated, has been considered, reviewed, and discussed.

Prof. Dr. Gianfranco Donelli
Guest Editor

Biomolecular Mechanisms of *Pseudomonas aeruginosa* and *Escherichia coli* Biofilm Formation

Garry Laverty, Sean P. Gorman and Brendan F. Gilmore

Abstract: *Pseudomonas aeruginosa* and *Escherichia coli* are the most prevalent Gram-negative biofilm forming medical device associated pathogens, particularly with respect to catheter associated urinary tract infections. In a similar manner to Gram-positive bacteria, Gram-negative biofilm formation is fundamentally determined by a series of steps outlined more fully in this review, namely adhesion, cellular aggregation, and the production of an extracellular polymeric matrix. More specifically this review will explore the biosynthesis and role of pili and flagella in Gram-negative adhesion and accumulation on surfaces in *Pseudomonas aeruginosa* and *Escherichia coli*. The process of biofilm maturation is compared and contrasted in both species, namely the production of the exopolysaccharides via the polysaccharide synthesis locus (*Psl*), pellicle Formation (*Pel*) and alginic acid synthesis in *Pseudomonas aeruginosa*, and UDP-4-amino-4-deoxy-L-arabinose and colonic acid synthesis in *Escherichia coli*. An emphasis is placed on the importance of the LuxR homologue *sdiA*; the *luxS*/autoinducer-II; an autoinducer-III/epinephrine/norepinephrine and indole mediated Quorum sensing systems in enabling Gram-negative bacteria to adapt to their environments. The majority of Gram-negative biofilms consist of polysaccharides of a simple sugar structure (either homo- or heteropolysaccharides) that provide an optimum environment for the survival and maturation of bacteria, allowing them to display increased resistance to antibiotics and predation.

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1. Introduction

Pseudomonas aeruginosa and *Escherichia coli* are the most prevalent Gram-negative biofilm forming medical device associated pathogens [1,2]. Nosocomial infections are estimated to occur annually in 1.75 million hospitalized patients throughout Europe, resulting in 175,000 deaths [3]. *Pseudomonas aeruginosa* accounts for 10%–20% of all hospital-acquired infections [4]. *Pseudomonas aeruginosa* is notoriously difficult to eradicate when colonizing the lungs of cystic fibrosis patients, forming thick antibiotic resistant biofilms that also guard from host immune defenses, lowering of the long-term prognosis of the infected patient [5]. *Escherichia coli* is the most frequently implicated bacteria in urinary catheter related infections, accounting for 50% of such all infections [6,7]. Urinary catheter related infections are the most common form of nosocomial infection with over one million cases a year in the United States alone [7]. In a similar manner to Gram-positive bacteria [8], Gram-negative biofilm formation is determined by the processes of adhesion, cellular aggregation, and the production of an extracellular polymeric matrix with the majority of Gram-negative polysaccharides having a simple structure consisting of either

homo- or heteropolysaccharides [9]. The following review will highlight the importance of these stages, and their control at a molecular level, in the production of highly antimicrobial resistant biofilm architectures.

2. Adhesion in the Gram-Negative Bacteria *Pseudomonas aeruginosa* and *Escherichia coli*

The successful adhesion of Gram-negative bacteria to surfaces is largely dependent on the presence of cell appendages such as flagella, pili, and fimbriae [10]. The presence of functional flagella enables the bacterium to swim and overcome repulsive electrostatic forces that may exist between the cell surface and the surface of material or the host's conditioning film [11]. In both *Pseudomonas aeruginosa* and *Escherichia coli* the flagellum-associated hook protein 1 is encoded by the *flgK* gene with a 40% correlation between the nucleotide sequences of the two species [12]. The processes of adhesion and accumulation in both species are outlined below.

2.1. Pseudomonas aeruginosa Adhesion and Accumulation

In *Pseudomonas aeruginosa*, type IV pili aid in surface adhesion. Type IV pili are constructed from a single protein subunit, PilA, that is exported out of the cell by the secretin, PilQ, to form a polymer fimbrial strand. PilA and PilQ are derived from preplins (molecules of short peptide sequences) whose synthesis is positively controlled by the *algR* regulator [13]. The *fimU-pilVWXYZIE* operon codes for type IV pili prepilins that gather in the periplasmic space to be cleaved and methylated by type IV prepilin peptidase [14]. Encoded in this sequence are PilY1, PilY2, and the six minor prepilins FimT, FimU, PilV, PilW, PilX, and PilE [15]. Required for pilus biosynthesis, the minor prepilins are located in the cell membrane, they are not incorporated into the pili structure and are normally associated with assembly, transport, localization, maturation, and secretion of bacterial proteins [16].

PilY1 and PilY2 are also required for the formation of pili [17]. PilY1 is a large protein located both in the membrane and as part of the pili, with involvement in fimbrial assembly. PilY2 is a small protein involved in fimbrial biosynthesis. The formation of genetic mutants that lack the necessary genes to form flagella and pili/fimbriae have been shown to be surface attachment deficient with little or no biofilm formation when compared to wild-type form, thus highlighting the importance of these bacterial appendages in the adhesion process [11,18].

In *Pseudomonas aeruginosa* type-IV pili are present to aid initial adhesion in combination with two forms of the O-polysaccharide chain of lipopolysaccharide, labeled A and B [19]. Makin *et al.*, utilizing *Pseudomonas aeruginosa* PAO1 discovered based on environmental factors that *Pseudomonas aeruginosa* could alter its phenotypic lipopolysaccharide composition to enhance adherence, thus favoring survival and biofilm formation on a variety of biomaterial surfaces. The production of lipopolysaccharide-A increased the hydrophobicity of the cell surface and increased adhesion to hydrophobic surfaces such as polystyrene [19]. The opposite was true of lipopolysaccharide-B with increased hydrophilicity and adhesion to hydrophilic glass observed. After initial adhesion, a monolayer of *Pseudomonas aeruginosa* forms at the material surface. Movement of bacteria across the surface continues via twitching motility carried out by extension and

contraction type IV pili [20]. The importance of type IV pili in biofilm architecture is demonstrated by the formation of a capped portion in the mushroom-shaped structures synonymous with *Pseudomonas aeruginosa* biofilms. These occur due to type IV pili-linked bacterial migration [21].

Intercellular adhesion of *Pseudomonas aeruginosa* cells is increased by the production of lectins, such as PA-IL and PA-IIL (also known as LecA and LecB) synthesized in the cytoplasm of planktonic cells [22]. These two internal lectins are synthesized when the cell population cannot support itself, as in the decline phase of bacterial growth or upon subjection to environmental stress. A proportion of the total bacterial population lyses, releasing these internal lectins. These newly available lectins weakly bind to healthy, uncompromised, bacterial cells with adherence to the glycoconjugate substrata. To aid in adherence PA-IL and PA-IIL are positioned in the outer membrane of biofilm bacteria [23]. PA-IL binds preferentially to galactose whereas PA-IIL has a high affinity for monosaccharides especially fucose, thus contributing to biofilm formation [24]. In *Pseudomonas aeruginosa* these lectins are soluble, with evidence to suggest they are involved in both strengthening of established biofilms and adhesion to the airways of cystic fibrosis patients [25]. Competitive inhibition of the lectin binding site, using alternative glycans such as fucose and galactose, has been studied as a potential strategy to reduce *Pseudomonas aeruginosa* exacerbations in cystic fibrosis patients [26]. Delivered as an inhalation therapy, fucose and galactose provided promising results when utilized as monotherapy or in conjunction with intravenous antibiotics. Improved lectin binding affinity was demonstrated when glycans were attached to multivalent dendrimers, suggesting a promising role as future therapeutics [27]. *Rhl* quorum sensing pathways and the stationary phase sigma factor RpoS both directly regulate the transcription of lectin-related genes (*lecA* and *lecB*) in *Pseudomonas aeruginosa* and also serve as potential therapeutic targets in the prevention of *Pseudomonas aeruginosa* biofilm formation [28].

2.2. *Escherichia Coli* Adhesion and Accumulation

Escherichia coli encode for pili via transcription of the *fim* gene operon with adhesion due partly to the production of type I, type IV and P pili [29]. *Escherichia coli* possess a mannose-specific FimH receptor on the tip of their type I pili that is responsible for invasion and persistence of bacteria in target cells [30]. Mannose-specific receptors aid adhesion to host tissue surfaces such as the bladder epithelium, resulting in cystitis [31]. Evidence provided by Mobley and colleagues showed that *Escherichia coli* isolates from established long-term bacteriuria, greater than 12 weeks, expressed more type I fimbriae (92% of isolates) than those in short term infections of a duration of less than 1 week (59% of isolates) [32]. A study of P fimbriae did not demonstrate persistence in the urinary tract, however proof was provided for an increase in adherence to ureteral stents when isolates possessing P fimbriae were present [33]. These results demonstrate the importance of the bacterial isolate/strain of *Escherichia coli* in the establishment of different infections. Strains of *Escherichia coli* with type I predominate in bladder infections, with P fimbriae strains usually present in kidney infections.

The assembly of type I pili is controlled by the periplasmic FimC protein. FimC accelerates the folding of pilus subunits in the periplasm for delivery to the outer membrane protein FimD, where these subunits then dissociate to form the mature pilus [34] (Figure 1). FimC is termed the periplasmic chaperone and FimD the outer membrane assembly platform or usher, based on how they control type I pili synthesis [35]. FimF and FimG are linear connective proteins present in the fibrillum tip allowing the projection of the adhesin FimH that occurs only on the outer surface of the pilus [36].

As discussed, other forms of pili exist in *Escherichia coli* namely: P pili and type IV pili. P pili are chaperone-usher assembly mediated pili, encoded by the *pap* locus and contain galabiose specific receptors (Gal(α 1-4)Gal-) on a distal PapG unit (Figure 2) [37]. This allows *Escherichia coli* to colonize the upper urinary tract, causing pyelonephritis, by binding of galabiose specific receptors (Gal(α 1-4)Gal-) to the glycolipid galabiose on urinary tract tissue [38]. The fibrillum tip of P pili is composed of repeating subunits of PapE protein, with the rod consisting of PapA. PapF, PapK, and PapH proteins are also present in low quantities. PapF and PapK act as protein initiators and coordinators for assembly. PapF also acts as a linker in the fibrillum tip to PapG and PapK, thus attaching the fibrillum tip to the rod protein PapA [39]. PapH acts as the rod terminus linking it to the outer membrane surface [40]. The protein PapD acts as the periplasmic chaperone in a similar manner to FimC in type I pili [41] with PapC, like FimD, acting as the outer membrane usher [42]. Type IV pili in *Escherichia coli* are formed independently of a chaperone-usher system and are coded for by the *bfp* operon [43]. Also termed bundle-forming pili, their properties are associated with swarming and twitching motility, unlike type I and P pili, as well as adhesion [44]. Relative to type I and P pili, the formation of type IV pili is less characterized. Ramer and colleagues discovered that a *bfp* encoded assembly complex spans the entire periplasmic space and associated proteins, such as BfpU and BfpL, are present at both the inner and outer membranes [45]. They observed that a type IV related assembly complex consisted of an inner membrane component composed of three pilin-like proteins, BfpI, BfpJ and BfpK. These proteins were localized with BfpE, BfpL, and BfpA forming the major pilin subunit. BfpI, BfpJ, and BfpK were also associated with an outer membrane, secretin-like component, BfpB and BfpG, and a periplasmic component composed of BfpU. Together they create the bundle-forming pilus.

Figure 1. The assembly of the type I pilus. The periplasmic protein FimC binds secreted pilus subunits, from the SecYEG translocon based in the internal membrane, to the periplasm. A process of accelerated subunit folding by FimC (periplasmic chaperone) occurs, followed by delivery to the usher outer assembly platform FimD, also performed by FimC. These FimC-subunit complexes are recognized and bind to the *N*-terminal domain of the usher: FimD_N. Uncomplexed FimC is then released to the periplasm when subunits are assembled into the pilus. The tip of the pilus (fibrillum) consists of the protein adhesins FimF, FimG, and FimH, with FimA forming the bulk of the pilus rod. Adapted from Capitani, 2006 [37].

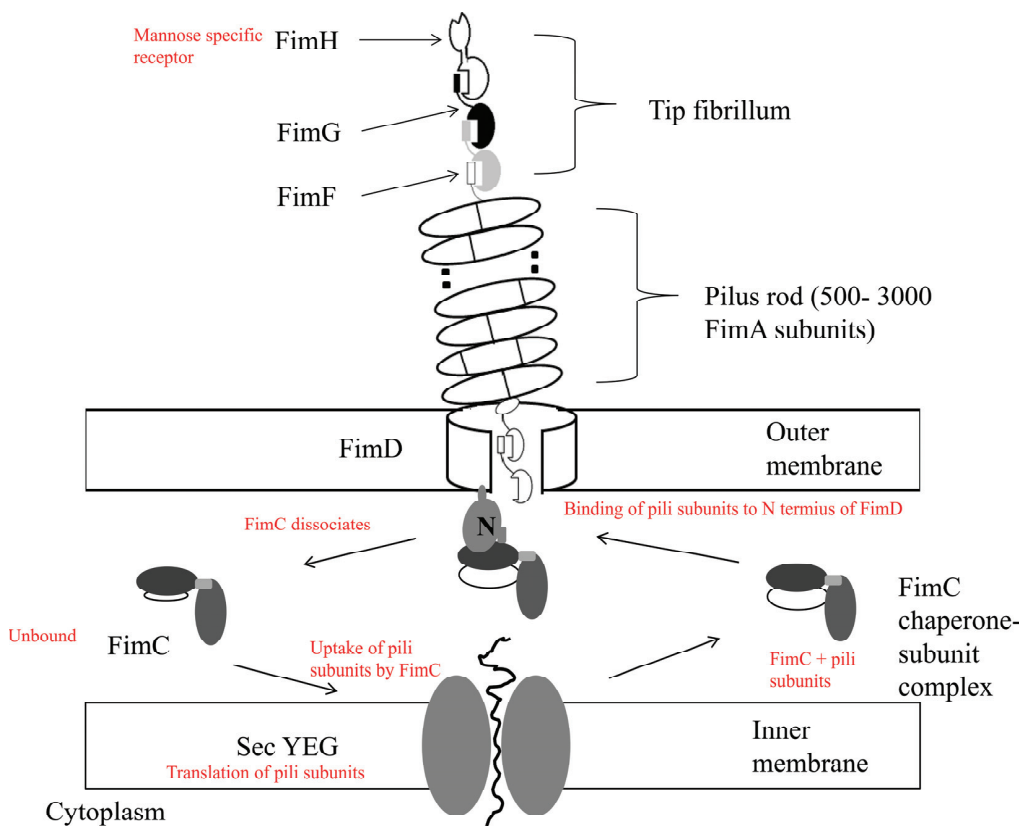
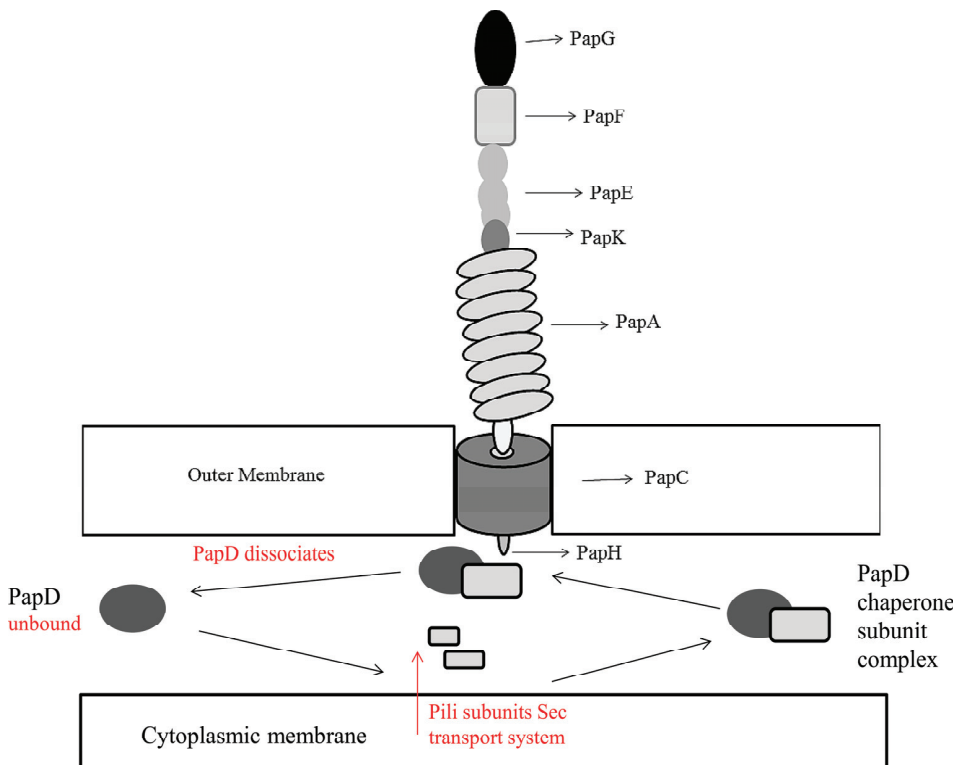


Figure 2. Structure of *Escherichia coli* Type P pili encompassing the PapG unit containing galabiose specific receptors (Gal(α 1–4)Gal-) for attachment to urinary tract tissue. The pilus is anchored to the membrane by PapH, whose location is yet to be characterized fully but has been hypothesized by Verger and colleagues to terminate the pilus structure at the base as shown, allowing anchoring to the membrane [46]. Type P pili subunits enter the periplasm by the Sec transport system. In the presence of PapD, stable chaperone-subunit complexes are formed via attachment to the hydrophobic C-terminus of pili subunits [47]. PapD acts as the chaperone to assemble and deliver pili subunits to the outer membrane usher PapC. PapC is a pore forming protein that facilitates pilus assembly by creating a narrow channel across the outer membrane. Assembly of subunits from the outer membrane PapC occurs through a donor strand exchange mechanism. PapA forms a tightly wound helix fiber on the external cell and provides a driving force for the translocation of pili subunits across the outer membrane, facilitating outward pilus growth [48]. Adapted from Mu and Bullitt, 2006 [48] and Mu, 2005 [49].



Curli fibers are organelles associated with the early stages of *Escherichia coli* adhesion and virulence. They consist of proteinaceous adhesive filaments that form a coil-like structure on the surface of *Salmonella* and *Escherichia coli*. They have an affinity for proteins such as fibronectin and are responsible for cell to cell adhesion [50]. The production of curli fibers is regulated by

transcription of the *csgD* gene. CsgD protein is derived from the LuxR family of transcriptional regulators and is the activator and transcription regulator of the *csgBAC* gene operon. It is at the *csgBAC* gene loci that the protein subunits that form curli fibers are encoded [51]. CsgD also controls cellulose production, through *adrA* gene transcription, which itself is linked to the formation of an extracellular matrix [52]. Control of curli production is a very complex process with two separate gene loci required for effective curli synthesis and multiple regulatory pathways controlling their expression [53,54]. The *csgDEFG* operon encodes both the transcription of CsgD and also a curli-specific transport system mediated by CsgEFG proteins. The curli structural subunits, encoded by upregulation of the *csgBAC* gene locus, are produced in the presence of cellular and environmental stress such as low temperature (<32 °C), lack of nutrients, low osmolarity, and iron shock [55–57]. Limited expression of curli related genes, such as the *csgA* gene, correlates to reduced biofilm formation due to a lowering in production of the main curlin protein subunit CsgA [58]. Their importance as therapeutic targets is demonstrated by the work of Cegelski and colleagues [59]. They produced a series of ring-fused 2-pyridone containing peptidomimetic molecules (FN075 and BibC6), which prevented macromolecular assembly of the major curli subunit protein CsgA and inhibited *Escherichia coli* curli biogenesis. This resulted in reduced *Escherichia coli* colonization in the bladder of *in vivo* mouse models. These so-called curlicides also prevented type I pilus biogenesis via blockage of the FimC chaperone. Delivery of such molecules at therapeutically relevant concentrations remains a challenge that prohibits their clinical development. Changes in environmental stresses affect biofilm formation in *Escherichia coli* via the two-component regulatory system CpxA/CpxR. The CpxA/CpxR system negatively controls the transcription of the *csg*, *pap*, *bfp*, and *flgM* (*flg* are involved in flagella protein transcription and motility together with *fli*) operons [60]. CpxA is a histidine kinase involved in the transfer of a phosphate group to the regulatory protein CpxR, allowing it to bind specifically to sequences of bacterial DNA that regulate gene transcription [61]. The CpxA/CpxR system senses changes in the environmental surroundings of the periplasm, outer membrane, and bacterial envelope. Activation occurs at low nutrient concentrations, high osmolarity and high temperatures due to their effects on lipopolysaccharide and exopolysaccharide biosynthesis and the outer membrane structure [62,63]. Transcription of *CpxA/CpxR* system genes is controlled by the general stress response factor RpoS (stationary phase sigma factor) [64]. RpoS, also known as the alternative subunit of RNA polymerase (σ^S), is a protein encoded by the *rpoS* gene that controls the overall response of *Escherichia coli* to environmental stress, with a sharp increase in concentrations shown at the onset of the stationary phase of growth [65]. Negative regulation of curli occurs by binding of phosphorylated CpxR to the *csgD* promoter therefore switching *csgD* expression off. In mature biofilm cells a majority of CpxA/CpxR are activated [66]. The events of initial adhesion have already occurred; therefore many of the adhesion-related appendages are not required. CpxA/CpxR expression correlates to an upregulation of genes corresponding to resistance pathways, such as the *mdtA* gene, responsible for the efflux and resistance against many β -lactam antibiotics [67]. Therefore the positive role of CpxA/CpxR is more likely to be associated with dormant or persister *Escherichia coli* cells. CpxA/CpxR is unlikely to be associated with the dispersal of biofilm cells, to facilitate recolonization of new surfaces, as genes related to motility

such as flagella-related genes (*flgM*) have also been shown to be downregulated by the CpxA/CpxR system [68].

Positive regulation of curli fiber production is controlled by the EnvZ/OmpR two-component regulatory system. The OmpR protein binds to the same promoter region of *csgD* as CpxR but it is still not fully established whether they actively compete for this binding site [69]. EnvZ is a histidine kinase that controls the phosphorylation and binding-affinity of OmpR to CsgD, with phosphorylation in the presence of environmental stimuli such as high osmolarity [70]. CpxA plays a similar activating role with CpxR via a process of phosphorylation [71]. Most recently Ogasawara and colleagues analyzed mRNA of mutant *Escherichia coli* and *csgD* to indicate that CpxR and H-NS acted as repressor molecules with OmpR, an acid-stress response regulator termed RstA and IHF acting as activators within a five component system. They concluded these five factors bonded to the same narrow gene operon region of approximately 200 base pairs, upstream from the *csgD* promoter [72]. Despite the promising results obtained, the biomolecular and transcription mechanism of the *csgD* operon has not been fully elucidated. Their work showed the presence of competitive positive and negative factors but also cooperation between the positive and negative factor groups. Regulation of the *csg* loci is also controlled by the global regulatory gene *hns* [73]. The gene regulator *hns* has an established negative effect on adhesion due to upregulation of genes responsible for flagella synthesis, in comparison to *ompR*, the conclusive positive regulator of curli production [69,74].

3. Biofilm Maturation in *Pseudomonas Aeruginosa* and *Escherichia Coli*

Accumulation and ultimately maturation of the biofilm corresponds to the increased production of the major extracellular polymeric substance alginate in *Pseudomonas aeruginosa* [75] and colanic acid in *Escherichia coli* [76]. These compounds are important in forming the respective biofilm architecture of these microorganisms but they are not essential for biofilm formation to occur. Both species exhibit similar three-dimensional structures possessing water channels; micro and macrocolonies of significant heterogeneity and a thick biofilm matrix. Both microorganisms display downregulation of genes required for motility apparatus, specifically flagella-related genes, and upregulation in genes for extracellular polymeric substance production in the maturation stage of growth [77]. Bacterial maturation in both these Gram-negative bacteria is tightly controlled by quorum sensing systems involving *N*-acyl-l-homoserine lactone as signaling molecules, together with long-chain hydrocarbon structures derived from fatty acids, fatty acid methyl esters, peptides, γ -butyrolactones, 2-alkyl-4-quinolones, furanones, and the 4,5-dihydroxy-2,3-pentandione derivatives, collectively referred to as autoinducer-II and autoinducer-III [78–82].

3.1. *Pseudomonas aeruginosa* Biofilm Maturation: Production of Exopolysaccharides via the Polysaccharide Synthesis Locus (*Psl*), Pellicle Formation (*Pel*), and Alginate Synthesis

The extracellular polymeric substance of *Pseudomonas aeruginosa* biofilm, in line with the majority of bacterial biofilms, consists mainly of polysaccharide, proteins, and nucleic acids [83–85]. In mucoid strains of *Pseudomonas aeruginosa*, isolated from cystic fibrosis patients, the most prevalent

exopolysaccharide produced is alginic acid, an *O*-acetylated linear polymer of β -1,4-linked D-mannuronic acid with a C-5 epimer, L-guluronic acid [86]. Interestingly non-mucoid strains have been shown to contain low levels of alginate, with biofilm formation retained [87]. Only 1% of strains isolated from sites other than the lungs of cystic fibrosis patients are mucoid [88], therefore in relation to medical device related infection, alginic acid is not necessarily the most common exopolysaccharide present.

3.1.1. Production of the Psl and Pel Exopolysaccharides by Non-Mucoid *Pseudomonas aeruginosa*

Adherence, aggregation, maturation, and formation of the biofilm architecture are also due to production the exopolysaccharides Psl and Pel. The proteins, enzymes, and transporter molecules required for Psl and Pel synthesis and pellicle formation (thin biofilm surrounding cells that assembles at the air-liquid interface) are encoded by the genes *pslA-O* and *pelA-G*, respectively, in *Pseudomonas aeruginosa* PAO1 [89]. Upon analysis of PelA-G proteins it was observed that PelA is a cytosolic protein and an oligogalacturonide lyase; Pel B functions as an outer membrane protein; PelC is a glycosyltransferase present in the periplasm; both PelD and PelE are large cytosolic proteins located on the inner membrane, with PelD an inner membrane located transmembrane protein; PelF is a glycosyltransferases and PelG is a 12-transmembrane inner membrane protein [90].

Psl proteins are not as well defined in the literature as Pel in terms of individual functions [91]. PslA was identified as a putative UDP-glucose carrier protein essential to biofilm formation in strains of *Pseudomonas aeruginosa* such as PAO1 [92]. Observations of the extracellular polymeric substances present in *Pseudomonas aeruginosa* PAO1 show that the main carbohydrate constituents are glucose, mannose, and rhamnose and not the alginic acid components mannuronate or guluronate [93]. Psl is rich in sugars, particularly mannose, with glucose, galactose, rhamnose, and a limited quantity of xylose also present [91]. The gene locus *pslA-G* is present in some strains, for example *Pseudomonas aeruginosa* PAO1, but not PA14 strains [94,95]. Pel is a glucose-rich polymer and although the genes encoding its production (*pel*) have been shown to be present in all identified strains of *Pseudomonas aeruginosa*, their expression is limited in laboratory conditions [94]. Psl is located mainly in the peripheral regions of the biofilm matrix and may have a role in attracting free-flowing planktonic bacteria to form part of the biofilm structure [84,96]. The reason for this peripheral localization is, as yet, unproven but an increase in nutrients; metabolism; DNA and protein synthesis at the outer extremities of the biofilm and/or a breakdown of Psl in the center of the matrix by the production of enzymes may contribute to this observation [97]. An interesting study by DiGiandomenico and co-workers highlighted the potential of monoclonal antibodies in combating exopolysaccharides such as Psl [98]. By performing phenotypic screening they discovered that Psl was an antibody-accessible antigen that allowed targeted monoclonal antibody mediated opsonophagocytic killing of *Pseudomonas aeruginosa*. Reduced bacterial attachment was shown with cultured lung epithelial cells and prophylactic protection provided in infected animal models. Use of such techniques may have potential for future prophylaxis against *Pseudomonas aeruginosa* infections in high-risk patients.

The production of the secondary messenger molecule bis-(3',5')-cyclic-dimeric-guanosine monophosphate (c-di-GMP) is linked to the maturation of biofilms and production of exopolysaccharides in many species of bacteria including *Pseudomonas aeruginosa*. Its production is regulated by the action of diguanylate cyclase enzymes. Cleavage of, or a decrease in, c-di-GMP production is linked to the expression of motility factors and virulence, with phosphodiesterases also linked to c-di-GMP degradation [99,100]. High levels of c-di-GMP are associated with an increase in biofilm-related traits (attachment and accumulation) [101]. A c-di-GMP binding site has also been identified on the cytosolic inner membrane protein PelD, therefore linking this molecule to Pel synthesis in *Pseudomonas aeruginosa* [90]. There has been an increasing interest in targeting c-di-GMP, or more specifically the proteins that are involved in the biosynthesis of c-di-GMP, in order to prevent biofilm formation [102]. C-di-GMP is produced from two guanosine triphosphate molecules and its synthesis is controlled by the enzyme diguanylate cyclase [103]. Irie and colleagues recently discovered that although Psl formation is controlled by c-di-GMP, it also acts as a positive feedback signal and stimulates the production of two diguanylate cyclases, SiaD and SadC, resulting in increased formation of c-di-GMP [104]. Eukaryotes do not express diguanylate cyclase, therefore it serves as an excellent target for antibacterial drug development [105]. Analogues of c-di-GMP, for example the monophosphorothioic acid of c-di-GMP (c-GpGps), display antibiofilm activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus in vitro* [106]. Biofilm dispersal and an increased sensitivity to antimicrobials have also been attributed to low concentrations of nitric oxide in *Pseudomonas aeruginosa*. Barraud *et al.* uncovered a possible molecular link between nitrous oxide, reduced levels of c-di-GMP and biofilm dispersion due to an increase in phosphodiesterase activity [107]. C-di-GMP also has potential as a vaccine molecule due to its immunostimulatory and adjuvant properties [108]. The translation of such therapies to clinical practice is limited at present, as the role of c-di-GMP in biofilm formation has not been fully established. Concerns may also exist with regard to the affect such therapies may have on commensal microorganisms.

Extracellular DNA also plays an important role in biofilm maturation and stabilization of non-mucoid *Pseudomonas aeruginosa* strains such as PAO1, compensating for a lack of alginate [109]. Matsukawa *et al.* showed that in the matrix of mature *Pseudomonas aeruginosa*, PAO1 extracellular DNA was the most prevalent polymer and that exopolysaccharides were of great importance with regard to structural integrity [85]. Whitchurch and colleagues demonstrated that DNase could dissolve young *Pseudomonas aeruginosa* PAO1 biofilms, but matured biofilms showed only small dissolution. This suggests that early biofilms are held together by extracellular DNA, but mature PAO1 biofilms are held together by other compounds, namely exopolysaccharides [110]. Research by Ma demonstrated extracellular DNA to be present mostly in the stalk region of mature biofilm colonies, and spatially separate from Psl proteins [111]. Differences in the prevalence of extracellular DNA in *in vitro* and *in vivo* biofilms may be attributed to the stage of biofilm growth. Extracellular DNA may also play a role in increasing resistance of biofilm forms of *Pseudomonas aeruginosa* toward cationic antimicrobials, such as antimicrobial peptides. Extracellular DNA is a cation chelator and acts to sequester cations from the surrounding environment. It also plays a role in the modification of the cationic antimicrobial

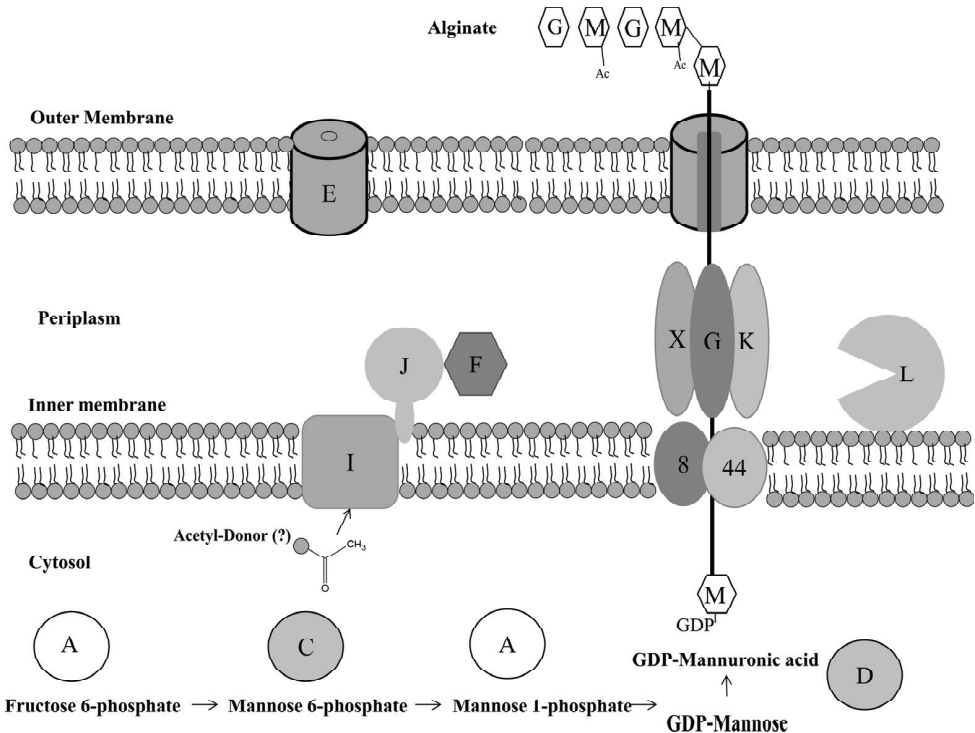
peptide binding site lipid A by the sugar dehydrogenases enzyme UDP-glucose dehydrogenase (Ugd) and covalent binding to 4-amino-4-deoxy-L-arabinose [112].

3.1.2. Production of the Exopolysaccharide Alginate by Mucoid *Pseudomonas aeruginosa*

Slime production by mucoid forming strains of *Pseudomonas aeruginosa* is important for the colonization of both medical devices and cell surfaces, such as the lungs in cystic fibrosis patients [113]. The formation of this slime, composed of mainly alginate, is important in protecting *Pseudomonas aeruginosa* from antimicrobials and host defense mechanisms by restricted penetration of these molecules through the biofilm matrix [114]. Synthesis of alginate, commonly known as alginate, is controlled by the *algACD* operon present in *Pseudomonas aeruginosa*. Upregulation of alginate-related genes is dependent on multiple environmental factors including: high oxygen concentration, high osmolarity, lack of nitrogen, and the presence of ethanol [115,116]. In general the production of alginate by the *algACD* gene locus is similar to the regulation of the *icaADBC* operon, responsible for polysaccharide intercellular adhesion production in staphylococci.

Of high importance to alginate production are the *algA*, *algC*, and *algD* genes that transcribe the enzymes required for the production of the alginate precursor guanosine diphosphate (GDP)-mannuronic acid [117]. A combination of the transmembrane transporter proteins Alg44 and Alg8, which are normally not active in non-mucoid *Pseudomonas aeruginosa*, allows the movement of this alginate precursor across the inner membrane for polymerization [118]. AlgA-X are alginate enzymes involved in the polymerization and biosynthesis processes that result in the formation of alginate (Figure 3). The role of alginate lyase, at the maturation stage, is unclear although it may allow the production of short oligomers that prime polymerization and may also allow the breakdown of alginate at the cell detachment phase of biofilm growth [119]. AlgG interacts with AlgK and AlgX. They have an important role in protecting the production of the alginate polymer by forming a scaffold in the periplasm surrounding newly formed polymer molecules [120]. Epimerization of polymerized mannuronate residues is controlled by AlgG, a C-5-epimerase enzyme [121]. Acetylation of these mannuronate residues also occurs via the enzymes AlgF, AlgJ, and AlgI at O2 and/or O3 positions [122]. AlgF is located in the periplasm. AlgJ is a type II membrane protein with an uncleaved signal peptide portion linked to the inner membrane with a remaining portion in the periplasm, whereas AlgI is an integral transmembrane helix that accepts an acetyl group from an unknown donor [123]. When the process of O-acetylation is concluded, transportation of alginate out of the cell is mediated by AlgE present on the outer membrane forming the majority of the extracellular polymeric matrix substance of mucoid producing *Pseudomonas aeruginosa* [124].

Figure 3. The synthesis and polymerization mechanism involved in the production of *Pseudomonas aeruginosa* alginate. The letters A–X and numbers 8 and 44 correlate to alginate biosynthetic enzymes that are preceded by Alg (for example A = AlgA). AlgA, AlgC, and AlgD control the production of the alginate precursor GDP-mannuronic acid. Both Alg8 and Alg44 transport this molecule for polymerization in the periplasm. Alginate lysase (AlgL) produces short oligomers that prime polymerization. AlgG interacts with AlgK and AlgX protecting the production of the alginate polymer by forming a scaffold in the periplasm. Epimerization of polymerized mannuronate residues is also controlled by AlgG, a C-5-epimerase. Acetylation of some mannuronate residues occurs via the enzymes AlgF, AlgJ, and AlgI at O2 and/or O3 positions, with AlgE transporting the formed alginate out of the cell. Adapted from Franklin and Ohman 2002 [122], Ramsey 2005 [125], and Gimmestad, 2003 [126].



AlgA, AlgC, and AlgD are important enzymes in the production of GDP-mannuronic acid. AlgA is involved in alginate biosynthesis catalyzing both the production of mannose-6-phosphate from fructose-6-phosphate and GDP-mannose from mannose-1-phosphate, as a phosphomannose isomerase and GDP-mannose pyrophosphorylase, respectively [127]. AlgC is a phosphomannomutase and phosphoglucomutase enzyme that catalyses the reversible production of mannose-6-phosphate to mannose-1-phosphate (Figure 3). AlgC has also been shown to be important in lipopolysaccharide synthesis, with its preference for both mannose and glucose containing substrates allowing it to possess a diverse mechanism of action [128].

AlgD is a rate-limiting GDP-mannose dehydrogenase that catalyses the production of GDP-mannuronic acid from GDP-mannose [129]. Control of alginate biosynthesis and the transcription of the Alg proteins are therefore mediated by the *algD* operon, which is responsible for the final production of GDP-mannuronic acid, the foundation molecule for polymerization and alginate synthesis. A regulatory cascade consisting of an alternative sigma factor AlgT, also known as AlgU or σ^{22} and encoded by *algT* controls the transcription of *algD* [130]. Autoregulation of *algT* and upregulation of the alginate-linked gene loci *algR*, *algB*, *algZ* are also performed by σ^{22} [131]. AlgR regulates both *algC* and *algD* transcription by binding to sites upstream of their genes [132]. AlgB also activates *algD* transcription but by an undefined method that has previously been thought to be related to indirect action of the promoter region of *algD* [133]. However, Leech and colleagues observed, through DNA binding and transcriptome analysis, that AlgB bound directly to the promoter region of the *algD* operon [134]. Transcription of *algZ*, also known as *amrZ*, leads to the activation of *algD* by binding to sequences upstream of the *algD* promoter [135]. It also inhibits transcription of the gene loci *fleQ* related to the control of flagella-related genes [136]. This demonstrates the complex systems involved in the transcription of alginate-related genes, such as *algD*, with multiple pathways involved in its transcription.

The action of σ^{22} is itself controlled by the regulatory protein products of *mucABCD* transcription [137]. The inner membrane protein MucA is an anti-sigma factor that complexes to the periplasmic protein MucB. The MucA portion then directly binds to σ^{22} after *algT* transcription to negatively regulate its activity [138]. Rowen *et al.* suggested that σ^{22} associates with the periphery of the inner membrane by interacting with RNA polymerase or MucA, or by an unknown independent mechanism blocking the action of σ^{22} [139]. MucC's role is relatively uncharacterized, however, it is hypothesized to be an inner membrane protein that may act synergistically with both MucA and MucB in the negative regulation of σ^{22} [140]. MucD is an endoprotease that negatively regulates σ^{22} by the removal of activating factors present in σ^{22} [141]. Inactivation of *mucA* or *mucB* via mutations in non-mucoid *Pseudomonas aeruginosa* strains has been shown to induce alginate synthesis leading to an overexpression in alginate-related genes. Clinical isolates from cystic fibrosis patients have also been shown to possess mutations in *mucA*, causing an exponential increase in alginate synthesis [142,143].

3.2. *Escherichia coli* Biofilm Maturation

The process of biofilm maturation in *Escherichia coli* is very similar to *Pseudomonas aeruginosa* in that genes that encode for flagella mediated motility (*fli* and *flg*) are downregulated with corresponding upregulation of genes corresponding to the exopolysaccharide colanic acid (*wca*), porin (*ompC*) production, tripeptidase T, and synthesis of a nickel and glycine betaine high-affinity transport system [144]. As previously discussed (Section 2.2.), the Cpx/CpxR two component regulatory system, controlled by the general stress response factor RpoS (stationary phase sigma factor) [64], is responsible for the upregulation of many of the genes implicated in biofilm maturation [66].

The production of the exopolysaccharide colanic acid is essential for the maturation and complex three-dimensional structure of *Escherichia coli* biofilms but not the process of initial

adhesion [145]. Colanic acid consists of hexasaccharide subunits with a high prevalence of fucose and glucuronic acid [146]. The physical barrier presented by colanic acid production and the negative charge that it possesses allows *Escherichia coli* biofilms to resist large changes in osmotic stress, oxidative stress (by hydrogen peroxide), and temperature [76]. Generally colanic acid is only produced at temperatures above 30 °C and is thought to be important in capsule formation and ultimately survival of *Escherichia coli* outside of the host [147]. The gene operon *wca*, also known as *cps*, encodes for polymerase enzymes that regulate colanic acid synthesis from sugars by a pathway that has not been elucidated fully. The transcription of the *wca* genes is controlled by the *rcaABC* gene loci, and this pathway is more fully understood. After phosphorylation of the response regulator RcsB by the sensor kinase RscC, the accessory positive regulator RcsA binds to RcsB to form the heterodimer RcsA-RcsB, which activates *wca* transcription [148]. RscF is hypothesized to promote the phosphorylation of RcsB by RscC [149]. The processes and signals that cause the activation of RscC are relatively unknown [147]. It has been suggested that environmental stimuli, such as osmotic shock, play a role in the upregulation of *rscC* via changes in membrane-bound protein MdoH, which is involved in the production of membrane-derived oligosaccharides [150]. RscC senses changes in these membrane-derived oligosaccharides to initiate a response [151]. RcsA is present normally in low amounts at 37 °C due to low levels of synthesis and the presence of the protease Lon, a negative regulator of *wca* transcription [152]. The minimal level of RcsA production is due to transcriptional silencing by the histone-like protein H-NS, that is negated by overproduction of a small RNA molecule known as DsrA [153].

The *wca* operon consists of 19 genes with the third gene in order of transcription being the *wzc* gene. *Wzc* has been shown to encode a membrane bound autophosphorylated protein-tyrosine kinase [154]. Upstream of this *wzc* locus, the *wca* operon codes for a phosphotyrosine phosphatase (PTP), *Wcz*, that has the ability to specifically dephosphorylate a corresponding protein-tyrosine kinase and is otherwise defined as a BY-kinase, a newly defined group of enzymes involved in protein-tyrosine phosphorylation [155]. Although the mechanism is unclear, dephosphorylation of *Wzc* tends to lead to increased colonic acid production and provides a means by which exopolysaccharides are transported out of the cell [156]. *Wzc* has also been shown to phosphorylate the sugar dehydrogenase enzyme Uridine diphosphate (UDP)-glucose dehydrogenase, allowing it to mediate the construction of extracellular polysaccharide precursors, such as UDP-glucuronic acid.

Etk, like *Wzc*, is a BY-kinase of *Escherichia coli* and is involved in the production of the group IV capsule surrounding the *Escherichia coli* bacterial cell membrane [157]. *Etk* is coded for by the *etk* gene present on the *ymc* operon of some pathogenic strains of *Escherichia coli* [158]. The mechanism and use of the *ymc* operon itself is unknown, although it could possibly be a promoter of *etk* expression [159]. *Etk* is also involved in the phosphorylation of UDP-glucose dehydrogenase allowing for the production of UDP-4-amino-4-deoxy-L-arabinose, a compound that allows *Escherichia coli* to become resistant to cationic antimicrobial peptides and polymyxin-B [159].

The two-component systems PhoP/PhoQ and PmrA/PmrB are induced by limitation of magnesium and calcium ions or the RcsA/RcsB/RcsC system leading to upregulation of the genes involved in UDP-4-amino-4-deoxy-L-arabinose biosynthesis. The gene loci *arn* is controlled by both the

PhoP/PhoQ and PmrA/PmrB pathways. Transcription of *arn* leads to the synthesis of UDP-4-amino-4-deoxy-L-arabinose via *arn*-linked enzymes, however synthesis of UDP-4-amino-4-deoxy-L-arabinose is also due to RcsA/RcsB/RcsC or PhoP/PhoQ and PmrA/PmrB mediated transcription of *ugd*. The protein Ugd, when phosphorylated via Etk, forms UDP-Glucuronic acid from the precursor UDP-Glucose. Formation of UDP-4-amino-4-deoxy-L-arabinose is mediated by a series of *arn* encoded enzymes [160]. Meredith *et al.* hypothesized that Ugd synthesis of colonic acid (in this case defined as M-antigen) also affects the production of lipopolysaccharides in the cell membrane via the formation of a complex lipopolysaccharide glycoform termed M_{LPS} [161]. Resistance develops against cationic antimicrobial peptides and polymyxin due to covalent modifications of lipid A, the hydrophobic anchor of the lipopolysaccharide membrane and the cationic antimicrobial binding site, by UDP-4-amino-4-deoxy-L-arabinose [160].

4. Quorum Sensing in Gram-Negative Bacteria: *Pseudomonas aeruginosa* and *Escherichia coli*

Quorum sensing is a system whereby bacterial cells communicate in order to act as a community of cells. This maximizes the potential of their mutualistic survival strategies, allowing selective benefits to be conferred to the bacterial population that would otherwise not be present as individual cells. Quorum sensing is of great importance in the production of bacterial biofilms and the up and down regulation of related genes [7].

4.1. Quorum Sensing in Pseudomonas aeruginosa

In *Pseudomonas aeruginosa* there are currently three identified quorum sensing systems. These are; the *las*-based system controlled via *N*-(3-oxododecanoyl)-L-homoserine lactone production by *lasRI* gene loci [162]; the *rhl* system regulated by *N*-butyryl-homoserine lactone produced by the *rhlRI* operon [163] and the *pqs* system that controls the *las* and *rhl* quorum sensing through 2-heptyl-3-hydroxy-4-quinolone production [164]. The *rhl* system is controlled by the *las* system as LasR-*N*-(3-oxododecanoyl)-L-homoserine lactone activates the transcription of *rhlR* and *rhlI* meaning these two systems are interlinked [165].

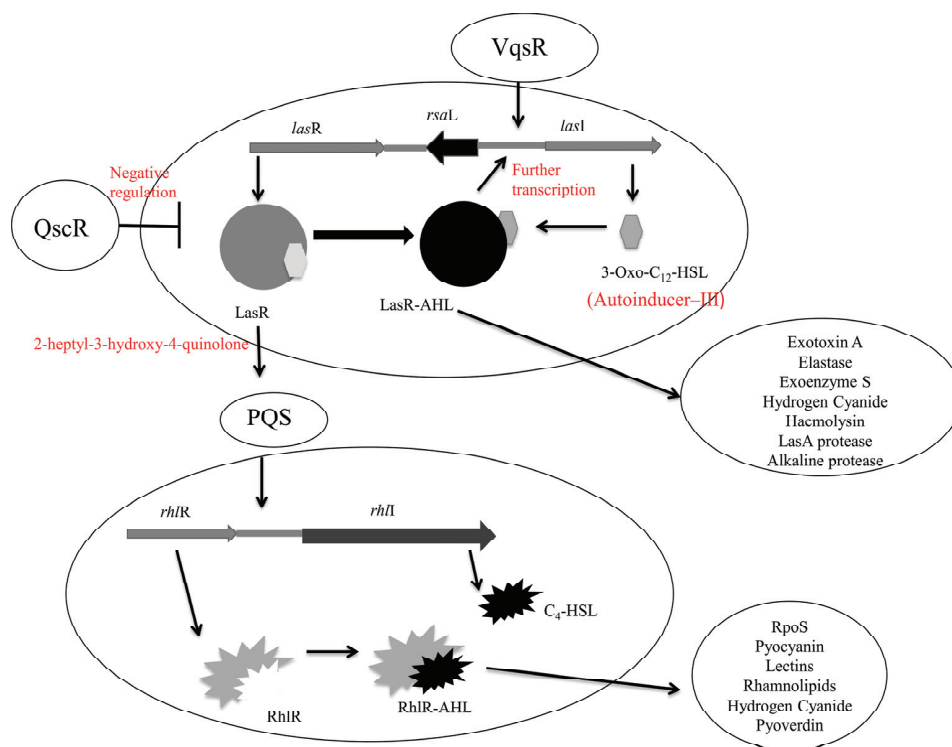
As for all microorganisms that demonstrate quorum sensing pathways, these signals allow for the control of phenotypic expressions such as virulence, biofilm formation, and resistance to antimicrobials [166]. In *Pseudomonas aeruginosa* quorum sensing controls the production of exoenzymes and secreted toxins, such as elastase and exotoxin A [162,167,168], and also directs biofilm formation [169]. Cells lacking in the *las* system have been shown to be flat rather than the atypical mushroom-like shape of mature *Pseudomonas aeruginosa* biofilms, with increased sensitivity to antibiotics also demonstrated [168]. It is hypothesized that the transcription of the exopolysaccharide-related *pelA-G* gene loci is controlled indirectly by *las* and *rhl* quorum sensing systems through transcriptional factors; however this process is as yet largely undefined [170]. Quorum sensing has also been shown to regulate the production of extracellular DNA in *Pseudomonas aeruginosa* biofilms [83]. The most defined quorum sensing pathways in *Pseudomonas aeruginosa* are the *las* and *rhl* systems.

The *las* system comprises of the transcriptional activator protein LasR and the autoinducer synthase enzyme LasI, coded for by *lasR* and *lasI*, respectively. The transcription of *lasI* results in the formation of the enzyme LasI that directs the synthesis of *N*-(3-oxododecanoyl)-L-homoserine lactone, an important acyl-homoserine lactone of *Pseudomonas aeruginosa*, otherwise known as autoinducer-III [171]. Increased cell density of *Pseudomonas aeruginosa* correlates to an increase in autoinducer-III concentration and when a threshold value is reached the binding of autoinducer-III to its specific target protein LasR will result in the transcription of multiple virulent and biofilm-related genes mediated by *las*. These genes include those responsible for the production of multiple enzymes such as exotoxin A (*toxA*), elastase (*lasB*), the LasA protease (*lasA*), and alkaline protease (*aprA*) [172]. The binding of autoinducer-III to LasR also results in further transcription of *lasI*, thus proving *N*-(3-oxododecanoyl)-L-homoserine lactone to be an autoinducing peptide [173]. The activation of the *las* system also results in activation of the *rhl* quorum sensing system, resulting in the production of a second autoinducer *N*-butyryl-homoserine lactone. It is therefore believed, through this mechanism, that the *las* system controls regulatory protein (RhlR) production both before and after transcription [163,173].

The *rhl* system consists of the transcriptional activator protein RhlR and the synthase RhlI that regulate *N*-butyryl-homoserine lactone production [165]. The *rhl* pathway is responsible for the production of amphiphilic biosurfactants, known as rhamnolipids (heat stable haemolysin), in the latter stages of biofilm development that aid in the maintenance of macrocolonies and fluid-filled channels [174]. The *rhl* system is also responsible for the production of multiple extracellular enzymes along with secondary metabolites such as pyocyanin, hydrogen cyanide and pyoverdine [175]. In *Pseudomonas aeruginosa* there is a high correlation between biofilm architecture and quorum sensing, with little or no influence on biofilm adhesion and motility [176].

The third quorum sensing system identified in *Pseudomonas aeruginosa* is the *pqs* system that utilizes 2-heptyl-3-hydroxy-4-quinolone, also known as *Pseudomonas* quinolone signal. The *pqs* system has particular importance for the production of *rhl*-dependent exoproducts at the beginning of the stationary phase of growth [177]. The production of 2-heptyl-3-hydroxy-4-quinolone is related to the activation of *lasR* and is therefore thought to operate between the *las* and *rhl* systems (Figure 4) [178]. The 2-heptyl-3-hydroxy-4-quinolone molecule differs from that of acyl-homoserine lactone in that it is capable of overcoming cell density dependent production of exoproducts but not growth-phase dependent production [179]. *Pseudomonas aeruginosa* mutants defective at producing 2-heptyl-3-hydroxy-4-quinolone have lower levels of exoproducts but similar levels of the lectin adhesin PA-IL as the wild type [180]. By supplying exogenous 2-heptyl-3-hydroxy-4-quinolone exoproduct levels are restored to wild type [181]. The similar levels of lectin adhesin (PA-IL) present in mutant defective *Pseudomonas aeruginosa* suggest its control via *lecA* transcription may be due in part to an alternative system, namely the stationary phase sigma factor RpoS [28].

Figure 4. The *las*, *pqs*, and *rhl* interlinked quorum sensing systems in *Pseudomonas aeruginosa*. The *las* system consists of the proteins LasR (transcriptional activator) and LasI (synthase enzyme) coded for by *lasR* and *lasI* respectively. The *rhl* system consists of RhlR (transcriptional activator) and RhlI (synthase enzyme) coded for by *rhlR* and *rhlI*. Adapted from Raina, 2009 [177].



The *las* system is regulated directly by a variety of complex factors. Two LuxR homologues, known as quorum sensing control repressor (QscR) and virulence quorum sensing regulator (VqsR), are responsible for the negative and positive regulation of the *las* system, respectively, by unquantified mechanisms [182]. It is hypothesized that QscR negatively regulates *las* by repressing *lasI* transcription in the lag phase of growth when the concentration of autoinducing signal molecules has not reached its threshold for quorum sensing activation. QscR is thought to achieve repression by forming heterodimers of both LasR and RglR that are inactive but compete with acyl-homoserine lactones for binding to their relative cognate response regulator up to the threshold concentration. Above the threshold concentration these heterodimers dissociate allowing the formation of active LasR and RhlR homodimers [183].

VqsR allows positive regulation of quorum sensing pathways. Mutant strains of *Pseudomonas aeruginosa*, with no *vqsR* gene, show a lack of acyl-homoserine lactone production corresponding to a reduction in virulence and pathogenicity by an as yet unestablished mechanism [182]. The complexity of quorum sensing control is shown by the multitude of factors controlling its regulation. RsaL is a transcriptional regulator that acts to block *lasI* transcription by competitively

binding to LasI and thus preventing LasR-*N*-(3-oxododecanoyl)-L-homoserine lactone complexes from autoinducing LasI production [172]. This is likely to occur below threshold concentrations of *N*-(3-oxododecanoyl)-L-homoserine lactone in response to an uncharacterized environmental or metabolic stimulus [184,185]. There are a variety of accounts in the literature where synthetic derivatives of *N*-L-homoserine lactones result in inhibition of quorum sensing pathways and reduced biofilm development. Of particular relevance is the study by Hentzer and colleagues [186]. They demonstrated that synthesized compounds based on natural furanones were shown to be potent antagonists of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. Similarly Geske *et al.* synthesized small molecular weight, non-native *N*-L-homoserine lactone derivatives that blocked natural signals via binding to LasR in *Pseudomonas aeruginosa* [187].

4.2. RpoS and Quorum Sensing

RpoS, also known as the alternative subunit of RNA polymerase or the stationary phase sigma factor, is a protein encoded by the *rpoS* gene and controls the overall response of both *Pseudomonas aeruginosa* and *Escherichia coli* to environmental stress. A sharp increase in RpoS concentration has been demonstrated at the onset of the stationary phase of growth [75]. In *Pseudomonas aeruginosa*, mutation of *rpoS* leads to enhanced susceptibility of stationary-phase cells to heat, high osmolarity, acidic (low) pH, hydrogen peroxide, and ethanol [188]. The stationary phase sigma factor RpoS is also controlled directly by the *rhl* system and therefore indirectly by *las*, enabling the quorum sensing control of genes at the stationary phase [165,188–190]. Their interaction is complex, a relationship has been observed but no obvious mechanism apparent. Whiteley *et al.* suggest RpoS is responsible for negatively regulating *rhlI* transcription, especially in hydrogen cyanide (*hcnABC*) and phenazine (*phzABC*) genes [190]. The production of the lectin adhesin PA-IL and possibly PA-III, encoded by *lecA* and *lecB*, respectively, are mediated both directly by the *rhl*-dependent regulation of the *lecA* and indirectly by *rhl* through RpoS [28]. Other studies, such as those by Medina *et al.*, suggest RpoS partially activates the *rhlAB* genes that transcribe rhamnolipid production with a possibility that RpoS activates quorum sensing linked genes required in the stationary phase only [191]. Schuster *et al.* proposed that RpoS acts by repressing *rhlI* at the log phase of growth thus decreasing *N*-butyryl-homoserine lactone production, with loss of this repression at the late logarithmic to stationary phase of growth [192].

4.3. Quorum Sensing in *Escherichia coli*

Quorum sensing in *Escherichia coli* occurs via four different systems; the LuxR homologue *sdiA* system [193,194]; the *luxS*/autoinducer-II system [195]; an autoinducer-III/epinephrine/norepinephrine system [78], and an indole mediated system [196].

4.3.1. *sdiA* Quorum Sensing System

The *sdiA* quorum sensing system provides a means by which *Escherichia coli* can sense the autoinducer *N*-acyl-L-homoserine lactone from other species of microorganisms. SdiA is a LuxR homologue that binds to *N*-acyl-L-homoserine lactone and has links to the upregulation of the


cell division gene operon *ftsQAZ* [197]. In other microorganisms, such as *Vibrio fischeri*, the LuxI protein [198] (encoded by *luxI*) allows the formation of *N*-acyl-l-homoserine lactone. The *luxI* gene is not present in *Escherichia coli* and thus *N*-acyl-l-homoserine lactone must be synthesized by other microorganisms [199]. This quorum sensing system of importance to *Escherichia coli* is present in the gut microflora, where other species of *N*-acyl-l-homoserine lactone producing bacteria are prevalent. Nature is proving to be a ubiquitous source of quorum sensing inhibitors. Research performed recently by Ravichandiran demonstrated that extracts from seeds of the *Melia dubia* plant contained compounds that competitively inhibited SdiA, resulting in reduced biofilm formation in *Escherichia coli* [200]. Other natural sources of quorum sensing inhibitors include garlic, marine algae, and soil bacteria [201–203]. Difficulties remain with regard to identification, structural elucidation, isolation, and purification of natural sourced components.

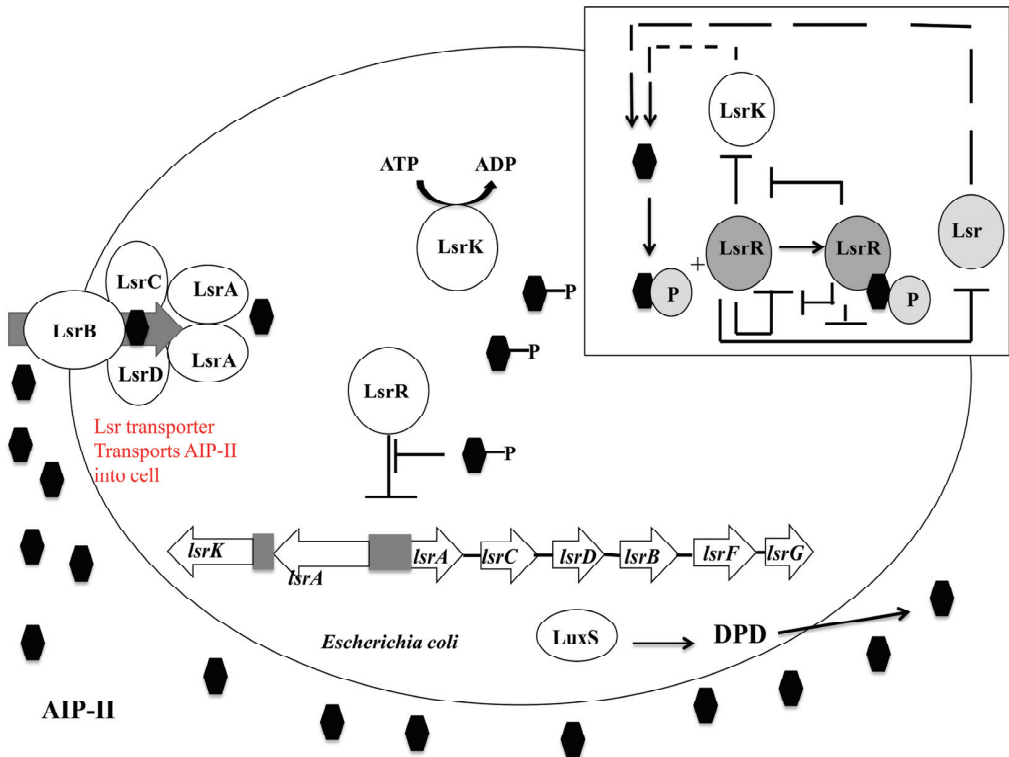
4.3.2. *luxS* Quorum Sensing System

The *luxS* system in *Escherichia coli* has autoinducer-II as its mediating quorum-sensing molecule, and synthesis of autoinducer-II is as described for the staphylococcal *luxS* system [7]. The *luxS* system may also have a role in cell metabolism through the intracellular activated methyl cycle, together with up and downregulation of quorum sensing-related genes [80,204]. Concentration of extracellular autoinducer-II reaches a peak at the mid- to late-exponential phase with a large decrease as bacteria enter the stationary phase, but there is no relative decrease in LuxS protein levels at the stationary phase of growth [205,206]. Decrease in the concentration of extracellular autoinducer-II, at the onset of the stationary phase, corresponds to an increase in autoinducer-II uptake into cells via an ATP-binding cassette deemed the Lsr transporter that is itself *luxS*-regulated [206]. Uptake of autoinducer-II, via an alternative active transport mechanism or diffusion, may be due to the bacteria requiring autoinducer-II to regulate gene expression and therefore switch off external metabolic and cellular responses. The Lsr transporter is encoded by the *lsrABCD* operon, with LsrB required for autoinducer-II transport into the cell, and whose transcription itself is regulated via the proteins LsrK and LsrR (transcribed from *lsrRK* operon) [207]. LsrK, present in the cytoplasm, is a kinase that donates a phosphate group to autoinducer-II, allowing the phosphorylated autoinducer-II to bind to the *lsr* repressor LsrR (Figure 5). Phosphorylation may act to sequester autoinducer-II activity within the cell, although its purpose is generally undefined [193]. Mutants of *lsrK*, deficient in LsrK production, have shown the Lsr transporter to be repressed, thus autoinducer-II remains in the extracellular fluid [207]. Mutants of *lsrR* express Lsr transporter and therefore autoinducer-II is imported into the cell cytoplasm continuously [208].

LsrK and LsrR are both quorum sensing regulators. A study of how the separate deletion of each corresponded to the resulting phenotypic profile was conducted by Li *et al.* [209]. They observed that genes associated with adhesion, such as the curli-related genes *CsgA*, *CsgE*, *CsgF*, and *CsgG*, together with the pili gene *htrE*, a homolog of *papD*, were all downregulated with increased intracellular autoinducer-II in *lsrR* mutants [210,211]. In *lsrK* mutants, fimbrial genes were downregulated with two putative fimbrial proteins, *yadK* and *yadN*, repressed. The large complexity of colonic acid synthesis and *luxS* quorum sensing in general is shown by upregulation of the *wza* gene in both mutants with *wcaA* upregulation in *lsrR* mutants corresponding to an

increase in intracellular autoinducer-II. The complexity involved in having extracellular and intracellular, as well as both quorum sensing and metabolic roles for autoinducer-II, is further demonstrated by the work of DeLisa *et al.* [212]. They observed that autoinducer-II upregulated a series of genes involved in fimbriae (*yadK* and *yadN*, putative fimbrial proteins), curli fiber (*crl*, the transcriptional regulator of cryptic *csaA* gene), and colonic acid production (*wzb*, a probable protein-tyrosine-phosphatase and *rscB*), with associated down regulation of genes linked to flagella synthesis (*flgN*). No link to either the intracellular or extracellular action of autoinducer-II was provided.

Figure 5. Summary of the *LuxS* Quorum sensing system of *Escherichia coli*. Autoinducer-II, represented by pentagons, is formed from a *LuxS* catalyzed cleavage reaction of *S*-ribosylhomocysteine to 4,5-dihydroxy 2,3-pentanedione and homocysteine [213]. Key: AIP-II: Autoinducer-II:  DPD: 4,5-dihydroxy-2,3-pentanedione. Adapted from Li, 2007 [184].



Further reactions form autoinducer-II. *LsrR* acts as the autoinducer-II uptake repressor repressing the *lsrACDBFG* and *lsrRK* operons [207]. At early and mid-exponential phases, the intracellular and extracellular autoinducer-II levels are low, thus *LsrR* can bind to and repress these genes [206]. As the levels of autoinducer-II increase extracellularly, active transport and diffusion of this molecule into the cell occurs by an uncharacterized non-*Lsr* related pathway [195].

Non-phosphorylated autoinducer-II binds to LsrR to depress many quorum sensing and biofilm forming genes including; *lsrR* itself; *flu* which is responsible for the phase-variable protein antigen 43 linked to autoaggregation; *wza* the gene linked to colonic acid synthesis; *dsrA* which encodes a small RNA molecule known as DsrA resulting in increased RcsA and upregulation of the colonic acid producing *wca* operon [153]. Lsr autoinducer-II uptake remains repressed until the late exponential phase whereby a threshold concentration of autoinducer-II is reached, corresponding to nutrient depletion, and leading to rapid autoinducer-II uptake by the Lsr transporter. Autoinducer-II is phosphorylated by LsrK (boxed section) with binding of this molecule to LsrR causing a cessation of *lsr* repression [214]. Transcription of the *lsr* operon acts as a positive feedback loop, importing more autoinducer-II in response to detection of phosphorylated autoinducer-II [215]. This leads to a relative decrease in LsrR/autoinducer-II quorum sensing and an increase in the LsrR/Phosphorylated autoinducer-II quorum sensing system, triggering the expression of biofilm linked genes.

4.3.3. Autoinducer-III/Epinephrine/Norepinephrine and Indole Quorum Sensing Systems

The LuxS enzyme is also responsible for the formation of another 4,5-dihydroxy-2,3-pentandione derivative, autoinducer-III [216]. This system is present within enterohemorrhagic *Escherichia coli*, which causes bloody diarrhoea linked to haemorrhagic colitis and haemolytic-uremic syndrome [217]. The formation of autoinducer-III is not fully reliant on LuxS. A *luxS* mutation leaves the cell with only one pathway to produce homocysteine, a molecule required for autoinducer-III synthesis, and produced by other microorganisms present as part of the normal microbial gut flora [218]. Autoinducer-III is not linked directly to biofilm formation in *Escherichia coli*. Its use is mainly focused on genes related to virulence factors, adhesion in the large intestine (*LEE* operon and attaching and effacing lesions) and motility (flagella) [219]. The catecholamine class of hormones also act as agonists to this system, in particular epinephrine and norepinephrine, which are produced by the colonized host [78,220]. The autoinducer-III/epinephrine/norepinephrine quorum sensing system involves a set of regulon. The QsecC regulon autophosphorylates in the presence of both autoinducer-III and epinephrine, phosphorylating the QseB response regulator to activate the genes responsible for flagella synthesis [221]. The attaching and effacing lesions required for intestinal attachment are controlled by a similar QseE and QseF system [222]. Two LysR transcriptional factors, QseA and QseD control *LEE* transcription [223]. The indole mediated quorum sensing signal is thought to allow *Escherichia coli* to adapt to environments where nutrients are poor and the breakdown of amino acids serves as an energy source. Indole itself is formed from the breakdown of tryptophan by the tryptophanase enzyme encoded by the *tna* gene. The production of indole activates this *tna* gene and also the *astD* and *gabT* genes, which code for enzymes that control the degradation of amino acids to pyruvate or succinate [196,224].

5. Conclusions

The biomolecular processes that govern biofilm formation in Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* display intricate differences and are excellent examples as to the

complexities that govern the survival of microbial communities. Antimicrobial resistance is increasing worldwide. This together with a relative lack of innovative antimicrobials being released to the pharmaceutical market has led to real concerns from the World Health Organization. Their 2014 global report on surveillance of antimicrobial resistance outlined the real possibility of a future whereby common infections and minor injuries can kill, including those by Gram-negative bacteria such as *Escherichia coli* [225]. Biofilm formation is one of the most important phenotypic traits in determining the resistance characteristics of microbial communities to current therapeutic strategies [226]. Therefore a comprehensive understanding of the biomolecular mechanisms allows targeted therapies to be developed in order to prevent the development of, or to eradicate, established biofilms. Thereby limiting pathogenicity in areas such as medical device related infections. O’Loughlin and colleagues recently demonstrated that small synthetically produced molecules, such as meta-bromo-thiolactone, were able to inhibit both *Pseudomonas aeruginosa* LasR and RhIR *in vitro* and *in vivo* resulting in inhibition of biofilm formation [227]. Major challenges still exist with regard to translating these molecules to produce effective therapeutic applications. One significant obstacle is that blocking a biofilm linked pathway may lead to upregulation of genes involved in the production of virulent factors [228]. Processes such as quorum sensing are often strain or isolate specific. Further research is required into control and feedback mechanisms of each biomolecular system and how they are inherently interlinked to produce phenotypic traits. Sophisticated control and modulation, rather than a policy of complete negation, of multiple pathways will be required tailored individual etiology of the infectious disease.

Author Contributions

G.L. conceived the study and wrote the review. S.P.G. and B.F.G. contributed to literature search, technical support and constructive discussions to form the review article.

Conflicts of Interest

The authors declare no conflict of interest.

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Iron and *Acinetobacter baumannii* Biofilm Formation

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Abstract: *Acinetobacter baumannii* is an emerging nosocomial pathogen, responsible for infection outbreaks worldwide. The pathogenicity of this bacterium is mainly due to its multidrug-resistance and ability to form biofilm on abiotic surfaces, which facilitate long-term persistence in the hospital setting. Given the crucial role of iron in *A. baumannii* nutrition and pathogenicity, iron metabolism has been considered as a possible target for chelation-based antibacterial chemotherapy. In this study, we investigated the effect of iron restriction on *A. baumannii* growth and biofilm formation using different iron chelators and culture conditions. We report substantial inter-strain variability and growth medium-dependence for biofilm formation by *A. baumannii* isolates from veterinary and clinical sources. Neither planktonic nor biofilm growth of *A. baumannii* was affected by exogenous chelators. Biofilm formation was either stimulated by iron or not responsive to iron in the majority of isolates tested, indicating that iron starvation is not sensed as an overall biofilm-inducing stimulus by *A. baumannii*. The impressive iron withholding capacity of this bacterium should be taken into account for future development of chelation-based antimicrobial and anti-biofilm therapies.

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1. Introduction

Acinetobacter baumannii has emerged worldwide as a leading cause of hospital-acquired infections, especially among severely ill patients in intensive care units (ICUs) [1]. Although *A. baumannii* was initially regarded to as a low-grade pathogen, evidence has been accumulated suggesting that *A. baumannii* infections are associated with increased mortality in critically ill patients [2]. *A. baumannii* causes a broad range of nosocomial infections, including ventilator-associated pneumonia, urinary tract infections, wound infection, bacteremia, endocarditis, meningitis [3], and has recently been associated with very severe community-acquired infections, especially among individuals with predisposing factors in Southern Asia and other tropical regions [4]. *A. baumannii* can also be isolated from veterinary sources, and show common characteristics with strains described in human infection [5].

Tendency to the epidemic spread, resistance to antibiotics and persistence in the hospital setting are hallmarks of *A. baumannii* infection [3]. Successful strains of multidrug-resistant (MDR) *A. baumannii* are notorious for their ability to rapidly spread among hospitalized patients, overcome geographical borders, and become epidemic worldwide [6]. Epidemiologic and population genetics studies indicate that the majority of *A. baumannii* infections are caused by strains belonging to three international clonal lineages (ICLs) [1,3,6]. *A. baumannii* strains belonging to the most widespread ICLs are invariably characterized by an MDR phenotype, which is progressively

evolving towards pandrug resistance, thereby challenging the current antimicrobial armamentarium [7,8]. This poses the urgent need for the development of novel treatment strategies to combat infections caused by MDR *A. baumannii* [9].

The capacity of MDR clinical isolates of *A. baumannii* to resist to desiccation and to form biofilms are regarded as crucial factors contributing to the clinical success and persistence of this species in healthcare facilities. *A. baumannii* can survive for up to months on the dry surface of inanimate objects [10,11], enabling transmission of infection for long times under both epidemic and endemic situations [12]. A number of reports have demonstrated that *A. baumannii* can form biofilms on several biotic and abiotic surfaces, providing the bacteria with protection against antibiotic/antiseptic treatment(s) and the host immune defenses *in vivo* (reviewed in [13,14]). Biofilm formation is crucial for several *A. baumannii* infections, since these are often associated with indwelling medical devices, e.g., vascular and urinary catheters, cerebrospinal fluid shunts, and endotracheal tubes [15]. While it is apparent that the capacity to form biofilms is a general phenotypic trait of *A. baumannii*, remarkable differences in the amount of biofilm formed by different strains have been reported, even if belonging to the same clonal lineage or epidemiological cluster [12,15–18]. A number of environmental factors can influence biofilm formation, including the presence of metal cations [16,19]. Among these, iron represents an essential nutrient for infecting bacteria, and a key determinant in host-pathogen interactions. This is because bacteria must counteract an iron-poor environment during infection, due to iron sequestration by iron carrier and storage proteins of the host and adaptive hypoferremia during infection [20]. *A. baumannii* has evolved an impressive capacity to acquire iron from the host, due to the production of multiple siderophores for Fe(III) transport, combined with uptake specificities for heme and Fe(II) [21,22].

Given the crucial role of iron in *A. baumannii*-host interactions [22–24], attention has recently been given to non-antibiotic approaches that target iron metabolism to achieve antibacterial activity, including chelation therapy and use of iron mimetics (reviewed in [9]). Interestingly, it was noted that: (i) high concentrations of deferiprone (DFP, (Sigma Aldrich, St. Louis, MO, USA)), a compound used for chelation therapy in humans, inhibited to some extent logarithmic growth of *A. baumannii* ATCC 17978 in a chemically defined medium [25]; (ii) gallium, an iron-mimetic drug, suppressed the growth of MDR *A. baumannii* strains both *in vitro* and *in vivo*, acting through disruption of bacterial iron metabolism [25–27]; (iii) mutants impaired in production of the acinetobactin siderophore show reduced fitness *in vivo* [23]. On the other hand, it was also reported that biofilm formation on plastic by the type strain *A. baumannii* ATCC 19606^T was stimulated under conditions of iron scarcity imposed by the addition of the chelator 2,2'-dipyridyl (DIP) [19]. Therefore, the effect of iron availability on both planktonic and biofilm mode of *A. baumannii* growth deserves more in-depth investigation.

In this report, strains and optimal growth conditions for the generation *A. baumannii* biofilms were preliminarily established. Then, the role of iron in *A. baumannii* biofilm formation was investigated. Lastly, the activity of a new therapeutic iron chelator was assessed in search for inhibitory drugs that could be repurposed as adjuvant antimicrobials in the treatment of biofilm-based *A. baumannii* infections.

2. Results and Discussion

2.1. Definition of Culture Conditions for *A. baumannii* Biofilm Formation

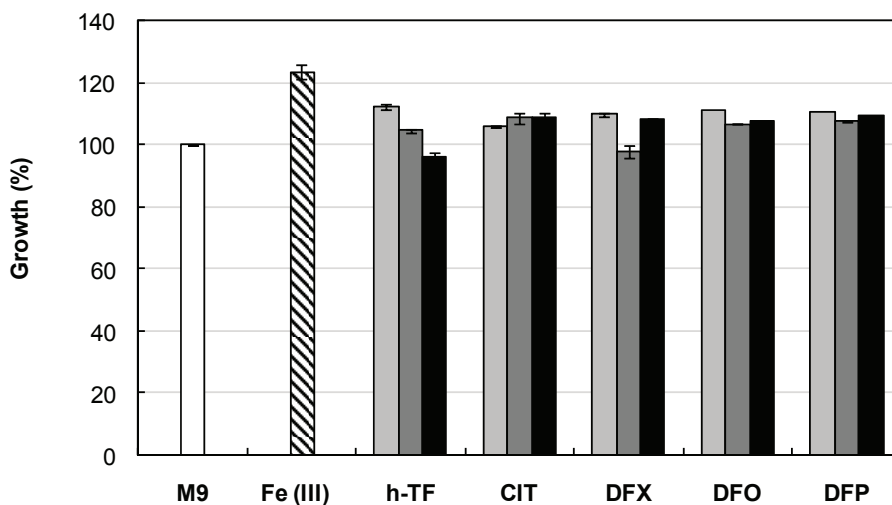
Biofilm formation is a multifactorial phenotype [13,14], and in *A. baumannii* it can be modulated by iron availability, carbon sources, growth temperature, and different expression levels of adhesive and cell-aggregating factors [13,14,16,18,19,28]. Therefore, as a preliminary step to the investigation of the effect of iron on *A. baumannii* biofilm formation, we determined the growth response of the reference strain *A. baumannii* ATCC 17978 [25,29] to iron restriction imposed by different chelators in M9 minimal medium [30] containing 20 mM sodium succinate as the carbon source [26]. In line with previous observations [25], *A. baumannii* ATCC 17978 showed an impressive ability to multiply under conditions of iron deficiency, as those imposed by the addition of up to 128 μM human apo-transferrin (h-TF (Sigma Aldrich)), trisodium citrate (CIT (Sigma Aldrich)), desferrioxamine (DFO (Ciba Geigy, Origgio, Italy)), deferasirox (DFX (Novartis, Basel, Switzerland)) and DFP (Figure 1).

None of the tested chelators reduced *A. baumannii* ATCC 17978 growth yields at 48 h, even when 100 μM DIP (a chelator of the intracellular Fe(II) pool) was added to further reduce iron availability. As expected, growth in M9 was stimulated by *ca.* 25% in the presence of 100 μM FeCl_3 . A similar resistance to exogenously supplied chelators in M9 was also observed for strains AYE [31] and ACICU [32], representatives for ICL-I and ICL-II, respectively (data not shown). These data can be explained by the presence in *A. baumannii* of very efficient iron uptake systems [11,21], capable of counteracting the iron withholding capacity of exogenously added chelators. The observation that DFX, DFO, and DFP do not stimulate bacterial growth in the presence of DIP (a chelator of the intracellular Fe(II) pool) suggests that these chelators are unlikely to serve as an iron source for *A. baumannii*.

Next, the inter-strain variability and the growth medium-dependence of biofilm formation was investigated. Five well-characterized *A. baumannii* strains (AYE, representative for ICL-I [31]; ACICU, representative for ICL-II [32]; 50C, ICL-II pandrug resistant isolate [33]; RUH5875, prototypic strain for ICL-III [34]; ATCC 17978 [29]) were grown in three iron-poor media [M9, M9 supplemented with 100 μM DIP, and Chelex-100-treated Tryptic Soy Broth dialysate, TSBD [35]) in order to determine both growth and biofilm levels at 24 and 48 h. Quantitative estimation of the bacterial biomass in biofilms was assessed in 96-well polystyrene microtiter plates (BD Falcon, Milano, Italy), using the crystal-violet (CV) staining method [36]. There was a wide range of variation in growth and biofilm levels between *A. baumannii*, depending on strains and culture media, though for some strains moderate correlation was observed between growth yields and biofilm levels (Figure 2).

Remarkably, all strains produced more abundant biofilm in TSBD than in the other iron-poor media, and biofilm levels in strain ACICU were significantly higher ($p < 0.05$ in the student's *t*-test) than all the other strains tested (Figure 2). These findings corroborate the notion that biofilm levels in *A. baumannii* can vary even between closely related isolates (e.g., strains ACICU and 50C belonging to the same genetic cluster according to ref. [33]), and that different media have a profound impact on biofilm yields [15–18].

Figure 1. Effect of different iron chelators on planktonic growth of *A. baumannii* ATCC 17978. Bacteria were grown for 48 h at 37 °C in 96-wells microtiter plates containing 100 μ L M9 supplemented with the indicated iron chelator at different concentrations: 32 μ M (light grey bars), 128 μ M (dark grey bars) or 128 μ M chelator + 100 μ M DIP (black bars). Growth was measured as OD₆₀₀ and expressed as percentage relative to the untreated control (*i.e.*, OD₆₀₀ in M9). The average of the OD₆₀₀ in control M9 was 0.318 ± 0.008 and represents 100% of growth (white bar). Relative growth in M9 supplemented with 100 μ M FeCl₃ is reported (striped bar). Data represent the average of three independent experiments \pm standard deviation. h-TF, transferrin; CIT, citrate; DFX, deferasirox; DFO, desferrioxamine; DFP, deferiprone.

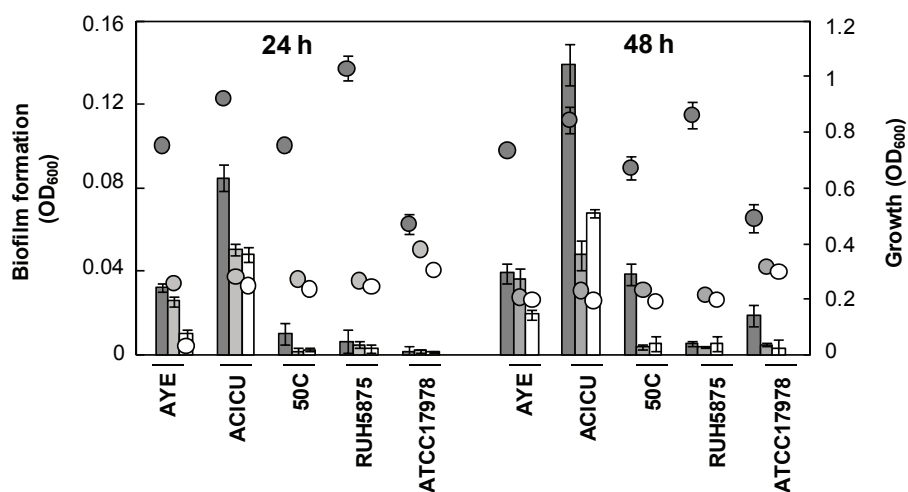


To rule out the possibility that differences in biofilm levels between TSBD and M9 or M9 plus DIP were due to different iron content of these media, an iron biosensor consisting of the Fur-controlled *basA* promoter fused to the reporter *lacZ* gene [26] was used as a probe to determine the intracellular iron level in *A. baumannii* ATCC17978. Since the Fur repressor protein acts as an iron sensor, the activity of the Fur-controlled *basA* promoter provides an indirect estimate of the intracellular iron levels of bacteria grown in the different media. The β -galactosidase (LacZ) expression was higher in TSBD than in M9 or M9 plus DIP (Figure 3), and it was invariably repressed by iron, indicating that TSBD is sensed by *A. baumannii* as an iron-poor medium.

To visualize differences in biofilm structure among the five representative *A. baumannii* strains, biofilm formation on glass slides was monitored during seven days growth in TSBD by means of confocal microscopy, according to a previously described procedure [37] (Figure 4). High biofilm levels with formation of large cellular aggregates were observed for *A. baumannii* ACICU, and to a much lesser extent for the other strains (Figure 4A). Interestingly, *A. baumannii* biofilm cells were found to be embedded in a blue fluorescent material upon staining with calcofluor white (Figure 4B). In line with previous findings [18,29], this observation denotes the presence of

exopolysaccharides in the matrix of *A. baumannii* biofilms, whose levels appear to be consistent with to the amount of biofilm formed in 96-well polystyrene microtiter plates (Figure 2).

Figure 2. Growth and biofilm formation by selected *A. baumannii* strains in different iron-poor media. Bacterial cells were inoculated at OD₆₀₀ of 0.01 in 100 μ L of the different growth media, dispensed in a 96-wells microtiter plate, and grown at 37 °C without shaking for 24 and 48 h. Growth (circles) was measured spectrophotometrically (OD₆₀₀) and biofilm formation (bars) was evaluated using the CV staining assay [36]. Dark grey, TSBD; light grey M9; white M9 supplemented with 100 μ M DIP. Data represent the average of three independent experiments \pm standard deviation.



Based on the above results, TSBD was considered as suitable iron-depleted medium that would allow robust biofilm formation and an easier evaluation of the effect of iron on this process. This is because the high biofilm levels achieved by *A. baumannii* in TSBD would facilitate the detection of biofilm variations in response to iron levels. Moreover, the high peptide content and balanced formula of TSBD (35, see also [38]) make it more similar to a biological fluid than the M9 mineral salt medium.

Figure 3. Regulatory mechanism and activity of the *basA::lacZ* iron biosensor in the reference *A. baumannii* strain ATCC 17978. (A) Schematic of the regulatory mechanism the *basA::lacZ* iron-regulated transcriptional fusion carried by plasmid pMP220::*P_{basA}* [26]. Under iron proficient conditions (left), the Fur repressor protein binds the *P_{basA}* promoter and inhibits β -galactosidase (LacZ) expression; under iron deficient conditions Fur repression is relieved and the LacZ enzyme is expressed. (B) Activity of the *basA::lacZ* iron-regulated fusion in *A. baumannii* ATCC 17978 grown for 24 and 48 h in different media, as indicated, in the absence (white bars) or presence (black bars) of 100 μ M FeCl₃. Data are the means (\pm standard deviations (SD)) of triplicate experiments.

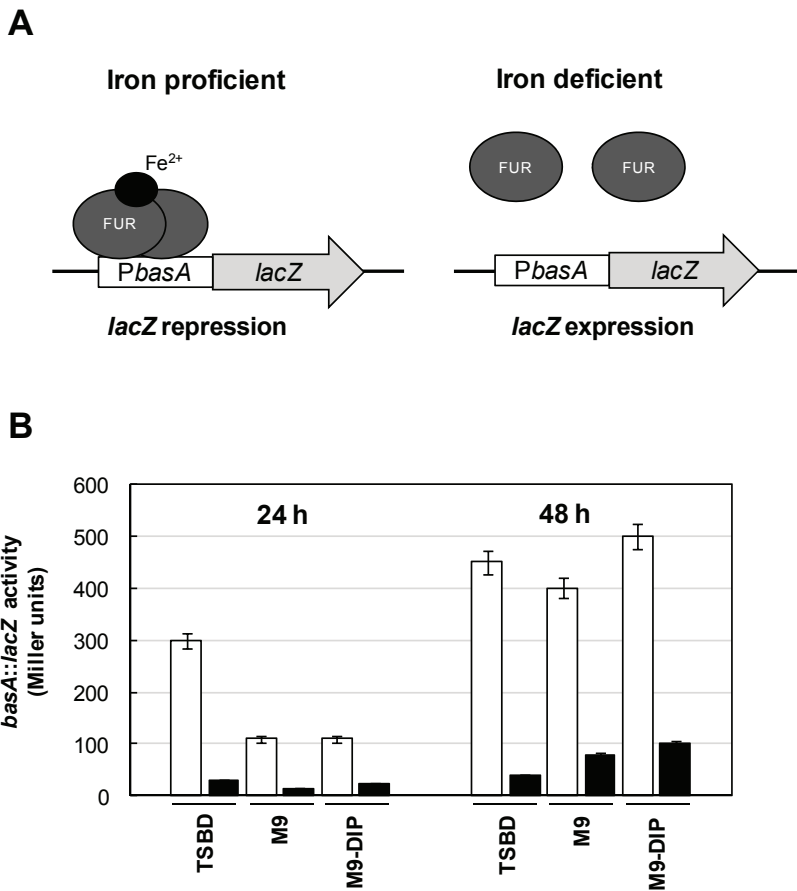
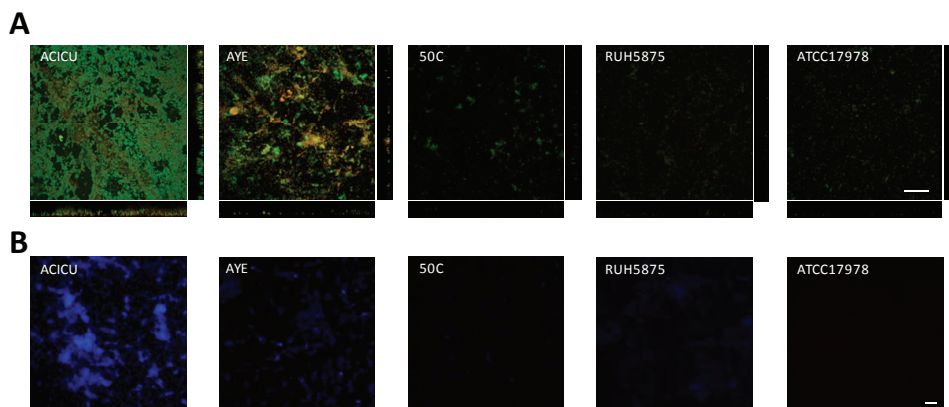


Figure 4. Seven-days biofilm of selected *A. baumannii* strains grown in TSBD. (A) Confocal microscope images (x-y plane and side view) of *A. baumannii* biofilms stained with acridine orange, a fluorescent dye which labels double-stranded nucleic acids (prevalently DNA) in green, and single-stranded nucleic acids (prevalently RNA) in red. (B) *A. baumannii* biofilms stained with the calcofluor white for exopolysaccharide labelling [19,28], and analyzed by fluorescence microscopy. Scale bar: 50 μ m.



2.2. Effect of Iron Levels on Biofilm Formation by a Collection of Diverse *A. baumannii* Isolates

We investigated the effect of iron on biofilm formation in a representative collection of 54 *A. baumannii* strains (Table S1 in Supplementary Material), including 27 isolates from veterinary sources (67% MDR) and 27 isolates from clinical sources (96% MDR). Isolates were selected so to maximize diversity, as inferred by Random Amplified Polymorphic DNA fingerprints with primer DAF4 ([21,33]; data not shown), and represent the most widespread ICLs and some emerging lineages (Table S1 in Supplementary Material). High growth yields were observed for almost all isolates in TSBD (median $OD_{600} = 0.716$), which were significantly increased by the addition of 100 μ M $FeCl_3$ (median $OD_{600} = 1.031$), consistent with iron being a nutritionally-limiting factor in TSBD (Figure 5A). Remarkably, biofilm formation was more abundant in $FeCl_3$ -supplemented TSBD (median $OD_{600} = 0.102$) than in TSBD (median $OD_{600} = 0.071$) (Figure 5B). After having excluded from the analysis 4 biofilm non-producing isolates (namely, 4297, 196-1, 82D, RUH5875, see Table S1), the normalization of biofilm formation by growth yields resulted in minor differences between the two growth conditions (median values were 0.104 and 0.098 for the iron-limited and iron-rich condition, Figure 5C). This result is due to somehow opposite responses of *A. baumannii* isolates to iron starvation (*i.e.*, TSBD vs. $FeCl_3$ -supplemented TSBD); in 21 isolates (42%) biofilm production was significantly enhanced by iron deficiency, in 12 (24%) it was significantly reduced, and in 17 (34%) iron had no effect on biofilm formation (significance in the Student's *t*-test was set at $p < 0.05$). Although stimulation of biofilm formation in response to iron-limited growth was observed for a minority (42%) of *A. baumannii* isolates, this may have relevant medical implications, since transition of these isolates from planktonic to biofilm-growing

cells could be favored *in vivo*, where infecting bacteria are normally challenged with iron shortage. However, this behavior cannot be generalized, since biofilm production was either unchanged or even inhibited by iron deficiency in the majority (66%) of the isolates.

2.3. Effect of Deferasirox on *A. baumannii* Biofilm Formation

We showed that planktonic *A. baumannii* has an impressive ability to grow in the presence of exogenously added therapeutic chelators DFP, DFO, and DFX (Figure 1), and previous data indicate only modest inhibition of *A. baumannii* growth at high DFP concentrations (*ca.* 200 μM , ref. [25]). To gain further insight into the effect of iron withholding on *A. baumannii* biofilm formation, we examined the effect of DFX, a newly developed orally active Fe(III) chelator, on our collection of 50 biofilm-producing isolates. DFX is a synthetic compound with high affinity and specificity for Fe(III) ($\log\beta_2 = 36.9$ according to ref. [39]), and is unlikely to serve as an iron carrier to *A. baumannii* based on growth assays (Figure 1). It was successfully used in combination therapy against murine staphylococemia [40], and in treatment of invasive fungal infections [41].

Here, biofilm formation was tested in a DFX concentration range 4–128 μM , in order to match the DFX plasma levels achievable during treatment of iron overload in humans [42]. Notably, biofilm formation by most of the isolates was not significantly affected by DFX up to 128 μM , either in TSBD or in TSBD plus 100 μM FeCl_3 (Figure 6A).

No obvious correlation could be observed between the biofilm response to iron starvation in TSBD (see Figure 5 and associated text) and in the presence of 128 μM DFX (Figure 6B). The observation that a minority of isolates (*i.e.*, Km1008-06, 132, P1697, P869, A472, and ATCC17978) showed opposite responses to iron starvation induced by TSBD with and without iron (Figure 5), compared with TSBD and TSBD plus DFX (Figure 6B), suggests that either DFX exerts iron chelation-independent effects, or that the iron deficiency threshold that determines the biofilm response in *A. baumannii* can vary for these isolates. Apart from this, the ability to generate biofilms is scarcely influenced by the presence of DFX for most *A. baumannii* isolates, indicating that, at least *in vitro*, this therapeutic chelator is incapable of overcoming the iron withholding capacity of *A. baumannii* biofilms.

Figure 5. Growth and biofilm formation in a representative collection of 54 *A. baumannii* strains from clinical and veterinary sources. **(A)** Growth of 54 *A. baumannii* strains for 48 h in 96-wells microtiter plates containing 100 μ L TSBD supplemented (black circles) or not (white circles) with 100 μ M FeCl₃, as indicated. **(B)** Absolute values of biofilm formation by the same isolates shown in panel A, evaluated by the CV staining assay (OD₆₀₀). Grey circles (B) represent the values for strains that in either or both conditions yielded negative biofilm values, and were excluded from calculations in panel C. **(C)** Relative values of biofilm formation (Biofilm formation (OD₆₀₀)/Growth (OD₆₀₀)) for a subset of 50 biofilm-producing isolates. The line bar represents the median value for each group. Values for each strain are the average of three independent experiments.

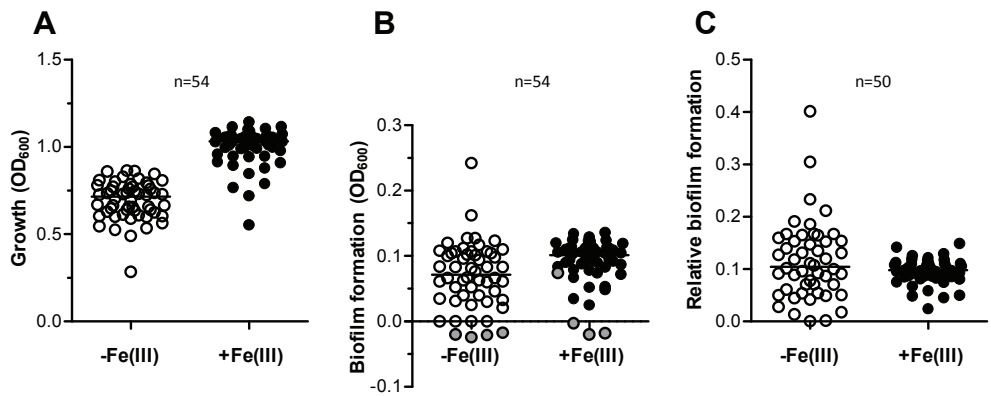
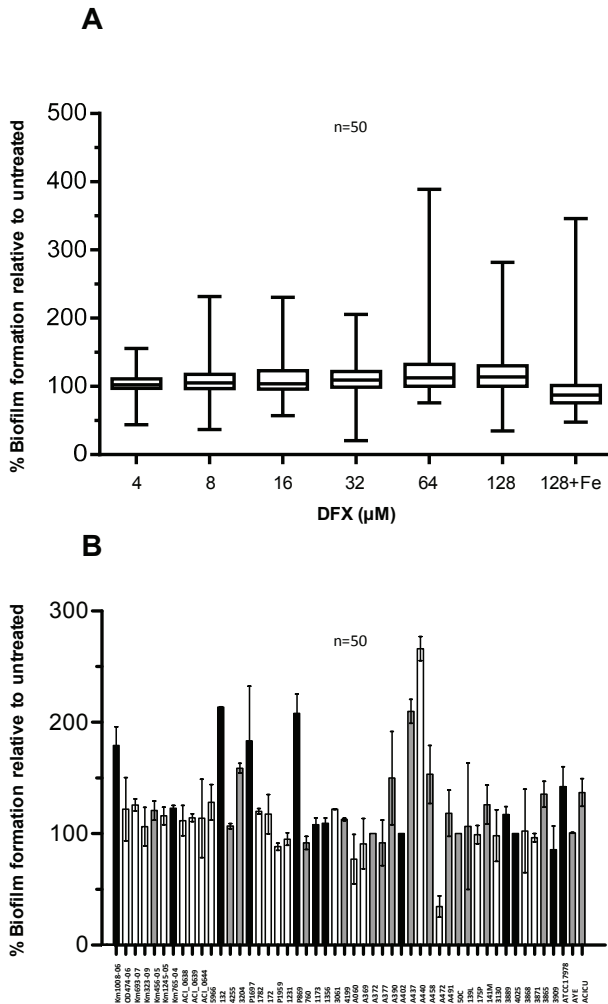


Figure 6. Effect of DFX on *A. baumannii* biofilm formation. **(A)** *A. baumannii* strains were grown statically for 48 h in microtiter plates containing 100 μL TSBD supplemented with DFX at indicated concentrations, or 128 μM DFX plus 100 μM FeCl_3 . Biofilm formation (OD_{600} in the CV staining assay) was normalized by the growth yield (OD_{600} of the culture) and expressed as percentage relative to the DFX-untreated control (TSBD). Boxes represent medians, second and third interquartiles; whiskers represent range of 50 isolates tested. **(B)** Relative biofilm levels produced by individual isolates in presence of 128 μM DFX, expressed as % of the untreated control in TSBD. With reference to Figure 5C, the bar filling denotes: isolates in which biofilm production was significantly enhanced by iron deficiency (white, 21 isolates), or significantly reduced (black, 12 isolates), or in which iron had no effect on biofilm formation (grey, 17 isolates). In both panels data represent the average of three independent experiments.



3. Experimental Section

3.1. Bacterial Strains and Growth Media

Relevant characteristics of the 54 *A. baumannii* strains used in this study are provided in Table S1 in the Supplementary Material. The collection includes representative strains for ICLs I, II, and III, namely AYE (ICL-I) [43], ACICU (ICL-II) [32], and RUH 5875 (ICL-III). Strain ATCC 17978 is a well-characterized clinical isolate dated 1950s and showing moderate antibiotic resistance [29]. Strain 50C is a pandrug resistant clinical isolate [33,44]. Other clinical and veterinary isolates were provided as part of the collections from various European laboratories [5,21,45–48]. Iron-poor culture media used in this study were the M9 minimal medium [30] supplemented with 20 mM sodium succinate as the carbon source, and TSBD, a Chelex 100-treated Trypticase Soy Broth dialysate [35]. When required, media were supplemented with either 100 μ M DIP or 100 μ M FeCl₃.

3.2. Chemicals

The chemicals used in this study were deferiprone (DFP, (Sigma Aldrich)), tri-sodium citrate [CIT, (Sigma Aldrich)], desferrioxamine (DFO, (Ciba Geigy)); human apo-transferrin (h-TF, iron content \leq 0.005%, (Sigma Aldrich)), and deferasirox (DFX, (Novartis)).

3.3. Growth Inhibitory Activity of Iron-Chelators

The activity of the iron chelators on bacterial growth was tested in 96-well microtiter plates (BD Falcon) containing increasing concentrations (4–128 μ M) of iron chelators. Plates were inoculated at OD₆₀₀ of 0.01 in a 100 μ L final volume of M9 supplemented or not with 100 μ M DIP at the highest iron-chelator concentration tested, or 100 μ M FeCl₃, and incubated at 37 °C for 48 h with moderate shaking (100 r.p.m.). Spectrophotometric readings were performed in a Wallac 1420 Victor3V multilabel plate reader (Perkin Elmer, Milano, Italy).

3.4. Biofilm Formation

Biofilm formation was measured according to the microtiter plate assay [36]. Briefly, bacterial cells were inoculated at OD₆₀₀ of 0.01 in 100 μ L of medium and grown at 37 °C for up to 48 h in 96-wells microtiter plates without shaking. Planktonic cells were removed and the attached cells were gently washed three times with sterile PBS, air dried, and stained with 150 μ L of 0.1% CV water solution for 20 min. The wells were gently washed four times with distilled water, and the surface-associated dye was eluted in 200 μ L of 95% ethanol. The OD₆₀₀ of the eluate was measured in a Wallac 1420 Victor3V multilabel plate reader (Perkin Elmer).

3.5. Biofilm Inhibition

To investigate the effect of DFX on biofilm formation, fifty biofilm-producing *A. baumannii* strains were inoculated (OD₆₀₀ of 0.01) into 96-well microtiter plates containing 100 μ L TSBD

supplemented with increasing DFX concentrations (4 to 128 μM) or 128 μM DFX plus 100 μM FeCl_3 . The assay was performed as described above.

3.6. Microscopy Analysis

For microscopic visualization of *A. baumannii* biofilms, strains were grown in an 8-well chamber slide as previously described [37]. Briefly, bacteria were inoculated at OD_{600} of 0.01 in 200 μL of TSBD and incubated at 37 $^\circ\text{C}$ for 48 h to allow the adhesion of the bacterial cells on the glass surface. To maintain bacterial viability, the medium was changed every 24 h until the seventh day. To visualize biofilms structure, *A. baumannii* biofilms were stained with the acridine orange (0.1% water solution), a fluorescent dye, which labels double-stranded nucleic acids (prevalently DNA) in green, and single-stranded nucleic acids (prevalently RNA) in red, and examined using Leica TCS SP5 confocal microscope. For detection of matrix exopolysaccharides, samples were stained with calcofluor white (Fluka) and analyzed with an epifluorescence microscope. The Image J software [49] was used for image analysis.

3.7. β -Galactosidase Activity Assay

The *basA* promoter was cloned upstream of the *lacZ* reporter gene in plasmid pMP220::*PbasA* (carrying the tetracycline-resistance (Tc^R) determinant) as previously described [26]. For reporter gene activity measurements, *A. baumannii* ATCC 17978 (Tc^S) was transformed with *PbasA*::*lacZ* (Tc^R) and grown for 16 h at 37 $^\circ\text{C}$ in M9 medium supplemented with 10 $\mu\text{g}/\text{mL}$ Tc. Cultures were then appropriately diluted in TSBD, M9 and M9 supplemented with 100 μM DIP, with or without 100 μM FeCl_3 to reach an initial cell concentration corresponding to $\text{OD}_{600} \sim 0.01$ and incubated at 37 $^\circ\text{C}$ with vigorous shaking. The β -galactosidase (*LacZ*) activity expressed by *A. baumannii* ATCC 17978 (pMP220::*PbasA*) after 24 and 48 h growth was determined spectrophotometrically on toluene/SDS-permeabilized cells using *o*-nitrophenyl- β -D-galactopyranoside as the substrate, and expressed in Miller units [50].

$$\text{Miller units} = 1,000 \times [\text{OD}_{420} - (1.75 \times \text{OD}_{550})] / \text{Volume (ml)} \times \text{Time (min)} \times \text{OD}_{600}$$

4. Conclusions

The formation and maturation of *A. baumannii* biofilms depend on the complex interplay of many environmental and cell-associated factors [13,14]. In this study, attention has been focused on the role of iron, since this metal is essential for bacterial nutrition and virulence [22–24], and plays a central role in host defense from bacterial infection [20]. In agreement with a previous study [25], we showed that planktonic *A. baumannii* cells can overcome iron restriction imposed by a variety of exogenous chelators, likely due to the presence in this species of multiple iron scavenging systems [21,22]. Then, we observed relevant differences in biofilm levels depending on *A. baumannii* strain and growth medium, and established suitable conditions for testing the effect of iron on biofilm formation. The two most relevant findings of these experiments were: (i) the strong influence of medium composition on biofilm yields; (ii) the high variability in biofilm levels produced by *A. baumannii* strains of clinical and veterinary origin, irrespective of their genetic

relatedness or epidemic potential; (iii) the strain-dependent response of *A. baumannii* biofilms to iron scarcity. Since biofilm formation was either stimulated by iron or not responsive to this metal in the majority of strains tested, we conclude that iron starvation is not sensed as an overall biofilm-inducing stimulus by *A. baumannii*. Consistent with these findings, a recently developed clinical chelator, endowed with extremely high affinity for iron, showed no significant anti-biofilm activity in *A. baumannii*. Thus, while iron metabolism continues to represent a promising target for *A. baumannii* inhibition, the impressive iron withholding capacity of this bacterium should be taken into account for future development of chelation-based antimicrobial and anti-biofilm therapies.

Supplementary Materials

Supplementary materials can be accessed at: <http://www.mdpi.com/2076-0817/3/3/704/s1>.

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Author Contributions

Conceived and designed the experiments: Emanuela Frangipani, Paolo Visca. Supervised the laboratory work: Emanuela Frangipani, Paolo Visca. Performed the experimental work: Valentina Gentile, Carlo Bonchi, Federica Runci, Fabrizia Minandri. Wrote the manuscript: Paolo Visca.

Conflicts of Interest

The authors declare no conflict of interest.

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***Pseudomonas aeruginosa* Diversification during Infection Development in Cystic Fibrosis Lungs—A Review**

Ana Margarida Sousa and Maria Olívia Pereira

Abstract: *Pseudomonas aeruginosa* is the most prevalent pathogen of cystic fibrosis (CF) lung disease. Its long persistence in CF airways is associated with sophisticated mechanisms of adaptation, including biofilm formation, resistance to antibiotics, hypermutability and customized pathogenicity in which virulence factors are expressed according the infection stage. CF adaptation is triggered by high selective pressure of inflamed CF lungs and by antibiotic treatments. Bacteria undergo genetic, phenotypic, and physiological variations that are fastened by the repeating interplay of mutation and selection. During CF infection development, *P. aeruginosa* gradually shifts from an acute virulent pathogen of early infection to a host-adapted pathogen of chronic infection. This paper reviews the most common changes undergone by *P. aeruginosa* at each stage of infection development in CF lungs. The comprehensive understanding of the adaptation process of *P. aeruginosa* may help to design more effective antimicrobial treatments and to identify new targets for future drugs to prevent the progression of infection to chronic stages.

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1. Introduction

Cystic fibrosis (CF) is an autosomal recessive disease caused by a defect in the cystic fibrosis conductance regulator (CFTR) gene located in human on chromosome 7 that mainly affects lungs, digestive and reproductive systems, but also the secretory glands, such as the endocrine and sweat glands [1]. Although CF is a multi-system disorder causing several complications on the human body, its effects on lungs are the best studied so far due to the severe symptoms that patients suffer and high mortality rate associated to poor lung function.

It is generally accepted that CFTR acts as a channel that pumps chloride from the intracellular to extracellular space through the membrane of the epithelial cells that produce sputum. Several hypotheses have been formulated attempting to explain the relationship between CFTR deficiency and sputum accumulation. It has been considered that the transport of chloride partially controls water movement and consequently influences the production of thin and flowing sputum, fundamental to keeping the lungs protected [1,2]. The CFTR lacks causes, thus, a defective chloride secretion creating an osmotic gradient that, consequently, provokes water hyper-reabsorption and abnormal thick and sticky sputum [1,3]. This sputum with altered pH interfere with, reducing or even inhibiting the activity of epithelial antimicrobial molecules of innate immune system and ciliary functions, both crucial for homeostasis.

Other functions are also associated with CFTR, including inhibition of sodium absorption, of which loss causes excessive sodium (and water) absorption, regulation of HCO_3^- and some proteins

transport through epithelial cell membranes [3,4]. The relevance of the latter mechanisms in CF airway is unclear, however, it is believed that reduced chloride secretion or sodium hyper-absorption can occur. Both mechanisms lead to airway-surface-liquid depletion and sputum viscosity increase, causing impaired cilia beats and accumulation of thick dehydrated airway sputum, which profoundly accounts for the typical symptoms suffered by CF patients [4,5]. The defective mucociliary transport and the compromised immune defenses predispose CF patients to the establishment of recurrent bronchopulmonary infections. Sputum retention leads to infection and consequently to inflammation, and this circle perpetuates itself since inflammatory products, such as elastase released by neutrophils, stimulate sputum secretion and breakdown [3,6]. The accumulated sputum is rich in nutrients being, thus, a good environment for microbial colonization [7,8]. CF lungs are infected with a complex microbial flora, mainly composed by bacteria, provoking acute and chronic infections that result in decline of the lung function, respiratory failure, and premature death of patients. Once bacterial infections are established, their eradication by antibiotic treatment is hardly ever achieved [9,10].

Some progress was made in this field extending the life expectancy of CF patients, however, it remains very reduced, around 37 years, mainly because of bacterial infections [11]. In the last decades, new therapies have emerged, based on the knowledge of CFTR dysfunction and airway CF microbiome, such as targeting CFTR replacement, stimulation of alternative chloride channels, inhibition of sodium absorption, and airway rehydration, in order to avoid sputum accumulation and, consequently, the establishment of bacterial infections [3–5,12]. None of these strategies has sufficient potential to stop CF infections development thus far. The actual and more effective approach to fight CF-associated infections relies on antimicrobial treatment. Currently, there is no consensual antimicrobial treatment to eradicate bacterial infection from CF lungs [13,14]. Treatments vary among clinics, countries, and even continents. Numerous strategies have been used varying in route of antibiotic administration (systemic, oral, inhaled antibiotics, or routes combination), classes of antibiotics, and treatment duration. Inhaled antibiotics, mainly aminoglycosides, have high success rates in bacteria eradication, in particular against *Pseudomonas aeruginosa*, due to the direct delivery of high-dose of antibiotic to the bronchial lumen space with limited systemic toxicity. For instance, a tobramycin inhalation solution has been used to treat long-term and chronic bacterial infection with significant benefits for lung function delaying re-infection and reduce mortality [15–17]. Oral and intravenous antibiotics have also attracted interest and currently quinolones, in particular ciprofloxacin, are the most used. However, ciprofloxacin usage is somewhat limited due to the rapid emergence of resistance. As a solution, ciprofloxacin is frequently combined with other antibiotics through other routes of administration. Combination of inhaled colistin or inhaled tobramycin with oral ciprofloxacin has been used successfully [13]. Some authors had suggested the still used broad-spectrum penicillins and cephalosporins in efforts to improve outcomes for CF patients infected with *P. aeruginosa* [18].

Other antibiotics have been introduced and used as alternative agents, such as inhaled amikacin, aztreonam lysine, and the combination of fosfomycin and tobramycin [12–14]. However, it has assisted to the failure of these antibiotic courses, making urgent the comprehension of the

mechanisms underlying antibiotic resistance to rapidly define effective strategies to eradicate those infections.

2. *Pseudomonas aeruginosa*

The microbial community resident in CF lungs is known to be complex and it has considerably changed, mainly due to alterations in antibiotic regimens. Nevertheless, *P. aeruginosa* is still the most common pathogen isolated from CF sputum, being more prevalent in adults [2,10,19].

P. aeruginosa is a versatile microorganism, ubiquitously distributed in different environments, including terrestrial, aquatic, animal, human, and plant. It is a Gram-negative opportunist pathogen in hospitalized or immune-compromised patients, causing infections, such as pneumonia, burn, wound, urinary tract and gastrointestinal infections, otitis media, and keratitis [19,20]. Its versatility arises from its large genome, with nearly 6000 genes that enclose, for instance, genes associated with diverse metabolic pathways, virulence factors, transport, efflux, and chemotaxis, conferring to *P. aeruginosa* great adaptive ability. Moreover, this bacterium is able to coordinate metabolic pathways, optimize nutritional and reproductive potential according to the surrounding conditions and resources and, thus, it can survive, grow and cause infection in different environments [20,21].

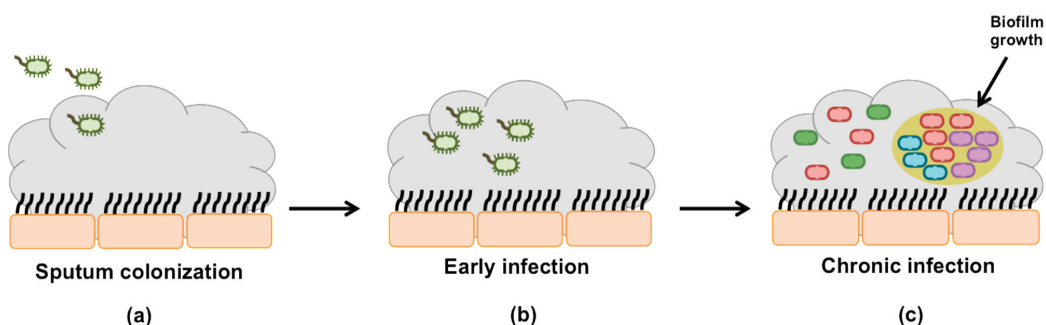
The presence of *P. aeruginosa* in CF airways is highly associated with poor lung function, morbidity and mortality of patients. Despite the inflammatory response and the long-term and intensive antibiotic treatments, infections caused by *P. aeruginosa* persist in CF lungs. Once entering in CF airways, *P. aeruginosa* is virtually impossible to eradicate due to its remarkable genome plasticity that allows it to rapidly adapt to the greatly stressful CF environment [2,22,23]. After *P. aeruginosa* colonization, patients may suffer of successive episodes of re-colonization until resulting in a chronic infection that can persist from years to decades, or even never eradicated [23,24]. Several factors can influence the infection course in CF airways and, unfortunately, there is limited knowledge about the characteristics of this microorganism that have impact on the severity of infection. Until now, it is just known that during CF infection development, *P. aeruginosa* switch from an acute environment virulent pathogen, characteristic from early infection stages, to a CF-adapted pathogen, typical of chronic infection stages [6,21,25]. This review aimed to provide an overview of the successive adaptations that *P. aeruginosa* undergo, and to describe their impact on long-term persistence in the airways. The identification of the genetic background, interactions, and strategies, used by *P. aeruginosa* are crucial, and a prerequisite to develop new approaches for effectively eradicate lung infections.

3. Sources of Phenotypic Diversification

The long-term persistence of *P. aeruginosa* infections in CF lung is associated with clonal diversification, or expansion, into specialized phenotypes (Figure 1). Driven by the challenging selective pressures imposed by the typical CF conditions, e.g., interspecies competition, deficient oxygen availability, biofilm growth, the immune system action, oxidative stress, and antibiotic treatment, *P. aeruginosa* progressively generates phenotypes specially adapted to CF airways conditions [2,9,26,27]. The CF selection forces are evident when clinical isolates of *P. aeruginosa*

are frequently mucoid and highly resistant to antibiotics. Indeed, mucoid variants are rarely isolated from non-CF environments, suggesting the existence of specific CF selective pressure [2,23]. For this reason, *P. aeruginosa* conversion from non- to mucoid form is considered the hallmark of CF airway.

Figure 1. Time course of *P. aeruginosa* infection development. (a) Sputum colonization stage - *P. aeruginosa* equipped with full virulence factors enter in CF sputum; (b) Early infection stage—*P. aeruginosa*, which exhibit the environmental or wild-phenotypes species characteristics, starts its adaptation to CF environmental conditions; (c) Chronic infection stage—*P. aeruginosa* is full adapted to CF environment. At this stage, there is high phenotypic and genotypic diversity and formation of biofilms.



However, other phenotypic features of clonal variants adapted to CF airways have been frequently observed, including small colony variants (SCV), non-pigmented variants, increased antibiotic resistance, altered metabolic pathways, and attenuated virulence potential [3,22,27]. This phenotypic variation occurs for adaptation to the different niches in CF airways. The repeated occurrence of these particular phenotypic features and/or gene expression in chronic isolates, sampled from different patients and clinical settings, suggests the existence of a parallel evolution of *P. aeruginosa* in CF airways [28,29]. This topic is discussed in detail in Section 4.2.

The biofilm-lifestyle represents a reservoir of high phenotypic diversity and it is considered one of the most important adaptive mechanisms of *P. aeruginosa* within CF sputum (Figure 1c) [22,30,31]. Worlitzsch *et al.* (2002) [32] have shown that in the conductive zone, the region from the trachea to the terminal bronchiolus, *P. aeruginosa* grows mainly in biofilms, whereas very few bacteria are localized at the epithelial surface of the bronchi. Biofilms are microbial communities encased in self-produced matrix composed of exopolysaccharides, proteins and DNA [33–35]. Nowadays, biofilms are recognized as an important issue in human disease management due to their notoriously resistance, achieving 10- to 1000-fold higher tolerance to antimicrobial agents than corresponding planktonic bacteria [35,36]. Biofilm resistance has multifactorial nature resulting from the combination of several mechanisms, including restricted penetration of antimicrobials through the exopolysaccharide matrix, slow growth of bacteria within biofilms caused by nutrient and oxygen restriction, and accumulated metabolic wastes, and quorum-sensing (QS) molecules [37–40].

The limited penetration of antibiotics and immune defenses through the exopolysaccharide matrix is definitely a great contributor for their ineffective action and *P. aeruginosa* persistence. Alginate is the major component of CF biofilm matrix providing structure and protection to bacteria from the stressful environmental conditions of CF lungs. Augmented levels of alginate are generally observed in CF patients chronically infected and it is associated with poor prognosis because alginate triggers a vigorous antibody response [41,42].

Airway CF biofilms are genetic, proteomic and physiologic different of surface-attached biofilms formed, for instance, on indwelling devices (catheters, prostheses, pacemakers, stents), and medical and clinical equipment. Instead of the direct surface colonization, clearly observed in device-associated infections, bacteria in the CF lungs preferentially form multicellular clusters or macrocolonies within the sputum and not on the surface epithelium in the bronchi and non-respiratory bronchioles as initially supposed [31,32,43,44]. Additionally, the environment in which CF-associated biofilms are formed is considered to be microaerophilic or anaerobic. Bacteria enter and colonize CF sputum, consume oxygen via respiration, and generate steep oxygen gradients within the sputum [9,30,44]. The limited oxygen availability to potentially anaerobic environments in CF sputum was confirmed by direct *in situ* oxygen measurements using a microelectrode [32]. The oxygen-limited and anaerobic growth conditions significantly increase antibiotic resistance of biofilm-forming bacteria [45].

Until now, it is not clear what time bacteria after CF airway colonization switch to sessile lifestyle, but it is known that biofilm formation enables bacteria to successfully establish chronic infections. Presumably, *P. aeruginosa* form biofilms in response to stressful conditions including microaerophily and/or antibiotic treatments [23,46].

To switch from planktonic to biofilm mode of growth, bacteria undergo a number of complex physiological, metabolic, and phenotypic differentiations. For instance, biofilm-growing bacteria undertake specific changes in protein regulation, especially those related with proteins involved in resistance to oxidative damage, exopolysaccharide production, phospholipid synthesis, and membrane transport [47–49]. Global gene expression analyses of mature *P. aeruginosa* biofilms have revealed 1% of differential gene expression between the planktonic and biofilm mode of growth, with 0.5% of the genes being activated and about 0.5% being repressed [50]. Among the transcription factors, repression of flagellar and pili genes and stress response regulator genes, such as *rpoS*, hyperexpression of genes for ribosomal proteins and metabolism and transport functions were the most identified. Interestingly, in the same study, QS-regulated genes were not identified. QS is a cell-cell communication system used by bacteria to regulate gene expression in response to fluctuations in cell-population density and it has been reported to play a role in early and later stages of biofilm development. *P. aeruginosa* has two distinct QS systems, termed *las* and *rhl* [51,52]. The lack of *las* QS system allowed the formation of biofilms, however, does not allow them to achieve the mature stage. The *rhl* QS system has been reported as active in the early stages of biofilm development and its block may prevent biofilm formation [47,53]. Other regulatory systems can influence early stages of biofilm development, such as the global virulence regulator GacA [54], the catabolite repression control protein Crc [55], and the response regulator proteins

AlgR [56]. The blockage by mutation of those factors has demonstrated a significant decreased of biofilm formation.

Gene expression may vary during biofilm development, which means that there is a stage-specific temporal and spatial gene expression patterns. This is particular relevant concerning the resistance of mature biofilms to antimicrobial treatment. The biofilm-specific phenotype can trigger mechanisms responsible for antimicrobial resistance and persistence and consequently enhanced pathogenicity. *P. aeruginosa* genome sequencing have revealed that a mature biofilm can express several cluster genes encoding efflux pump involved in resistance to some antibiotics [50].

The great variability or heterogeneity of phenotypes included and developed within biofilms is certainly one of the major contributors for sessile bacteria recalcitrance that it is not observed in planktonic state [57–59]. Within biofilms, various heterogeneous environments exist as a result of the distinct levels of nutrients and oxygen availability and accumulated metabolic wastes that bacteria have to face and adapt in a process similar to CF airway adaptation [60]. This range of microniches with specific biological activities may somewhat be translated by the several distinct colony morphologies that biofilm-growing bacteria adopt when grown in a solid media. Such trait diversification profits the whole population, with diverse abilities to face environmental challenges, as long as bacteria coordinate with each other. Bacterial cooperation and differentiation is facilitated through the production and perception of QS small signaling molecules called autoinducers. This interbacterial communication is mediated by two types of molecules, *N*-acylhomoserine lactones (AHL) and 4-quinolones, allowing bacteria to perceive their density and regulate their gene expressions properly. For instance, up-regulating genes encoding virulence factors such as those related to the production of enzymes or toxins, optimizes the metabolic and behavioral activities of bacteria within the community [61,62].

Biofilm heterogeneity is also reflected in distinct antibiotic susceptibility profiles. Due to the different biofilm-cell physiological states, biofilms have typically a top-to-bottom decreasing susceptibility profile. Antibiotics are effective against the cells located in the top of the biofilm, generally in active state, in contrast to the middle and bottom zones, in which cells have reduced or even an absence of metabolic activity. Even when antibiotics reach the middle and/or bottom biofilm zones, the majority of them have no activity against dormant cells and, thus, are unsuccessful in biofilm eradication [39,60,63].

Planktonic *P. aeruginosa* cells are also found in CF sputum [31]. Due to alterations in CF environment, such as pH and oxygen and nutrients availability, biofilm-cells dispersion may occur [64]. The dispersal of biofilm population provides to *P. aeruginosa* an opportunity to colonize new zones or niches and, thus, perpetuate infection. In fact, dispersal events can be responsible for the acute exacerbations observed in chronic infections [46,58,65].

The whole adaptation process to CF airways can be accelerated by the emergence of mutator phenotypes (or hypermutable phenotypes) which have high mutation rates up to 1000-fold than non-mutator phenotypes [66–68]. In extreme selective conditions, such as those occurring in CF airways, this sophisticated mechanism improves the microevolution of *P. aeruginosa* accelerating its intraclonal diversification. The emergence of phenotypic variants and mutators can be intrinsic, relying on mutations (or recombinations) caused by defects on one of the several DNA repair or

error avoidance systems, combined, or not, with extrinsic or environmental factors, such as competition for different niches in a spatially heterogeneous environment as CF airways, and/or selection that favors any mutant as a better “fitter” to CF airways [67,69,70]. Mutators can also be stimulated by environmental factors, such as the presence of reactive oxygen species (ROS) generated from inflammatory responses [71]. ROS can trigger the generation of phenotypic variants damaging DNA and cause mutations in bacteria. Further, sub-inhibitory or sub-lethal concentrations of antibiotics can induce mutations and recombinations and, consequently, supporting the emergence of phenotypic variants and mutators [26,67,72]. The genes mainly affected are the antimutator genes *mutS*, *mutL*, and *uvrD* but it can be observed defects, as well in the genes *mutT*, *mutM*, and *mutY* [66,73,74].

The amount of mutators in biofilms is significantly higher than in planktonic state. This condition may explain why biofilm-associated bacteria exhibited enhanced antibiotic resistance, and frequently multidrug resistant, and high genetic diversity leading the emergence of diverse phenotypic variants [75,76].

The generation of various subclonal variants represents a huge biological advantage because it prepares the *P. aeruginosa* population for extreme and unpredictable stresses (insurance hypothesis) supporting the long-term survival of this pathogen [59,77]. Mutators achieve more quickly CF adaptation due to the expression of virulence traits, antibiotic resistance, metabolic functions, and increased ability to form biofilms, all these features representing a serious clinical problem [67,78,79]. In effect, mutators can increase the transcription of genes involved in the metabolism of fatty acids and amino acids crucial for obtaining energy in CF ecological niches where aerobic respiration is not possible [80]. On the other hand, mutators may have a reduced ability to survive in other distinct environments, indicating they can reach high levels of habitat- or niche-specialization spending their biological fitness [23,67]. During infection development non- and mutators coexist in CF airways, however, mutators prevail at chronic stage, which may be indicative that they have an adaptive advantage.

The combined action of all these sources of clonal diversification may achieve impressive levels of diversification, adaptation, and evolution, promoting the persistence of the bacterial populations in CF airways. Therefore, these sources should be intensively studied in order to understand the underlying mechanisms to further block them and combat the recalcitrant infections.

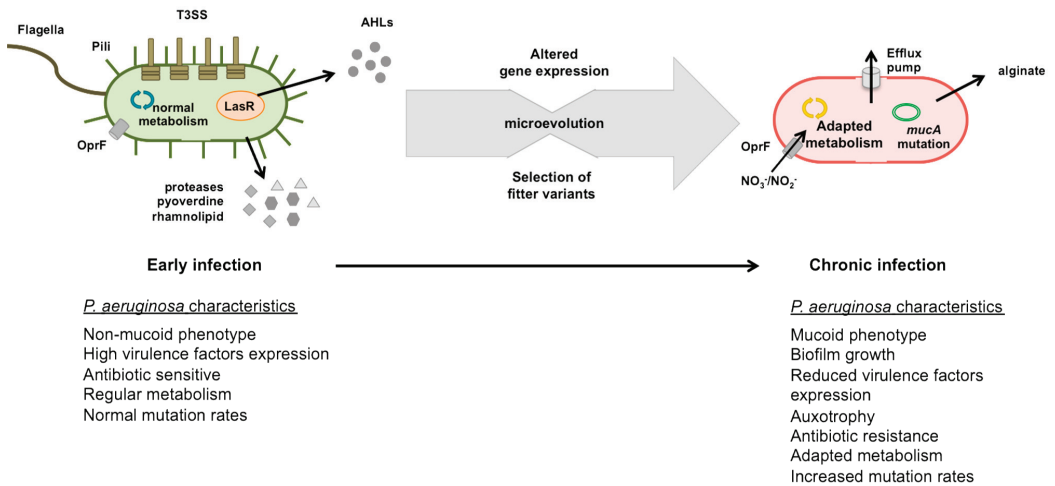
4. *P. aeruginosa* Evolution and Adaptation during Infection Development

The regular sampling of CF sputum has allowed performing a detailed characterization of *P. aeruginosa* over infection development through DNA sequencing and other approaches, such as transcriptomic, metabolomics and proteomic techniques. Therefore, it is now possible to start drawing an evolutionary trajectory of *P. aeruginosa* within CF airways.

During infection development genotypes and phenotypes differ markedly from those that initially colonized CF airways (Figure 2). Microbiological studies have reported changes in *P. aeruginosa* phenotypic and genetic traits, relevant in the context of bacterial pathogenesis, and different antibiotic resistance patterns along infection development, as well as after antibiotic treatments. Similar evolution and adaptation profiles were observed in distinct clonal lineages of

CF-adapted strains, suggesting that, in fact, there is a similar selective pressure in CF airways. This evolution and adaptation processes lead to the generation of several phenotypes varying in characteristics, such as colony morphology with distinct consistency, size, texture and color, the inactivation of QS, hypermutation, loss of the O-antigen components of the lipopolysaccharide (LPS), loss of motility, resistance to antibiotics, changes in nutritional requirements, and other virulence-associated traits [2,26,81,82]. In fact, some of those factors have been considered the hallmark of CF disease and can determine the infection stage, such as the conversion of *P. aeruginosa* to mucoid phenotype, loss of motility, and the emergence of SCV. However, many other characteristics have been described across all phenotypes isolated so far.

Figure 2. Representation of *P. aeruginosa* microevolution during infection in CF airways. At early stage of infection, *P. aeruginosa* is full equipped with cell-associated virulence factors, including flagella, pili, type 3 secretion systems (T3SS) and secreted virulence factors (e.g., proteases, pyoverdine, and rhamnolipid) and exhibit antibiotic sensitivity. At the chronic stage of infection, *P. aeruginosa* is fully adapted to CF environment and exhibits a variety of adaptations, including overproduction of alginate, loss of the implicated virulence factors for initial infection establishment, are resistant to antibiotics (expression of efflux pumps), and adapted metabolism. This microevolution occurs by the repeated interplay of mutation and selection.



Antibiotics have provided significant control of bacterial infections in CF airways, however, the occurrence of antibiotic resistance and the lack of new drugs or therapeutic strategies make imperative the identification of alternative targets for treatment. For understanding the mechanisms underlying bacterial adaptation to CF environment and the resistance to antibiotic treatments, an overall picture of the actual knowledge about the *P. aeruginosa* populations, resident in CF lungs, is needed.

The compilation of the phenotypic traits exhibited by bacteria according the infection stage is a hard task due to the lack of agreement on the definitions of early, intermediate, and chronic

colonization and infection stages. In this paper, the evolution, adaptation, and diversification profiles of *P. aeruginosa* were reviewed and compared agreed by the “European Consensus” that just considers two infection stages, early and chronic stages, according to the presence of *P. aeruginosa* being lower or higher than six months, respectively [83].

4.1. Early Infection

Most CF patients acquired pathogens mainly from environment, especially in clinical settings where CF patients remain for long periods of time. For this reason, respiratory infections associated to CF patients can be in somewhat considered nosocomial infections. As CF patients acquired environmental pathogens, early CF isolates exhibited identical microbial characteristics of their environmental or wild-phenotypes species (Figure 2) [21,84,85]. At the first colonization of CF airways, *P. aeruginosa* have to regulate properly its gene expression to quickly adapt to this new environment, including host immune defenses, antibiotics, and different substrate composition.

The bacterial characteristics among CF acute isolates significantly vary, however, there is a trend towards high virulence potential and cytotoxicity and lower frequency of mutators strains [86]. The expression of virulence factors, including cell-associated and secreted virulence factors, is considered to be fundamental at early stage for the success of infection establishment. For instance, (i) the increased production of pyoverdine, haemolysin, and phospholipase C; (ii) the augmented production of rhamnolipid, regulated by QS, helps biofilm formation that protects cells against oxidative stress, decreases liquid surface tension, due to its biosurfactant feature, and facilitates the access to nutrients within biofilms; (iii) the increased production of total protease that promotes mucoidy essential for long-term bacterial persistence; (iv) the swimming and twitching motilities; and (v) the expression of the type III secretion system that augments cell cytotoxicity potential and facilitates infection development [23,84,85,87,88].

Typically, *P. aeruginosa* exhibited a non-mucoid phenotype, sensibility to antibiotics and have low bacterial density in lungs in contrast to chronic infections [24,85]. Acute CF isolates produced AHL suggesting that QS circuit plays a role for *P. aeruginosa* pathogenesis at this stage of infection. Afterwards, QS seems be no longer needed and *lasR* mutants are frequently isolated. Mutator strains are not prevalent at this stage because they are not efficient to establish a primary infection [51,89].

At the early stage, eradication is still possible whether an antibiotic treatment was started as soon as possible. Otherwise, 20% of those first *P. aeruginosa* colonisations could become directly chronic infections and may persist up to the end of patient life [24,85]. Following *P. aeruginosa* eradication, it is common a new acquisition event with a different genotype or a re-colonization with the same genotype. Re-colonization with the same genotype may occur due to the persistence of the environmental source or due to the colonization of the upper airways, such as the paranasal sinuses [90,91]. Upper airways can function as reservoirs of pathogens, and interchange of *P. aeruginosa* can be possible. Colonization of the CF airways with mucoid strains is associated with an accelerated rate of decline in pulmonary function, however, there is some evidence that early acquisition of mucoid strains could be successfully achieved [24,92].

In summary, although the virulence potential of early CF isolates is higher than chronic isolates, they exhibited increased antibiotic sensitivity. Therefore, *P. aeruginosa* early detection and eradication are currently the main goal to avoid infection progression to chronic stage. Early infections are intensively treated with antimicrobial therapy resulting in *P. aeruginosa* eradication, at least temporal eradication in the majority of patients [24]. In cases of antimicrobial therapy failure, infection can shortly evolve to chronic infection. Identification of the CF patients who may evolve to chronic infections based on the acute bacterial characteristics is still not possible because of factors related to host-pathogen and pathogen-pathogen interactions may play a role and their impact is, thus far, unknown [86,93].

4.2. Chronic Infection

The continuous and selective pressure over the population leads to the emergence of diverse phenotypic and genetic variants specially adapted to CF airways. It has been observed, among chronic *P. aeruginosa* isolates, that there are alterations in colony morphology, namely the conversion to the mucoid morphotype, to SCV and non-pigmented variants, changes in surface antigens, lack of some virulence factors expression, increased antibiotic resistance, overproduction of exopolysaccharides, and modulation of microaerobic and anaerobic metabolic pathways (Figure 2). These alterations suggest a survival strategy to switch off or, at least, to reduce the expression of some traditional virulence factors. In fact, this *P. aeruginosa* strategy consists in saving or reducing energy costs with virulence factors expression in favor of alternative metabolic pathways crucial at this stage.

The repeated occurrence of these phenotypic features in chronic isolates of *P. aeruginosa* indicates that they may be a result of parallel evolution, which means that related microorganisms develop the same adaptive features in identical, but independent, environments [28]. Several transcriptomic studies have profiled isolates of *P. aeruginosa* in attempt to find the common route towards the chronic phenotype and the mechanisms underlying such a route. Longitudinal studies using transcriptomic approaches have provided relevant information regarding the genetic changes undergone by *P. aeruginosa* and allowed comparing the expression of specific set of genes among patients in different periods of time. Gene expression changes in multidrug efflux pumps and regulators of quorum sensing and alginate biosynthesis have been identified, being the two latter hotspots of mutations [21,28,94,95].

In the scope of genomic evolution in chronic CF lung infection, it should be highlighted that the notorious work performed by the Copenhagen and Hanover clinics, which had regularly collected *P. aeruginosa* from the CF lungs of all their patients in the 1970s and 1980s, and performed the genome sequencing of the isolates. They started their investigation with the most prevalent clones, the C and PA14, and observed that both clones convert their phenotype becoming deficient in the LPS O-antigen, with impaired motility and decreased siderophores secretion, as well as in other virulence factors expression and remaining non-mucoid [96]. Those isolates later collected just exhibited impaired competitive growth. These cases demonstrated that the evolutionary transition might be through the additive effects of various mutations. However, a single loss-of-function

mutation can induce dramatic changes in *P. aeruginosa*, as those observed through the mucoid variant, due to the pleiotropic effects of *mucA* mutation [2,21].

Mucoid colony morphology results from alginate overproduction, absence of flagellin and pilin and expression of other virulence factors. Within the mucoid form, *P. aeruginosa* is more difficult to eradicate because it is highly resistance to antibiotics, as well as to the actions of host immune defenses, for instance, to phagocytosis, mediated by macrophages and neutrophils and to antibodies opsonization [3,30,97]. Alginate overproduction is on the basis of such protection and resistance. Alginate promotes *P. aeruginosa* encapsulation and biofilm formation protecting sessile bacteria from the action of ROS, antibiotics and host immune defenses persisting in CF lungs [22,23]. Because mucoid *P. aeruginosa* raise a vigorous antibody response, its presence contributes to tissue damage, decreased lung function, and a decline in health [41,42]. The genetic mechanisms underlying *P. aeruginosa* transition to the mucoid form have been intensively studied and conversion is mainly caused by mutational inactivation of the *mucA* gene and rarely of *mucB* or *mucD* genes [98,99]. *mucA* gene encodes a cytoplasmatic membrane bound protein that acts as anti- σ -factor, σ^{22} , limiting the expression of the *algD* operon required for alginate synthesis. MucA binds to AlgT (also termed AlgU) that negatively controls the transcription of the *algD* gene. Inactivation of *mucA* results in upregulation of AlgT and production of alginate [2,100,101]. In fact, σ^{22} can also activate the transcription of several other genes related to virulence factor expression and to stress response, including heat shock, and osmotic and oxidative stress [21,102]. Additionally, it can repress the expression of type III secretion system (T3SS) genes through activation of AlgU that activates the regulatory genes *algP*, *algQ*, *algB*, and *algR*. AlgR, a global regulator, affects the expression of multiple genes including T3SS [103]. This suggests an impressive coordination of two high-cost energy systems in order to bacteria persist in CF airways.

Although mucoid phenotype is very successful at chronic infection stage, non- and mucoid phenotypes can coexist [31]. Non-mucoid isolates can occur from persistence of *P. aeruginosa* wild-type or re-conversion of mucoid phenotypes (revertants). Mucoid phenotypes can revert to non-mucoid form in the absence of *in vitro* selective pressure or through secondary mutations. Non-mucoid phenotypes can also carry *mucA* mutation, suggesting that mutation occurred when selective pressure occurs and when its vanished secondary mutation takes place [21,104]. This suggests that the production of alginate represents a high-energy cost and, thus, its unstable feature. At this stage, non-mucoid phenotypes have its alginate production at minimal levels [52].

The conversion to mucoid phenotype also promotes the biofilm mode of growth. The presence of biofilms is a key factor for the persistence of infection in CF airways. Biofilm-cell differentiation and dispersal events contribute to the generation of higher diversity that consequently increases the ability of *P. aeruginosa* to colonize new niches in CF airways, thus, perpetuating infection [46,58,59].

Another variant frequently isolated from chronic CF lung infections are the SCV. SCV designation comes from their small-colony size, typically 1–3 mm after 24–48 h of growth on agar media [105]. SCV are normally hyperpilated, hyperadherent, excellent biofilm formers, and exhibit autoaggregative behavior and increased twitching motility [106–109]. In addition, SCV display augmented resistance to several classes of antibiotics, notably to aminoglycosides,

contributing to their persistence in CF airways and poor lung function. SCV are generally selected after prolonged antibiotic treatments [105,110]. In contrast with the mucoid phenotype, the mutations that arise in SCV appear to be very diverse and a challenge for the understanding of the underlying molecular mechanisms [111]. This phenotype may arise from the increased expression of the *pel* and *psl* polysaccharide gene loci and elevated intracellular c-di-GMP levels that enhance the ability to form biofilms, motility, and the expression of the type 3 secretion system, persisting, thus, more efficiently in the CF airways [109,111]. Until now, SCV were mostly studied regarding *Staphylococcus aureus* but, currently, it has been equally assumed that *P. aeruginosa* SCV is, as well, a cause of infection persistence [35,112–114].

Other colony morphologies have been isolated from CF airways typically exhibiting rough texture due to alteration of the lipid A moiety of LPS. Those variants contain a few, short, or no O side chains and exhibited augmented proinflammatory activity [23].

In chronic infections, CF isolates typically exhibited impaired motility, namely swimming and twitching, due to the absence of flagella and pili, respectively. Lacking flagella (e.g., *fliC* mutant), *P. aeruginosa* isolates are hardly phagocytosed by alveolar macrophages and neutrophils helping to evade the host immune defenses, allowing its persistence in CF airways [23,52]. Moreover, *P. aeruginosa* lives in this chronic stage in biofilm-growth mode in which cells downregulate flagellum and type IV pili since they are no longer needed to move across sputum and along epithelial cell surfaces [115]. Nonpiliation may arise from mutations of *pilB*, encoding an ATPase needed for the extension and retraction of pili, or defects in *pilQ* gene, required to extrude the pilus through the bacterial outer membrane [116]. Nevertheless, the majority of CF isolates exhibited *rpoN* mutations that provoke the loss of both pili and flagella [117].

Chronic CF isolates show other attenuated virulence factors such as reduced production of AHL, proteases, phospholipase C, loss of pyoverdine, pyocyanin, and elastase and decreased cytotoxicity potential, due to the switching off of the T3SS. These alterations reduce the efficacy of the immune system to recognize *P. aeruginosa* helping, thus, its persistence in CF airways [23,52].

Chronic *P. aeruginosa* isolates are commonly *lasR* mutants. *lasR* gene encodes QS transcriptional regulator LasR and its downregulation may explain the reduced or absent production of AHL at this infection stage, the autolysis and the iridescent gloss of *P. aeruginosa* colonies, the growth advantage on amino acids and decreased virulence potential [94,118]. In addition, *lasR* mutants can use nitrate (NO_3^-) and nitrite (NO_2^-) as the terminal acceptor of electrons allowing *P. aeruginosa* growth in anaerobic niches. The loss of social and cooperative behavior may confer an adaptive advantage since the production of QS signal molecules, such as *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), *N*-butanoyl-L-homoserine lactone (C4-HSL), 2-heptyl-4(1H)-quinolone (C7-HHQ), and 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS), are costly. Avoiding these costs, *P. aeruginosa* can ensure its persistence for the long-term [52,81]. QS seems just contribute to *P. aeruginosa* pathogenesis at colonization or acute stages [81,119].

To survive and adapt to CF airways, *P. aeruginosa* has, as well, to adapt its metabolic pathways. In fact, those alterations are also considered a marker of the chronic stage. The generation of energy is mainly based on oxidative substrate catabolism, however, *P. aeruginosa* is able to use alternative electron acceptors. The carbon metabolism of *P. aeruginosa* is mediated by catabolite

repression control, which determines the catabolism of substrates in a preferred order. Short-chain fatty acids, amino acids, and polyamines are generally the preferred carbon sources and sugars the less favored [120].

CF sputum contains high amount of mucin, DNA, lipids, amino acids, and proteins that *P. aeruginosa* can uptake. Several studies have reported that peptides, amino acids, and fatty acids belonging to host defenses, such as prostaglandins and phosphatidylcholine, supports *P. aeruginosa* growth in CF airways [7,80]. The increased availability of those components is highlighted by the frequent isolation of auxotrophic variants for different amino acids, however the adaptive advantage of those variants in CF airways is unclear thus far [121]. Arginine and methionine are the most common auxotrophisms detected among CF isolates [122–124]. Auxotrophic variants may be more common than actually reported because those variants may be less cultivable *in vitro* and, consequently, under-estimated.

As mentioned earlier, the distinct oxygen availability in CF sputum represents a challenge for *P. aeruginosa*, which undergoes metabolic and physiologic changes with a high impact on antibiotic treatments. Along chronic infection progress, *P. aeruginosa* can face aerobic, microaerophilic, and anaerobic zones within the CF sputum and different enzymes, transporters, and regulators for different metabolic pathways are up-regulated to achieve this adaptation [86]. *P. aeruginosa* preferentially uses oxygen as terminal electron acceptor to obtain maximum energy. However, *P. aeruginosa* is able as well to survive and growth in hypoxic and anoxic CF niches adapting its metabolic pathways. Under anaerobic conditions, *P. aeruginosa* can obtain energy to grow from the denitrification or fermentation of arginine [30,80]. Denitrification or anaerobic respiration allows the detoxification of NO, generated during infection development. The outer membrane protein, OprF, represents a crucial factor in anaerobic metabolism since it allows the permeation of the ions $\text{NO}_3^-/\text{NO}_2^-$ fundamental to perform denitrification [30,44,80]. In niches where oxygen and N-oxides are unavailable, but amino acids are in high amounts, *P. aeruginosa* can use fermentation of arginine, converting it into ornithine [80]. In cases of arginine limitation, *P. aeruginosa* can still convert pyruvate into acetate and, thus, obtain energy. In this way, anaerobic biofilms can be formed and support *P. aeruginosa* survival, growth, and persistence in CF airways. Anaerobic environments increase *P. aeruginosa* antibiotic tolerance and the robustness of biofilms through the increased production of alginate, typically via mutation in *algT/algU* [45,125–127]. Consequently, CF mucoid strains, that are alginate producers, are selected at this chronic stage. Despite all these findings about the metabolic pathways used by *P. aeruginosa* during chronic infections, information about the regulation and the mechanisms underlying each metabolic pathway and the specific effects on virulence, antibiotic resistance and persistence in CF lungs is still scarce. Certainly, the understanding of those mechanisms could help new therapeutic solutions to arise.

The presence of mutators within the populations are characteristic of chronic infections, considered a virulence determinant of *P. aeruginosa* and often associated with parallel occurrence of subpopulations with distinct phenotypic characteristics [66]. Mutators ensure *P. aeruginosa* survival against various CF stress conditions and other unpredictable stress factors, and being, moreover, a key factor in the development of multi-antimicrobial resistance [73]. At the chronic

stage, hypermutability increases, also due to the presence of biofilms, in which the frequency of mutators is higher than the free-living mode of growth [75,128]. The transcriptome comparison of a non- and mutator revealed significantly transcriptional changes among them. In fact, it was observed that mutators exhibited increased levels of genes involved in amino acid and fatty acid metabolism [80].

Chronic infections are usually punctuated with acute exacerbations in which *P. aeruginosa* may regain the increased levels of acute virulence of early stages, suggesting that the expression of some virulence factors can be reversible [65].

Despite the intensive and long antibiotic treatment, chronic infections of *P. aeruginosa* are rarely eradicated due to the occurrence of antibiotic resistance. It is frequently observed β -lactam-resistant *P. aeruginosa* phenotypes, due to the derepression of chromosomal β -lactamase [129], as well as ciprofloxacin [130], colistin [131], and tobramycin-resistant phenotypes, and even multi-drug resistance [132]. The main reasons for such increased antibiotic resistance is the biofilm-growth style and the presence of mutators [73,133–136].

In summary, the exhibition of certain characteristics, including alginate overproduction (mucoid phenotype), slow growth, alternative metabolic pathways, antibiotic resistance, and loss of virulence factors expression, is currently considered a chronic phenotypic profile and the end-point result of *P. aeruginosa* evolution in CF airways. *P. aeruginosa* clearly adopts a strategy aiming to reduce its energy costs in favor of activation of other biological pathways that ensure its long-term persistence. The actual evolutionary “model” of *P. aeruginosa* within CF airways consists in an initial and rapid adaptation period dominated by positive selection and adaptive mutations, followed by a period with minor phenotypic changes dominated by negative selection and fewer adaptive mutations [2]. This evolutionary process ends with an advent of a lineage of highly adapted bacteria with impressive ability to persist in CF lungs for long-term. Despite the assumption of parallel evolution to CF-well adapted phenotypes and the limited number of adaptive features, it is important to highlight that the actual evolutionary route towards a common profile among different patients is still not well understood. In fact, genomic and transcriptomic studies have just begun tracking *P. aeruginosa* evolution.

5. Conclusions

The study of the adaptation process and dynamical evolution of *P. aeruginosa* within CF lungs, and its impact on bacterial pathogenicity and virulence, is currently a topic of most importance in disease management. In this paper, the most common evolutionary profile of *P. aeruginosa* reported by researchers and clinicians were reviewed, however, other evolutionary, phenotypic, and genotypic profiles can be found in different demographic locations, clinics, and patients.

Longitudinal studies of clonal variants of different CF patients have tried to identify a common “expression signature” of *P. aeruginosa* over time. Genome sequencing, transcriptomic, and proteomic analysis have advanced the understanding of *P. aeruginosa* evolution, epidemiology and response to CF stress conditions, however there is still limited information about such “expression signature”. During adaptation, *P. aeruginosa* undergoes complex, structural and dynamic changes over the time. CF isolates from acute infections differs poorly from non-CF environment in

contrast to the isolates from chronic infections that have been interpreted as the result of *P. aeruginosa* adaptation to CF airways. Depending on the early antibiotic treatment, infection, sooner or later, will evolve to chronic infection. The CF lung adaptation of *P. aeruginosa* is characterized by the transition from an acute environmental pathogen to a chronic CF-well-adapted pathogen, and the emergence of a phenotypically heterogeneous population. To establish chronic infections, *P. aeruginosa* loses some of its virulence potential (production of enzymes and toxins and lack of QS), slows down its growth rate, increases its antibiotic resistance (often multi-drug resistance), and/or reduces the stimulation of the immune system mainly due to a switch to the biofilm mode of growth, favored by overproduction of alginate. Typically, chronic *P. aeruginosa* isolates exhibited mucoid phenotype due to the overproduction of alginate and lack of flagella and pili. The presence of mutators in CF resident population is not strictly necessary to achieve adaptation, however, it represents a very large biological advantage in contrast with irreversible and accumulative mutations. Diversification of the metabolic pathways plays a fundamental role in the establishment of chronic infections in CF airways. In effect, *P. aeruginosa* can grow in microaerophilic and anaerobic zones by adjusting its metabolism. Denitrification, arginine fermentation and consumption of fatty acids are alternatives pathways to survive and growth in CF.

Future investigations should address the mechanisms underlying *P. aeruginosa* adaptation to CF airways to better understand them and to help design new therapeutic strategies. Studies about *P. aeruginosa* pathogenesis at early stages should be, especially, investigated more as the better way to avoid chronic infections is not allow their progression.

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Author Contributions

Ana Margarida Sousa and Maria Olivia were all involved in the writing and editing of this manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Antibiotic Resistance Related to Biofilm Formation in *Klebsiella pneumoniae*

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Abstract: The Gram-negative opportunistic pathogen, *Klebsiella pneumoniae*, is responsible for causing a spectrum of community-acquired and nosocomial infections and typically infects patients with indwelling medical devices, especially urinary catheters, on which this microorganism is able to grow as a biofilm. The increasingly frequent acquisition of antibiotic resistance by *K. pneumoniae* strains has given rise to a global spread of this multidrug-resistant pathogen, mostly at the hospital level. This scenario is exacerbated when it is noted that intrinsic resistance to antimicrobial agents dramatically increases when *K. pneumoniae* strains grow as a biofilm. This review will summarize the findings about the antibiotic resistance related to biofilm formation in *K. pneumoniae*.

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1. Introduction

Klebsiella pneumoniae was isolated for the first time in 1882 by Friedlander from the lungs of patients who died after pneumonia. This encapsulated bacterium, initially named Friedlander's bacillus, was renamed *Klebsiella* in 1886. Later, it was described as a saprophyte microorganism, not only colonizing the human gastrointestinal tract, skin and nasopharynx, but also able to cause urinary and biliary tract infections, osteomyelitis and bacteremia [1–3].

The virulence factors playing an important role in the severity of *K. pneumoniae* infections are capsular polysaccharides, type 1 and type 3 pili, factors involved in aggregative adhesions and siderophores [3–6], with those studied in greater depth being capsular polysaccharides and type 1 and type 3 pili.

Capsules, whose subunits can be classified into 77 serological types [7], are essential to the virulence of *Klebsiella* [8]. The capsular material, forming fibrillous structures that cover the bacterial surface [9], protects the bacterium from phagocytosis, on the one hand [10], and prevents killing of the bacteria by bactericidal serum factors, on the other [11].

Type 1 and type 3 pili [12] (otherwise known as fimbriae), instead, are non-flagellar, filamentous fimbrial adhesins, often detected on the bacterial surface, that consist of polymeric globular protein subunits (pilin) [13]. On the basis of their ability to agglutinate erythrocytes and depending on whether the reaction is inhibited by D-mannose, these adhesins are designated as mannose-sensitive (MSHA) or mannose-resistant hemagglutinins (MRHA), respectively [14]. Type 1 fimbriae are encoded by an operon (*fim*) containing all of the genes required for the fimbrial structure and assembly, with assembly occurring via the chaperone-usheer pathway [15]. Type 1 fimbriae in *K. pneumoniae* are regulated via phase variation in a similar way to the regulation of

type 1 fimbriae in *E. coli* [16,17]. Alcántar-Curiel and colleagues demonstrated that the *fim* operon was found in 100% of 69 *K. pneumoniae* isolates and that type 1 fimbriae were detected in 96% of these strains [18]. Conversely, the type 3 fimbriae are encoded by the *mrk* operon and are predicted to be assembled via a chaperone-usher pathway, too, with the *mrk* gene cluster being chromosome or plasmid borne [19–21].

Due to its high pathogenicity, *K. pneumoniae*, in the pre-antibiotic era, was considered as an important causative agent of community-acquired (CA) infections, including a severe form of pneumonia, especially in alcoholics and in diabetic patients. In recent years, while CA pneumonia due to this pathogen has become rare, novel manifestations of *K. pneumoniae* CA infections, including liver abscess complicated by endophthalmitis, different metastatic infections [22], often caused by highly virulent strains of specific serotypes, such as K1 [23], as well as urinary tract infections [24], have been described.

The greater adhesiveness and presumably also the invasiveness of strains may play an important role in the recurrent infections, *K. pneumoniae* strains being able to persist despite appropriate antibiotic treatment [25]. However, unlike the adhesion ability, the invasive capacity of *K. pneumoniae* to cause liver infections [26,27] and urinary tract infections [28] is still controversial and requires further study.

In contrast, starting from the early 1970s, *K. pneumoniae* epidemiology and its spectrum of infections significantly changed when this microorganism was established in the hospital environment and became a leading cause of nosocomial infections, particularly in developed Western countries. In fact, its considerable efficiency of colonization, accompanied by acquired resistance to antibiotics, has enabled *K. pneumoniae* to persist and spread rapidly in healthcare settings, the most common healthcare-associated infections caused by this agent involving the urinary tract, wounds, lungs, abdominal cavity, intra-vascular devices, surgical sites, soft tissues and subsequent bacteremia [3,29–31].

Klebsiella is second only to *Escherichia coli* in nosocomial Gram-negative bacteremia [32], as well as in urinary tract infections (UTIs), affecting catheterized patients (16% and 70%, respectively) [33]. In fact, *K. pneumoniae* has been reported as a prominent cause of infections in individuals with indwelling urinary catheters [34,35].

Of interest, a high incidence of *K. pneumoniae* in UTIs (from 6% to 17%) was reported in previous studies carried out in specific groups of patients at risk, e.g., patients with diabetes mellitus or with neuropathic bladders [36,37].

As concerns the bacteremia associated with intravascular catheters, an epidemiological study on bloodstream infections carried out in Israel revealed that *Staphylococcus aureus* was the most common species (30%), followed by *K. pneumoniae* (10%) [38].

In general, a cohort study indicated that the majority of infections associated with different medical devices, including both urinary and intravascular catheters, was caused by *K. pneumoniae* followed by staphylococcal biofilms, and a high percentage (about 90%) of biofilm-producing bacterial isolates causing infection were multidrug resistant [39].

In 2013, the incidence of *K. pneumoniae* clinical infections was estimated in the United States to be higher in long-term acute care hospitals, compared to short-stay hospital intensive care units [40].

In a prospective study on hospital-acquired infections carried out in Rome in the period January, 2002–December, 2004, *K. pneumoniae* was reported as the second most frequent Gram-negative species (11%) after *Pseudomonas* (25%) [41]. In a countrywide cross-sectional survey carried out in collaboration with twenty-five large clinical microbiology laboratories from 23 Italian cities, Klebsiella pneumoniae carbapenemase (KPC)-producing *K. pneumoniae* (KPC-KP) were revealed to majorly contribute to the epidemic dissemination of carbapenem-resistant *Enterobacteriaceae*, their spreading being mostly sustained by strains of clonal complex 258 (ST-258 producing KPC-2 or KPC-3 and ST-512 producing KPC-3) [42].

In a recent meta-analysis covering studies (2000–2010) on Gram-negative wound infections in hospitalized adult burn patients, *K. pneumoniae* has been reported as one of the most common Gram-negative pathogens, after *P. aeruginosa* [43].

2. Antibiotic Resistance

Although *K. pneumoniae* possesses only moderate amounts of chromosomal penicillinases, it is a well-known “collector” of multidrug resistance plasmids that commonly encoded resistance to aminoglycosides, till the end of 1980s, while, later, encoding extended-spectrum β -lactamases (ESBLs), mostly Temoniera (TEMs) and Sulfhydryl variable (SHVs) active against last generation cephalosporins, as well as a variety of genes conferring resistance to drugs other than β -lactams [3,44].

The acquisition of these plasmids and the occurrence of chromosomal mutations that confer resistance to fluoroquinolones often makes the treatment of *K. pneumoniae* healthcare-associated infections possible only by using carbapenems as “last-line of defense” antibiotics [45–47].

Unfortunately, from the early 2000s, multidrug-resistant (MDR) *K. pneumoniae* strains started to produce also “carbapenemases” encoded by transmissible plasmids and rapidly disseminated within both acute hospitals and long-term care facilities. Later, other enterobacterial species, including *E. coli*, acquired carbapenemase genes, thus suggesting that *K. pneumoniae* may have acted as a pool of β -lactamases [48].

Over the past decade, carbapenemases of Classes A, B and D, which are β -lactamases able to efficiently hydrolyze penicillins, cephalosporins, monobactams, carbapenems and β -lactamase inhibitors, have progressively disseminated among *Enterobacteriaceae* [49]. The Class A KPCs, firstly discovered in the USA in 1996, are the most worrying carbapenemases for their spreading across countries and continents, even if their expansion is dependent on the geographical area [50,51]. Furthermore, the zinc-dependent Class B metallo- β -lactamases (M β LS), mainly represented by the Verona integron-encoded metallo- β -lactamase (VIM), imipenemase metallo- β -lactamase (IMP) and New Delhi metallo- β -lactamase (NDM) types, are encoded on highly transmissible plasmids that spread rapidly between bacteria, rather than relying on clonal proliferation. In particular, NDM-1, originated in Asia, was found in almost every continent within one year from its emergence in India [52]. The picture is completed by the plasmid-expressed Class D carbapenemases of the oxacillinase-48 (OXA-48) type [48,53–55].

KPC enzyme-producing *K. pneumoniae* is generally susceptible to few antibiotics, and it is associated with a high mortality rate among patients with bloodstream infections. In fact, many of

these strains are susceptible to colistin, tigecycline and one or more aminoglycosides, but some of them are resistant even to these drugs [56].

3. Biofilm

K. pneumoniae was reported to be able to grow *in vitro* as a biofilm since the end of the 1980s [57], but clear evidence of an *in vivo* biofilm was provided only in 1992 by Reid and coworkers, who investigated by scanning electron microscopy some bladder epithelial cells of a spinal cord injured patient with an asymptomatic urinary tract infection caused by *K. pneumoniae* [58].

Later, *in vitro* studies have demonstrated that about 40% of *K. pneumoniae* isolated not only from urine, but also from sputum, blood and wound swabs, were able to produce biofilm [59], as well as that about 63% of *K. pneumoniae* isolates from urine samples of catheterized patients suffering from UTIs were positive for *in vitro* biofilm production [33]. Recently, also a high rate of *K. pneumoniae* strains isolated from endotracheal tubes (ETT) of patients affected by ventilator-associated pneumonia (VAP) were reported to be able to form an *in vitro* biofilm [39].

Biofilm formation on abiotic surfaces was shown to be more consistent at 40 °C than 35 °C, using atomic force and high-vacuum SEM [60]. The ability of *K. pneumoniae* clinical strains to adhere and form biofilm *in vitro* was recently investigated by field emission scanning electron microscopy (FESEM) [61] and by confocal laser scanning microscopy (Figure 1).

3.1. Virulence and Biofilm Formation

Type 1 or type 3 fimbriae, as well as the capsule and the LPS are the virulence factors mostly involved in the ability of *K. pneumoniae* to grow as biofilm.

Type 3 fimbriae have been demonstrated to be the major appendages that mediate the formation of biofilms on biotic and abiotic surfaces and the attachment to endothelial and bladder epithelial cell lines [62–67]. In particular, growth of *K. pneumoniae* on abiotic surfaces is facilitated by the MrkA type 3 fimbrial protein, whereas growth on surfaces coated with a human extracellular matrix (HECM) requires the presence of the type 3 fimbrial adhesin MrkD [62,64].

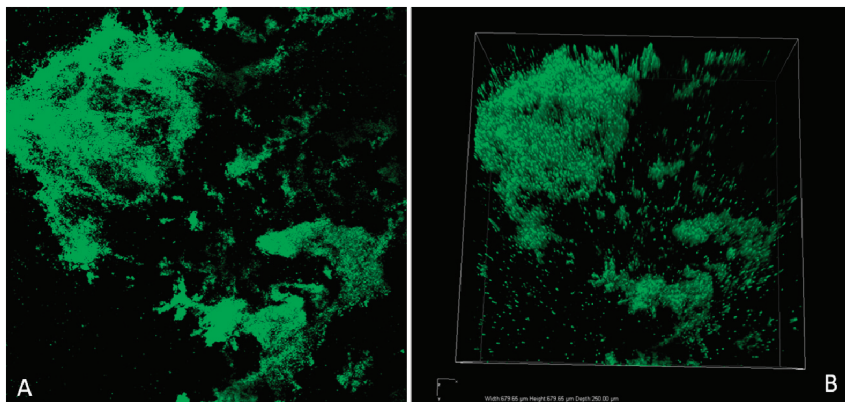
Recent investigations have verified that type 3 fimbrial gene expression is regulated, at least in part, by the intracellular levels of cyclic di-GMP [68].

Type 3 fimbriae have been confirmed by Murphy and colleagues (2013) to be a very important colonization factor in biofilm-mediated infections associated with catheter-associated urinary tract infections (CAUTIs) by obtaining mutants lacking the ability to produce type 1 or type 3 fimbriae or a combined double mutant. These mutants were impaired in colonization and had subsequent persistence under specific experimental conditions [69].

Alcántar-Curiel and colleagues demonstrated that, among the 69 examined *K. pneumoniae* isolates, 55 were able to produce biofilm and all of them contained *mrkA*, but only 57% of them produced MR/K fimbriae [18]. The same group also revealed that 96% of 69 *K. pneumoniae* isolates harbored the *ecpABCDE* operon homolog of the operon encoding the *E. coli* adhesive structure common pilus (ECP), with 94% of them producing ECP during adhesion to cultured

epithelial cells and 8% during the formation of biofilms on glass. These results suggest that ECP also seems to be required for biofilm formation, at least *in vitro* [18].

Figure 1. Bidimensional (A) and three-dimensional (B) images of a *K. pneumoniae in vitro* biofilm obtained by Confocal Laser Scanning Microscopy (CLSM) on different areas of a biofilm-covered glass coverslip. SYTO ® 9 green fluorescent nucleic acid stain has been used to detect both live and dead bacteria (Life Technologies, Monza (MB), Italy).



Regarding the involvement of capsule and LPS in *K. pneumoniae* biofilm formation, it has been proven that both of them contribute to the structure of biofilm communities of *K. pneumoniae*. In fact, gene disruption and microscopic analyses showed that LPS is involved in the initial adhesion on abiotic surfaces and that the capsule is necessary for a proper initial coverage of substratum and construction of mature biofilm architecture [70].

Furthermore, *treC* and *sugE* genes have been demonstrated to affect biofilm formation by modulating CPS production [71].

However, conflicting results on the involvement of CPS production in biofilm formation arise from a paper of Huang and coworkers (2014). In fact, the authors found that two knockout mutants, the first one with the entire gene cluster responsible for biosynthesis of the extracellular polysaccharide capsule deleted and the other one with the capsule export subsystem deleted, have lower amounts of capsule, but produce greater amounts of biofilm [72].

3.2. Involvement in Mixed Biofilms

The first investigation of mixed microbial populations including *K. pneumoniae* dates back to 1991. Siebel and colleagues demonstrated the ability of *K. pneumoniae* to form *in vitro* a dual-species biofilm together with *P. aeruginosa*. Authors determined that, although the *K. pneumoniae* specific cellular growth rate was five times that of *P. aeruginosa*, it did not dominate the microbial population, with results indicating that neither the specific cellular product formation rate nor the glucose-oxygen stoichiometric ratio of *K. pneumoniae* or *P. aeruginosa* when grown together were affected by the presence of the other species [73].

Later, *K. pneumoniae* was shown to form a biofilm more successfully in a mixture than in isolation with an increased resistance to disinfection [74].

In the same year, Stickler and colleagues, by using a model of a catheterized bladder, firstly investigated the possible role of *K. pneumoniae* and other uropathogens in the development of crystalline biofilm on catheter surfaces, demonstrating that this microorganism is not able to raise the urinary pH and, thus, to contribute to the crystalline biofilm formation [75].

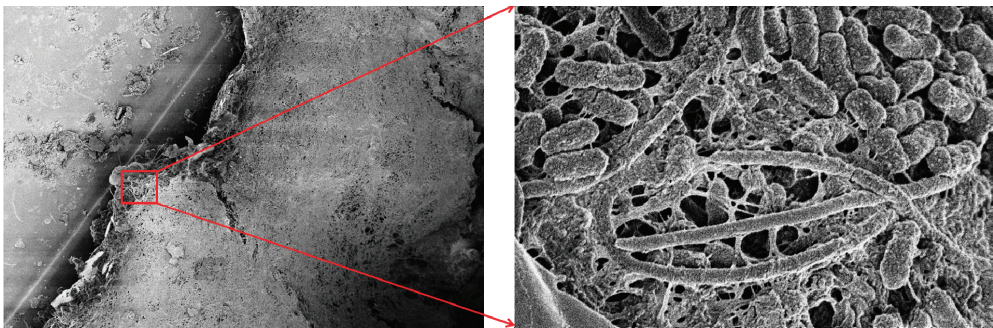
In 2007, the same research team analyzed 106 biofilms samples developed on urinary catheters, finding that *K. pneumoniae* was able to form a mixed biofilm with *Proteus mirabilis*, when *E. coli*, *Morganella morganii* or *Enterobacter cloacae* were also present [76].

The ability of *K. pneumoniae* to form a mixed-species biofilm *in vitro* with *P. aeruginosa* and *Pseudomonas protegens* was confirmed by adding a fluorescent tag to each species in order to determine the abundance and spatial localization of each of them within the biofilm. The development of the mixed-species biofilm was delayed 1–2 days compared with the single-species biofilms, and the composition and spatial organization of the mixed-species biofilm changed along the flow cell channel. Furthermore, the mixed-species biofilm resulted in being more resistant to sodium dodecyl sulfate and tobramycin with respect to the single-species biofilms [77].

Recent investigations on 35 ETTs obtained from 26 neonates with mechanical ventilation demonstrated that *K. pneumoniae*, together with *Pseudomonas* and *Streptococcus*, was the most common bacteria isolated from ETT-bacterial biofilms, and it was hypothesized that there were interactions among these species in the biofilm [78].

Our recent findings demonstrated that *K. pneumoniae* is able to form a multi-species biofilm together with *Candida albicans* within a urinary catheter removed from a patient hospitalized at the neuromotor rehabilitation hospital, Fondazione Santa Lucia in Rome, Italy (Figure 2).

Figure 2. FESEM micrographs (**a** = 2000×; **b** = 25,000×) of a polymicrobial biofilm grown in the lumen of a urinary catheter removed from a patient recovered at the research hospital for neuromotor rehabilitation, Fondazione Santa Lucia in Rome. The species identified by culture methods were *Klebsiella pneumoniae* and *Candida albicans*.



4. Antibiotic Resistance of Biofilm-Growing Strains

The response of *K. pneumoniae* biofilm to different antimicrobial agents has been investigated in several studies in the last decade.

One of the first studies that addressed the issue of the penetration of antimicrobials through *K. pneumoniae* biofilm was in 2000, when Anderl and co-workers used an *in vitro* model system to evaluate the effect of ampicillin and ciprofloxacin on *K. pneumoniae* biofilm developed on microporous membranes with agar nutrient medium. *K. pneumoniae* biofilms resisted killing during prolonged exposure to both antibiotics. The authors directly measured the antibiotics' diffusion, demonstrating that ampicillin did not penetrate wild-type *K. pneumoniae* biofilms, whereas ciprofloxacin and a nonreactive tracer (chloride ion) penetrated the biofilms quickly. Ampicillin was able to penetrate biofilms only when formed by a β -lactamase-deficient mutant, thus demonstrating that the increased resistance of both wild-type and mutant *K. pneumoniae* biofilm to ampicillin and ciprofloxacin could not be attributed to slow diffusion [79].

The penetration of ampicillin and ciprofloxacin through *K. pneumoniae* biofilms was then confirmed using transmission electron microscopy (TEM). The authors visualized cells in biofilm after antibiotic exposure, identifying those regions of the biofilm contained on an agar plate that were able to reach 10-fold minimum inhibitory concentration (MIC) with ciprofloxacin or ampicillin [80].

This topic was further addressed one year later by Andrei and colleagues, assuming that the already observed mechanism might be due to the presence, in internal zones of biofilm with nutrient limitations, of stationary-phase bacteria that become tolerant to ampicillin and ciprofloxacin, as observed in free-floating bacteria [81].

A number of other antibiotics, including piperacillin, piperacillin-tazobactam, cefoperazone, ceftazidime, cefepime, meropenem, ciprofloxacin, netilmicin and amikacin, were also evaluated on *K. pneumoniae* biofilm, confirming that adherent bacterial populations exhibited reduced antimicrobial susceptibility with respect to their planktonic counterpart [82].

On the contrary, in 2009, tetracycline and chloramphenicol at a five-fold MIC were demonstrated to affect *K. pneumoniae* biofilms, even if to a different extent ($p < 0.05$ and $p < 0.01$, respectively) [83], and their inhibitory effects could be explained by the fact that both of these antibiotics are protein synthesis inhibitors, which act on the bacterial ribosome [84,85].

The higher resistance of *K. pneumoniae* biofilm to ciprofloxacin, amikacin and piperacillin was confirmed by testing them on different phases relevant for biofilm formation, including planktonic cells at mid-log phase, planktonic cells at stationary phase, adherent monolayers and mature biofilms. *K. pneumoniae* in a biofilm growth mode resulted in being more resistant to all antibiotics. The effect of amikacin and ciprofloxacin on young and older biofilms, at the highest achievable serum concentrations, was also examined, observing that amikacin was able to eradicate the young biofilms, but became completely ineffective when biofilm increased in age. A possible explanation for the increasing resistance during the time of growth was given by calcofluor staining, an enhanced production of exopolysaccharide in older biofilms being observed [86].

Bellifa and coworkers investigated the response of biofilm-growing *K. pneumoniae* strains isolated from medical devices to gentamicin, cefotaxime and ciprofloxacin, detecting that isolates were at least 10–25-times more resistant when grown as a biofilm than in the planktonic form [87].

Recently, differences in the antibiotic resistance of biofilm-growing *K. pneumoniae* strains to gentamicin, depending on the resistance or susceptibility of the planktonic-growing isolates to this antibiotic, have been observed. In fact, gentamicin-resistant isolates dramatically increased their resistance when grown as a biofilm (up to 234-fold), whereas gentamicin-susceptible isolates preserved their susceptibility also in biofilm, thus supporting the use of this antibiotic to successfully treat gentamicin-susceptible biofilm-growing *K. pneumoniae* strains [88].

The efficacy of amikacin has been evaluated also by developing a biofilm model of *K. pneumoniae* B5055, mimicking *in vivo* biofilm system. By using the BacLight viability staining kit, the antibiotic was effective against younger biofilm, but ineffective against older biofilm, possibly due to the heterogeneity and thickness of the biofilm itself [89].

Contrariwise, imipenem recently has shown a potent activity against established *K. pneumoniae* biofilms under both static and flow conditions *in vitro* and *in vivo*, by using a rabbit ear wound model [90].

5. Correlation between Biofilm and Antibiotic Resistance

To date, it has been demonstrated that some correlations exist between antibiotic resistance and the biofilm-forming ability of *K. pneumoniae* strains.

For instance, the ability of 150 *K. pneumoniae* strains, isolated from sputum and urine, to form biofilm exhibited a significant association with their ESBL production. In fact, among the 44.7% biofilm formers, 45.3% of them produced ESBLs [59].

Later, a NDM-1 carrying a *K. pneumoniae* isolate has been demonstrated to be the most virulent in the murine sepsis model and the stronger biofilm producer, as tested with the Calgary device method, with respect to the non-NDM-1 carrying isolates, but there was no clear correlation with *in vitro* virulence factors, such as biofilm formation ability or killing in *Caenorhabditis elegans* [91].

In 2012, a prospective analysis revealed that 80% of the biofilm-producing strains collected from 100 urine samples from catheterized patients with symptoms of UTI over a period of six months, exhibited the MDR phenotype. In particular, biofilm-positive isolates showed 93.3%, 83.3%, 73.3% and 80% resistance to nalidixic acid, ampicillin, cefotaxime and co-trimoxazole, respectively, compared to the 70%, 60%, 35% and 60% resistance shown by biofilm non-producers for the respective antibiotics [92].

Afterwards, Sanchez and coworkers confirmed the propensity of MDR *K. pneumoniae* strains to form a richer biofilm with respect to the susceptible ones, with special reference to those resistant to cephalosporins [93].

The link between antibiotic resistance and biofilm formation has been also examined by growing *K. pneumoniae* strains under antibiotic pressure, mostly with a sub-inhibitory concentration of antimicrobials. Hennequin and colleagues monitored the bactericidal effect of cefotaxime (MIC 516 mg/L) and ofloxacin (MIC 2 mg/L) on CTX-M-15-producing *K. pneumoniae*. While in the presence of sub-MICs of ofloxacin, the biomass decreased in inverse proportion to the

antibiotic concentrations; in the presence of cefotaxime at sub-MIC concentrations, the biofilm formation enhanced [94].

Finally, looking more specifically at antibiotic resistance genes responsible for this correlation, *AmpR*, a regulator of *K. pneumoniae* virulence, particularly regulating a cephalosporins resistance gene (*DHA-1*) carried on a plasmid, has been demonstrated to modulate biofilm formation and type 3 fimbrial gene expression, as well as the adhesion to HT-29 intestinal epithelial cells [95].

6. Conclusions

The opportunistic pathogen, *K. pneumoniae*, can give rise to severe diseases, typically nosocomial infections, such as septicemia, pneumonia, UTI and soft tissue infection. *Klebsiella* infections are often considered as a paradigm of hospital-acquired infections. The indiscriminate use of antibiotics has revealed a considerable increase in outbreaks caused by microorganisms resistant to antimicrobial drugs, such as KPC-producing *K. pneumoniae*.

Nosocomial *Klebsiella* infections continue to be a heavy burden on the economy and on the life expectancy of patients in developed countries. Thus, further progress in the prevention of hospital-acquired infections will require new approaches to infection control.

The increasing evidence on the ability of *K. pneumoniae* to form biofilm, mostly on medical devices and the recent data supporting the correlation of such a behavior with the antibiotic resistance acquisition should alert even more regarding the hazard of this pathogen in hospital settings.

The exploration of these virulence factors and the study of new mechanisms to control them could be an important way to counteract *K. pneumoniae* nosocomial infections. In particular, the biofilm mode of growth makes bacteria up to 1,000-times more resistant to antibiotic therapy. In *K. pneumoniae*, many studies were performed in order to better highlight the mechanisms underlying this resistance, demonstrating that the limitation of the penetration of antibiotic molecules through the biofilm matrix is not the main reason for the increasing resistance, but rather the slow growth rate in the center of biofilm is. In any case, other mechanisms are involved, and further studies are requested as a future challenge to elaborate new concepts in the preventive measures against nosocomial *K. pneumoniae* infections.

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Author Contributions

Claudia Vuotto wrote the manuscript. Francesca Longo selected references and contributed to the manuscript drafting. Maria Pia Balice supplied investigated urinary catheters and *K. pneumoniae* strains. Gianfranco Donelli critically revised the manuscript. Pietro Emanuele Varaldo provided insightful comments.

Conflicts of Interest

The authors declare no conflict of interest.

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Exploring Dangerous Connections between *Klebsiella pneumoniae* Biofilms and Healthcare-Associated Infections

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Abstract: Healthcare-associated infections (HAI) are a huge public health concern, particularly when the etiological agents are multidrug resistant. The ability of bacteria to develop biofilm is a helpful skill, both to persist within hospital units and to increase antibiotic resistance. Although the links between antibiotic resistance, biofilms assembly and HAI are consensual, little is known about biofilms. Here, electron microscopy was adopted as a tool to investigate biofilm structures associated with increased antibiotic resistance. The *K. pneumoniae* strains investigated are able to assemble biofilms, albeit with different kinetics. The biofilm structure and the relative area fractions of bacteria and extracellular matrix depend on the particular strain, as well as the minimal inhibitory concentration (MIC) for the antibiotics. Increased values were found for bacteria organized in biofilms when compared to the respective planktonic forms, except for isolates Kp45 and Kp2948, the MIC values for which remained unchanged for fosfomycin. Altogether, these results showed that the emergence of antimicrobial resistance among bacteria responsible for HAI is a multifactorial phenomenon dependent on antibiotics and on bacteria/biofilm features.

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1. Introduction

In recent years, healthcare-associated infections (HAI), defined as infections occurring after exposure to healthcare, but not always as a consequence of this exposure, have been reported as a major public health problem. In 2012, the European Centre for Disease Control (ECDC) has estimated that at least 2.6 million cases of HAI occur annually in long-term care facilities. This number adds to the previously estimated 4.1 million cases in acute-care hospitals, which result in 37,000 annual deaths in Europe [1].

The incidence of HAI varies with body site and is determined to a large extent by the underlying disease condition of patients (e.g., immunosuppression) in addition to their exposure to high-risk medical interventions, such as surgery or invasive diagnostic procedures. Nevertheless, the main infection sites are the urinary and lower respiratory tracts, surgical sites and the bloodstream. Treatment of HAI is challenging due to the prevalence of antibiotic-resistant bacteria [2], and their ability to assemble biofilms can worsen the situation turning HAI refractory to antibiotherapy. Biofilm is defined as a thin layer of microorganisms and secreted polymers adhering to a biotic or abiotic surface, presenting an internal organization that evolves over time. Biofilms tend to increase bacterial resistance to host defense mechanisms, antibiotics, sterilization procedures (other than autoclaving) and the persistence in water distribution systems and in generic surfaces [3].

Biofilm-associated microorganisms have been related with several human diseases and are known to colonize a wide variety of medical devices [4]. *Klebsiella pneumoniae* is a worldwide leading cause of hospital-acquired urinary tract infections, as well as pneumonia, being responsible for many cases of pyogenic liver abscess or endophthalmitis contracted in community patients [5]. The incidence of *K. pneumoniae* infections increased in hospitals and, according to the latest data from ECDC, was included among the six ESKAPE bacteria responsible for two-thirds of all HAIs. The ESKAPE pathogens are multi-drug resistant strains of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella* species, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species [6]. In spite of their close relation, one notable difference between *K. pneumoniae* and the other members of HAIs is the extremely thick, hypermucoviscous, extracellular polysaccharide capsule. Virulent strains have been predominantly associated with the K:1 and K:2 capsular serotypes. The capsule is believed to be a major virulence determinant by protecting *K. pneumoniae* against phagocytosis and destruction by antimicrobial peptides [7,8]. Other virulence factors have provided new insights into the pathogenic strategies of *K. pneumoniae*, such as fimbriae type 1 and type 3, which mediate attachment to the host mucosal surfaces and inert surfaces [9,10]. In addition, the incidence of isolation of antibiotic-resistant *K. pneumoniae* has increased in recent years, thus complicating the therapy of HAIs and community infections. Research into new antibiotics, phage therapy and vaccines has been some of the features of the past decade. There is now a pressing need for new therapeutic approaches, given the increased number of multi-resistant bacteria (MRB). Here, we focus on the role played by biofilm organization on antibiotic resistance. A deeper understanding of this topic is the first step towards the development of more effective, either preventive or curative, approaches to minimize the impact of HAI.

2. Results and Discussion

2.1. Bacteria Characterization

Ten *K. pneumoniae* MRB were collected at Lisboa hospitals, between 1980 and 2011 (Table 1). Kp45 and Kp26 strains were isolated, from a nurse neck swab and from a newborn rectal swab, respectively, during a colonization study at a neonatology ward. These isolates showed the same capsular type, K:2. The non-capsulated Kp703 strain was isolated from the urine of a burn patient, and its bacterial surface carbohydrates (O antigen) were typed as O:1. From the remaining isolates, only Kp2948 showed capsular type K:2.

Various fimbrial adhesins have been shown to play a role in biofilm formation [11]. Most *K. pneumoniae* isolates express two types of fimbrial adhesins: type 1 and type 3. In this study, all strains, except Kp26 and Kp3466, were amplified for *fimH* and *mrkD* gene subunits of the type 1 and type 3 fimbrial adhesins, respectively. It should be emphasized that the *fimH* gene was not found in the non-capsulated *K. pneumoniae* Kp703 strain (Table 1).

The main difference between *K. pneumoniae* strains isolated in 1980 and twenty years later is the antimicrobial resistance; isolates from 1980 were susceptible to all cephalosporins and produced a broad spectrum β -lactamase TEM-1, while isolates from 2010 to 2011 have evolved to encode extended-spectrum β -lactamases CTX-M-15 and TEM-163, which confer resistance to

extended-spectrum cephalosporins, with the hallmark being the resistance to ceftazidime and cefotaxime. The Kp2948 and Kp3385 isolates exhibit increased resistance to carbapenem antibiotics, producing KPC-3 carbapenemase. All strains have acquired resistance mechanisms to other classes of antibiotics and are considered MRB.

Table 1. Characteristics of *Klebsiella pneumoniae* multidrug-resistant isolates producing β -lactamases.

Strain	Source	Year	Serologic Group	Fimbriae		β -lactamases
Kp45	Neck swab	1980	K:2	fimH	mrkD	TEM-1
Kp26	Rectal swab		K:2	n.a.	n.a.	TEM-1
Kp703	Urine		O:1	n.a.	mrkD	TEM-1
Kp3921			n.a.	fimH	mrkD	CTX-M-15
Kp2948	Wound	2010	K:2	fimH	mrkD	KPC-3; TEM-1
Kp3421	Urine		n.a.	fimH	mrkD	CTX-M-15
Kp3407			n.a.	fimH	mrkD	KPC-3
Kp3466	Urine	2011	n.a.	n.a.	n.a.	TEM-163
Kp3385			n.a.	fimH	mrkD	KPC-3

n.a.: no amplification.

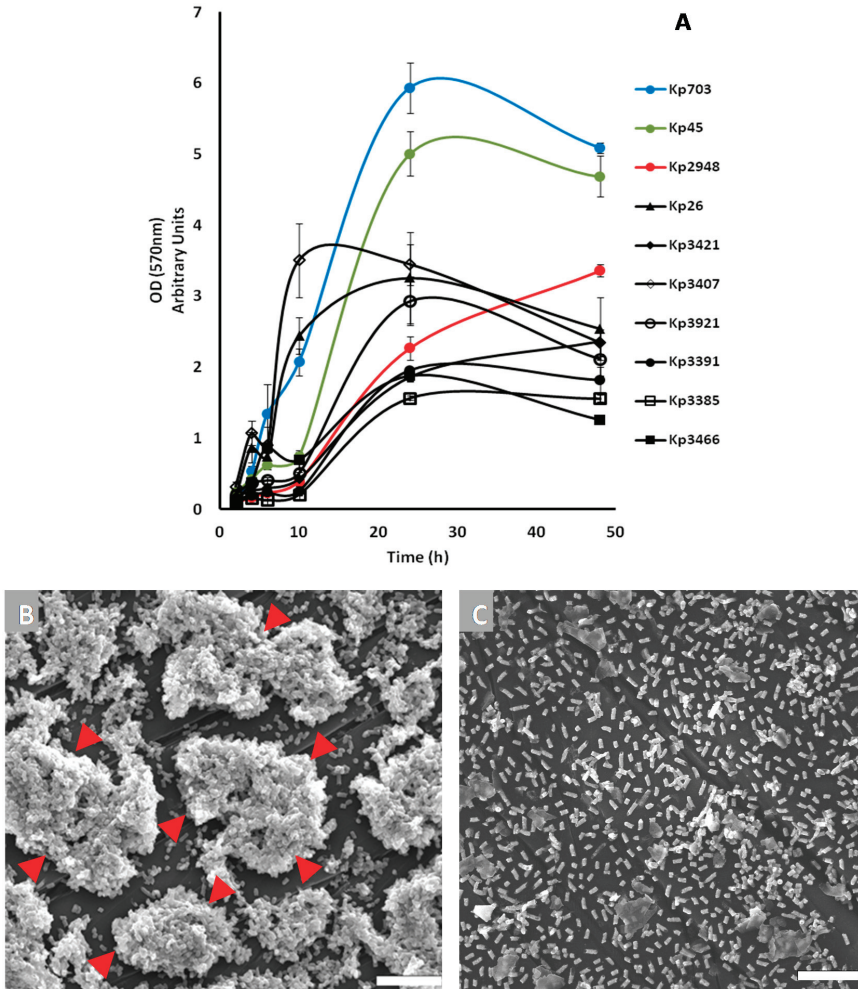
2.2. Biofilm Assembly

The bacterial ability to assemble biofilms on cell culture plates was evaluated. All strains were able to assemble biofilms, although following different kinetics (Figure 1A). The strains were divided into two main groups according to biofilm evolution. Group 1 includes the strains for which the three major biofilm assembly stages could be clearly identified before 48 h of culture, namely adhesion, maturation and dispersion: Kp703, Kp45, Kp3921, Kp3466, Kp3391, Kp3407 and Kp26. Group 2 comprises Kp2948, Kp3421 and Kp3385, which did not reach the dispersion stage at 48 h.

The most efficient biofilm assemblers were selected from each group for detailed study: Kp703 and Kp45 from Group 1 and Kp2948 from Group 2. *K. pneumoniae* strains Kp703 and Kp45 followed similar kinetics with comparable biomass increase (Figure 1A), although these strains differ in capsule expression; Kp703 is non-capsulated (O:1), while Kp45 is capsulated (K:2) (Table 1).

The Kp2948 strain selected from Group 2 shared several features with Kp45, such as the presence of a K:2 capsule and type 1 (fimH) and type 3 (mrkD) fimbriae; nonetheless Kp2948 exhibited distinct biofilm assembly kinetics (Table 1). Both capsulated strains exhibited longer adaptation phases, supporting the previous findings of the downregulation of type 1 fimbriae [9]. On the other hand, the non-capsulated Kp703 strain had a shorter adaptation phase with a faster increase of biomass. The absence of type 1 fimbriae and the presence of type 3 fimbriae may account for the observed difference in kinetics. Type 3 fimbriae are known to be important, both for initial cell-surface attachment and for the cell-cell adherence mediation in the biofilm [9].

Figure 1. The kinetics of biofilm assembly by 10 strains of *K. pneumoniae* was followed over 48 h using a spectrophotometric assay (A); in parallel, scanning electron microscopy (B,C) was used to illustrate biofilm assembly. Representative micrographs of 12 h-old biofilms of kp703 (B) and Kp2948 (C) show the different ability to assemble biofilms exhibited by these strains. The presence of organized bacterial structures is highlighted by arrowheads (B). Scale bar: 10 μ m.



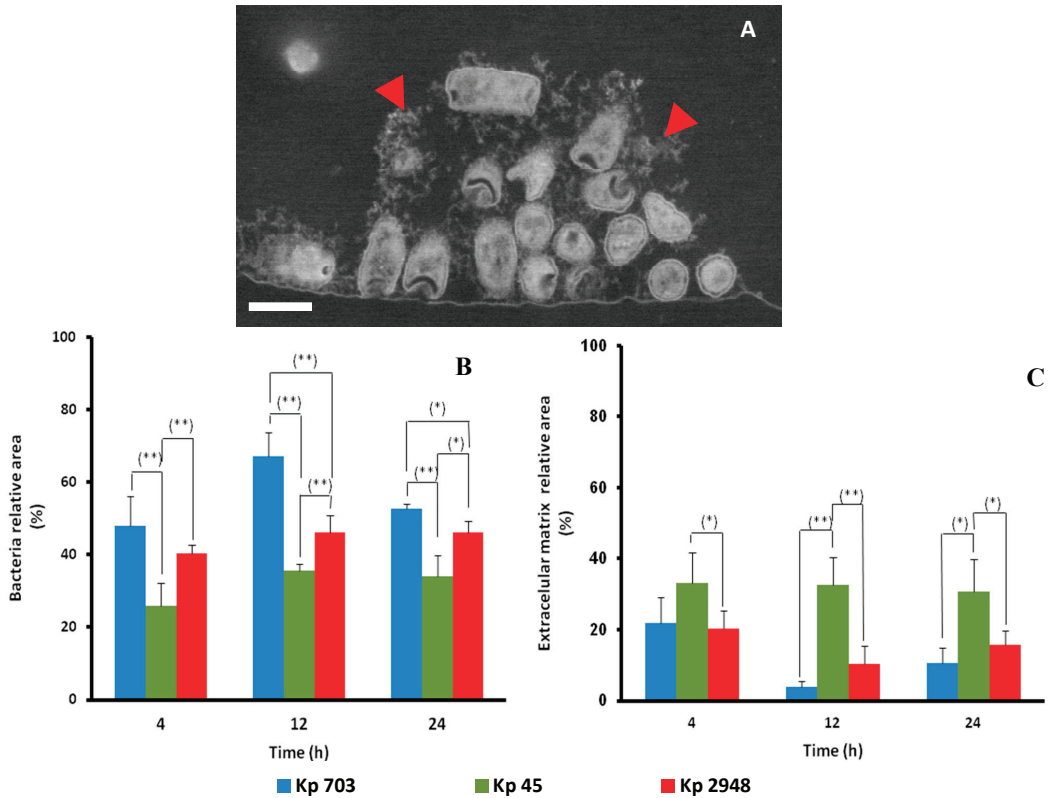
2.3. Scanning Electron Microscopy

Scanning electron microscopy (SEM) highlighted the differences between the biofilms assembled by the selected bacterial strains. Micrographs of 12 h-old biofilms are shown in Figure 1. The Kp703 biofilm is fully mature with clear bacterial complexes highlighted by arrowheads in Figure 1B. The Kp2948 biofilm showed few bacteria attached to the surface (Figure 1C), supporting the assumption that this isolate is in a different stage of biofilm assembly (Figure 1A).

In order to further characterize the internal organization, biofilm cross-sections were observed in backscattered electron mode after staining with heavy metals. This experimental approach has advantages and disadvantages when compared with transmission electron microscopy (TEM) observations; on the one hand, it is less time consuming and allows larger fields of view; on the other hand, the resolution attained is lower [12]. The method was used to assess the evolution of the relative area fractions of bacteria and extracellular matrix (EPS) as inferred from the cross-sectional areas. A representative micrograph of a 4 h-old Kp703 biofilm is shown in Figure 2A, where EPS is indicated by arrowheads. The results obtained from the analysis of biofilms at different maturation stages are shown in Figure 2B,C, where it is evident that the relative area fraction of bacteria depends consistently on the strain: Kp703 biofilms have higher amounts of bacteria (Figure 2B) than Kp45 biofilms (Figure 2C). In good agreement with the biofilm assay (Figure 1A) and the SEM micrographs shown in Figure 1B, the Kp703 strain was associated with the highest biomass at all stages of biofilm assembly (Figure 2B). At 4, 12 and 24 h, the relative area occupied by Kp45 biofilms is statistically smaller ($p < 0.010$) than in both Kp703 and Kp2948 biofilms. In fact, the relative bacterial areas for the most and least efficient biofilm assembler (Kp703 and Kp2948, respectively) only differ at later stages, when the relative area occupied by Kp703 becomes bigger than the one taken by Kp2948 ($p < 0.039$). These data suggest that Kp703 and Kp2948 assembled similar biofilms, although following different kinetics. The intermediate biofilm assembler (Kp45) registered the lowest bacterial areas for all culture times. Altogether, these results showed that Kp45 biofilm is unique although assembled with a kinetic similar to Kp703 biofilm (Figure 1A).

The area fraction of EPS (Figure 2C), a key player in biofilm assembly and persistence, was also determined from cross-sectional observations. Kp45 excreted significantly more EPS when compared to Kp2948 for all culture times ($p < 0.048$). The EPS relative area fraction in the Kp703 biofilm was always lower than in the Kp45 biofilm; nevertheless, the difference was significant only at 12 and 24 h. No differences between the EPS relative area fractions of Kp703 and Kp2948 were detected. This fact supports the previous assumption that these two strains form similar biofilms, although following different kinetics. The EPS amount was not uniform in each biofilm and may be altered in space and time, proving that different bacteria produce it in different amounts [3]. The EPS content within biofilm varies over time, with the highest amount at the attachment and dispersion phases. Studies have shown that EPS plays an important role in attachment and dispersion phases, binding cells to a surface [3,13]. It should be remembered that the dispersion stage occurs due to a local nutrient depletion, triggering bacteria to colonize other areas from the surface [3]. Extracellular matrix contributes to the increased virulence of microorganisms, blocking mass transport of antibiotics through the biofilm [3] and hampering the elimination of infection by the immune system and antimicrobials.

Figure 2. The biofilms assembled by three *K. pneumoniae* strains (Kp45, Kp703 and Kp2948) were characterized using SEM in backscattered electron mode. The existence of extracellular matrix (arrow heads) surrounding bacteria from early stages is highlighted by arrow heads in a 4 h-old Kp703 micrograph (A). The relative areas occupied by bacteria (B) and extracellular matrix (C) during the different phases of biofilm assembly were assessed. The differences were considered significant for $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$). Scale bar: 1 μm .



2.4. Antimicrobial Activity of Planktonic and Biofilm-Embedded *K. pneumoniae* Strains

Biofilms can physically protect bacteria from antimicrobial exposure when compared with planktonic forms, it being important to develop accurate methodologies to determine the MIC for *K. pneumoniae* strains embedded in the respective biofilms.

The MIC values found were different according to antibiotics and bacteria. In general, an increase of MICs for bacteria in biofilm form was verified (Table 2). The increase in MIC values for amoxicillin ranged from five- to 10-fold. The source of β -lactamases in biofilms has been attributed to bacteria lysis promoted by antibiotics. These enzymes once secreted maintain their activity within the biofilm matrix, hydrolyzing β -lactam antibiotics before these drugs reach the bacterial cells [14]. Antibiotics penetration within biofilms could be hampered by other factors,

such as the presence of negatively-charged surfaces, particularly for large polar molecules that are positively charged, e.g., aminoglycosides [15]. In addition, limited oxygen and metabolic rates are probably important factors contributing to increased bacterial aminoglycosides tolerance. Altogether, these factors could account for the variation of two-, eight- and 250-fold in MIC values for gentamicin for *K. pneumoniae* isolate Kp2948, Kp45 and Kp703 biofilms when compared to the planktonic forms, respectively. In contrast to amoxicillin and gentamicin, fosfomycin is known to penetrate through biofilm layers [16]. In the present study, only Kp703 increased the MIC value 1000-fold for fosfomycin between planktonic and biofilm forms. The other *K. pneumoniae* strains retained unchanged MIC values for this antibiotic. This apparently unexpected result could be explained by the presence/absence of a capsule. Both capsulated strains (Kp45 and Kp2948) expressing capsular type K:2 showed unchanged MIC values of 0.781 µg/mL, whereas the non-capsulated Kp703 strain registered the most significant increase. The isolate Kp2948 producing carbapenemase KPC-3 is resistant *in vitro* to all β-lactams, including β-lactam/β-lactamase inhibitor combinations, quinolones and aminoglycosides [17]. The therapeutic options are often limited to tigecycline and colistin, although this strain has moderate susceptibility to these antibiotics and shows susceptibility to fosfomycin (data not shown). In summary, fosfomycin demonstrated *in vitro* activity against the KPC-producing *K. pneumoniae* isolate in planktonic and biofilm forms. Fosfomycin is an “old” antimicrobial that rapidly penetrates tissues [18] and could represent a possible alternative to tigecycline and colistin, minimizing the impact of HAI.

Factors affecting biofilm formation were not evaluated in this study. Several genes involved in biofilm formation by *K. pneumoniae* were described, which account for the production of exopolysaccharides, lipopolysaccharides and capsule, as well as the cell density-dependent regulation by quorum sensing [19].

Table 2. Comparison of minimal inhibitory concentration (MIC) obtained for planktonic and biofilm-organized *K. pneumoniae*.

A: MIC (Planktonic)			
Drug Strain	Amoxicillin (µg/mL)	Fosfomycin (µg/mL)	Gentamicin (µg/mL)
Kp45	250	0.781	3.05
Kp703	250	<0.488	0.760
Kp2948	>500	0.781	1.52
B: MIC (Biofilm)			
Drug Strain	Amoxicillin (µg/mL)	Fosfomycin (µg/mL)	Gentamicin (µg/mL)
Kp45	>2,500	0.781	24.4
Kp703	>2,500	500	195
Kp2948	2,500	0.781	3.05

3. Experimental Section

3.1. Bacterial Strains

Ten *Klebsiella pneumoniae* multiresistant strains were isolated from biological products and are part of the FFUL (Faculty of Pharmacy ULisboa, Lisboa, Portugal) collection. All strains were from healthcare-associated infections.

3.2. Capsular Type

Three *K. pneumoniae* isolates identified in 1980 (Kp 45, Kp26, Kp703) were typed at the World Health Organization International *Escherichia* and *Klebsiella* Centre, Copenhagen, Denmark. The detection of capsular type K:1 and K:2 for remaining isolates were performed by PCR using specific primers for *rmpA* [20] and *K2a* [21] genes, respectively.

3.3. Biofilm Assay

The assay was performed in triplicate using 96-well flat-bottomed cell culture plates (Nunc, New York, NY, USA) as described previously with small modifications [22]. Briefly, *K. pneumoniae* suspensions at a final concentration of 10^7 CFU/mL were prepared in 0.9% sodium chloride from overnight cultures in Mueller–Hinton (MH) agar and ten-fold diluted in MH broth (Oxoid, Basingstoke, UK). Two-hundred microliters were distributed to each well, MH broth being used as the negative control. The plates were incubated at 37 °C to allow biofilm formation for different time periods. Then, the content of each well was removed, and each well was vigorously washed three times with sterile distilled water to remove non-adherent bacteria. The attached bacteria were stained for 15 min with 100 μ L of violet crystal at room temperature, washed with distilled water three times to remove excess dye and allowed to dry at room temperature. The violet crystal was dissolved in 100 μ L of 95% ethanol (Merck, Darmstadt, Germany), and the optical density at 570 nm was read using a (SpectraMax 340PC; Molecular Devices, Sunnyvale, CA, USA).

3.4. Scanning Electron Microscopy (SEM)

For SEM analysis, biofilms were allowed to form on six-well cell culture plates (Nunc) for 12 h at 37 °C. The biofilm was fixed with 2.5% glutaraldehyde (EMS, Hatfield, PA, USA), 4% paraformaldehyde (Sigma, St Louis, MO, USA) and 0.05% ruthenium red (Sigma) in 0.1 M cacodylate buffer, pH 7.2, overnight, at 4 °C. This was followed by post-fixation in the dark with 1% osmium tetroxide (EMS), 0.05% ruthenium red and then washed twice with cacodylate and water [23], dehydrated, transferred to glass slides (bioMérieux, Marcy l'Etoile, France) and allowed to dry at room temperature. For backscattered electron analysis, samples were further embedded in Epon812 epoxy resin (EMS) and allowed to polymerize at 65 °C for 3 days. Once polymerized, the blocks were trimmed and sectioned using an ultramicrotome (Leica, Solms, Germany). Thin sections were transferred to coverslips coated with 0.5% (m/v) gelatine (Sigma) and 0.05% (m/v) chromium potassium sulfate dodecahydrate (Panreac, St Loius, MO, USA) and

allowed to dry at room temperature. The sections were contrasted with saturated uranyl acetate in water, for 30 min, followed by Reynolds lead citrate for 3 min.

Samples were mounted on the sample holder with carbon tape, sputter-coated with carbon (20 nm) using a Sputter Coater QISOT ES (Quorum Technologies, Laughton, UK) and analyzed under an electron microscope, JSM-7100F (JEOL, Tokyo, Japan).

3.5. Antibiotic Susceptibility Test

The antimicrobial activity was evaluated by the micro-dilution method according to Clinical and Laboratory Standards Institute guidelines, using amoxicillin (Bio-Rad, Hercules, CA, USA), fosfomycin (Bio-Rad) and gentamicin (Gibco, New York, NY, USA). Briefly, antibiotics were diluted in MH broth to produce a two-fold dilution in the concentration ranges from 10,000 to 0.0048 $\mu\text{g}/\text{mL}$ for amoxicillin, 500 to 0.244 $\mu\text{g}/\text{mL}$ for fosfomycin and 12,500 to 0.191 $\mu\text{g}/\text{mL}$ for gentamicin. A positive control containing a suspension of bacteria in MH broth without antibiotics was performed in parallel. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic resulting in the absence of turbidity after over-night incubation at 37 °C.

The MIC for biofilm was performed using the same antibiotics and concentrations. Briefly, a biofilm was allowed to form as described in Section 3.2 during 12 h. After removing the non-adherent bacteria, the antibiotic solutions were added, and the plate was sonicated in a water table sonicator for 5 min and incubated over-night at 37 °C.

3.6. Statistical Analysis

The results of at least three independent experiments were expressed as the means \pm standard deviation (SD). SEM micrographs were analyzed using Image J software with the statistical significance assessed by the Student *t*-test (two-tailed). A *p*-value of <0.05 (*) and <0.01 (**) were considered statistically significant.

4. Conclusions

This study supports the hypothesis that biofilms formed on medical devices can promote the onset and enhance the spread of HAIs. The biofilm assembly process is bacterial type specific. Biofilm-forming bacteria are generally more resistant to antibiotics; however, fosfomycin demonstrated *in vitro* activity against KPC-producing *K. pneumoniae* isolate, both in planktonic and biofilm forms. The emergence of antimicrobial resistance among bacteria responsible for HAIs is a multifactorial phenomenon dependent on antibiotics and bacteria/biofilm features. Therefore, elucidating the biofilm-forming ability and evaluating the structural organization of biofilms and the susceptibility to antimicrobials are crucial for the development of treatment regimens.

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Author Contributions

Conceived and designed the experiments and data analysis: Luisa Jordao, Patricia Almeida Carvalho and Aida Duarte. Performed experiments: Maria Bandeira and Luisa Jordao. Wrote the paper: Luisa Jordao and Aida Duarte.

Conflicts of Interest

The authors declare no conflict of interest.

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Biofilms in Infections of the Eye

Paulo J. M. Bispo, Wolfgang Haas and Michael S. Gilmore

Abstract: The ability to form biofilms in a variety of environments is a common trait of bacteria, and may represent one of the earliest defenses against predation. Biofilms are multicellular communities usually held together by a polymeric matrix, ranging from capsular material to cell lysate. In a structure that imposes diffusion limits, environmental microgradients arise to which individual bacteria adapt their physiologies, resulting in the gamut of physiological diversity. Additionally, the proximity of cells within the biofilm creates the opportunity for coordinated behaviors through cell–cell communication using diffusible signals, the most well documented being quorum sensing. Biofilms form on abiotic or biotic surfaces, and because of that are associated with a large proportion of human infections. Biofilm formation imposes a limitation on the uses and design of ocular devices, such as intraocular lenses, posterior contact lenses, scleral buckles, conjunctival plugs, lacrimal intubation devices and orbital implants. In the absence of abiotic materials, biofilms have been observed on the capsule, and in the corneal stroma. As the evidence for the involvement of microbial biofilms in many ocular infections has become compelling, developing new strategies to prevent their formation or to eradicate them at the site of infection, has become a priority.

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1. Introduction

Ever since Robert Koch and Louis Pasteur in the 1860's established the modern field of bacteriology, studies employing pure bacterial cultures, often grown in liquid media (planktonic growth), have shaped our understanding of bacterial physiology and behavior. Pure cultures were required to establish microbial causes of disease, and growth in liquid media ensured that all cells were exposed to similar conditions and behaved in the same manner. As a result, most of the measures to control pathogenic bacteria (e.g., vaccines and antimicrobial agents) have been developed based on knowledge of bacteria grown as planktonic cells.

An appreciation for the fact that in nature, bacteria adhere to many abiotic or biotic surfaces and form communities of differentiated, interacting communities known as “biofilms”, emerged over the past few decades [1], and this concept was enthusiastically promoted by William (Bill) Costerton among others. Evidence of biofilm formation has been found in the analysis of microbial fossils including those from deep-sea hydrothermal sediments. This suggests that the ability to form biofilm is an ancient adaptation that dates back more than 3 billion years [2,3]. Biofilm formation conferred to individual bacteria the ability to collaborate and to adapt to a range of harsh environmental conditions, perhaps most of all, to evade predation by phagocytic microbes. The formation of a biofilm provides a microbe with a small measure of control over the local

environment, including fluctuations in temperatures, pH, ultraviolet light, starvation, and exposure to toxic agents [4,5].

The ubiquity of biofilm formation in natural ecosystems, industrial systems, and medical settings has accelerated the pace of biofilm research. Advances in medical biofilm research have led to an understanding that biofilms represent the prevalent form of bacterial life during tissue colonization, and may occur in over 80% of microbial infections in the body [6]. Biofilms play important roles in human infections including native valve endocarditis, otitis media, chronic bacterial prostatitis, lung infections in patients with cystic fibrosis and periodontitis [7,8]. In addition, biofilms form on indwelling devices including prosthetic heart valves, coronary stents, intravascular catheters, urinary catheters, intrauterine devices, ventricular assist devices, neurosurgical ventricular shunts, prosthetic joint, cochlear implants, intraocular and contact lenses [7,8]. Due to their medical importance, development of anti-biofilm compounds for clinical use are of vital interest [9].

2. Microbial Biofilms

The very first description of a biofilm dates back to the 17th century when Anthony van Leeuwenhoek examined his own teeth scrapings with one of the first microscopes and found a large amount of small living “animalcules” in his dental plaque matter. He concluded in his report to the Royal Society of London in 1684 that the thick white material found between his teeth protected the bacteria embedded in this substance against the action of the vinegar that he used to wash his mouth [10]. At the time, miasmatic and humoral theories of disease were dominant, and it took an additional 200 years until the germ theory of disease was advanced by Robert Koch before a connection between microbes and disease was made.

Today, biofilms are generally defined as a community of sessile microbes held together by a polymeric extracellular matrix, adherent to a surface, interface or to other cells that are phenotypically distinct from their planktonic counterparts [8]. This definition, although reflective of many biofilms, is in our view restrictive, as there is no particular requirement that microbes be held together by an extracellular matrix as opposed to any other adherence principle (surface charge, a network of surface attached proteins, *etc.*), or that they even adhere to a surface (as a raft consisting of only microbes could achieve all of the behaviors usually ascribed to a biofilm, e.g., microbes transiting the lumen of the colon).

Members of a biofilm community, which can be of the same or multiple species, show varying stages of differentiation and exchange information, metabolites, and genes with each other. As a result, members of the biofilm community are in a diversity of physiologies influenced by the unequal sharing of nutrients and metabolic byproducts, which results in subpopulations with increased tolerance to antimicrobials and environmental stresses, the host immune system, and predatory microorganisms [8,11–14].

Canonically, biofilm development has been grouped into five stages that are reflective of conditions in many, but not all biofilms: (1) reversible aggregation of planktonic cells on a surface; (2) irreversible adhesion; (3) formation of microcolonies; (4) biofilm maturation; and (5) detachment and dispersion of cells [11,15]. The events that are of special significance for ocular

infections and the treatment of biofilm infections will be discussed in greater detail below, while the reader is referred to several excellent reviews for details on other biofilm-related subjects [8,11–16].

In the established view of biofilm formation, planktonic cells initially adhere to a surface in a reversible, non-specific manner due to electrostatic interactions between the bacterial cell and the surface. Water contact angle measurements on bacterial cell lawns have shown that the surface of *P. aeruginosa* is highly hydrophobic, while *S. aureus* is highly hydrophilic [17]. Therefore, surface properties of a solid object can favor colonization by one microorganism over another.

Surfaces exposed to liquid solutions are generally coated with a conditioning layer consisting of molecules present in the solution. Antimicrobial agents that are present in multipurpose solutions, for example, bind non-specifically to the contact lenses [18]. Once the lens is placed on the surface of the eye, the disinfectant diffuses off the lens and is replaced by lipids and proteins present in the tear fluid [17,19,20]. The lens material plays an important role in this interaction, as it has been shown that hydrophilic contact lenses preferentially adsorb lysozyme from the tear film, while hydrophobic contact lenses accumulate more lipocalin and lactoferrin [17].

In addition to determining the local antimicrobial properties, this unique conditioning layer also provides specific anchor points for bacterial adhesion. Microbial adhesion to surfaces coated by proteins and other biomolecules is often accomplished by a class of molecules termed Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM), as well as other adhesive surface proteins [21]. As an example of the latter, in *S. epidermidis* and other staphylococci the bifunctional autolysin/adhesion protein AtlE, an abundant surface protein, mediates first attachment to abiotic surfaces and also matrix protein-covered devices [22].

In a moving suspension, cells are exposed to fairly uniform conditions. However, following attachment, the individual experience of a cell begins to differ from its neighbors (*i.e.*, a cell in the middle of a group will experience more excreted products and fewer factors from the environment than a cell on the periphery of a population), and as a result, cells begin to differentiate [23]. Many biofilms involve production of an extracellular matrix (ECM) that encases the cells, and in some cases, binds the cells together and that can be composed of polysaccharides, lipopolysaccharides, proteins, or extracellular DNA [24]. This process may be active or passive, in that cells on the surface of an adherent colony that are lysed by the ejection of neutrophil antimicrobial factors may encase and protect siblings below in a matrix consisting simply of cell lysate. Whatever the nature of the matrix, its chemical and physical properties contribute to the differentiation of cells within the encased population, a process that can protect the bacteria from the action of antimicrobial agents, host immune responses, bacteriophages and phagocytic amoeba [8]. In staphylococci, it appears that polysaccharide intercellular adhesin (PIA, encoded by the *icaADBC* locus), matrix proteins including the accumulation-associated protein (Aap) [25], and possibly the biofilm-associated homologue protein (Bhp, termed Bap in *S. aureus*) contribute to this matrix [26]. Commensal isolates of *S. epidermidis* and other coagulase-negative staphylococci (CoNS) recovered from healthy conjunctiva carry most of the genes related to biofilm maturation, suggesting that the ability to form biofilms is an integral part of their life-style [27–29].

As the microcolony grows through cell division or recruitment of more planktonic cells, the biofilm grows and takes on a three-dimensional structure that often includes open water channels [8,23]. Growing biofilms on various types of contact lenses have shown differences in cell densities and three-dimensional structures *in vitro*, suggesting an effect of the substrate on the development of the biofilm [30]. However, while several studies have measured biofilm thickness on various contact lens materials, with the underlying assumption that thicker biofilms are more likely to result in disease, the biological significance of these results remains unclear. In one experiment, Tam *et al.* [31] grew *P. aeruginosa* biofilms on custom contact lenses and tested them in a rat model of contact lens-associated keratitis [31]. Biofilms grown *in vitro* to low and to high cell densities both caused disease symptoms within 7–8 days, indicating that initial biofilm thickness did not matter [31]. In contrast, contaminated contact lenses that were transferred from one rat to a different healthy animal resulted in keratitis symptoms within 2 days [31]. These results suggest that adaptation to the host environment is a critical step in the pathogenesis of biofilm-related infections.

The three-dimensional organization of the biofilm causes gradients of oxygen, pH, and nutrients, resulting in the development of different microniches [32,33]. The cell's individual physiological adaptations to these microniches results in physiological heterogeneity [13]. Cells near the surface of the biofilm will be exposed to more nutrients and oxygen and are therefore more metabolically active, while cells in the deep regions will be less active or even dormant. This heterogeneity results in a range of responses to antimicrobial agents, with metabolically active cells at the surface being rapidly killed while more internal, dormant cells are comparatively unaffected [32]. This, together with potential effects on diffusion of antimicrobial molecules within the biofilm, causes some cells in a biofilm to be recalcitrant to antimicrobial treatment, with antibiotic susceptibilities reduced by 10 to 1000-fold compared to planktonic counterparts [32].

The high local concentration of cells in a biofilm creates an ideal environment for information exchange through cell-to-cell communication and lateral gene transfer. Cell signaling mediated by secreted, accumulating messenger molecules, known as quorum sensing, allows bacteria to sense and respond to their environment and couple cell-density and other environmental cues with gene expression in ways that allow adaptive phenotypic responses. Quorum sensing has been shown to be involved in the control of biofilm formation and production of virulence and colonization factors in a variety of organisms of medical importance [34]. Cell-to-cell signaling is also involved in biofilm dispersion, which is of general and medical interest [35].

Bacterial cells can leave or be shed from the biofilm and revert to a planktonic life-style, often by degrading the ECM that holds the cells together [35]. For example, thermonuclease is a bacterial enzyme that degrades the extracellular DNA that holds *S. aureus* biofilms together, while alginate lyase degrades the alginate matrix important for *P. aeruginosa* biofilms [36,37]. These processes are coordinated by small signaling molecules that induce the expression of genes for biofilm dispersal. For example, *N*-butanoyl-L-homoserine lactone (C4HSL) belongs to the family of cell-density dependent autoinducers and has been implicated in the dispersal of *P. aeruginosa* biofilms [38]. Other small molecules implicated in biofilm dispersal include the *Pseudomonas*

quinolone signal PQS, the furanosylborate autoinducer AI-2 from *Vibrio cholerae*, and the staphylococcal peptides δ -toxin and AIP-I [35].

3. Ocular Infections

The two leading causes of vision impairment worldwide are uncorrected refractive errors and cataract [39]. Measures to manage those eye abnormalities frequently include the use of contact lenses and the placement of intraocular lenses, and have enhanced the quality of life of millions of patients. Although use of such devices is of the utmost importance for correction of a variety of visual aberrations, they also provide a new surface on which many microbial pathogens can form biofilms (Table 1). As a result, device-related ocular infections are an important limitation of the success of such procedures. Moreover, many infections progress to secondary permanent sequelae that may lead to poor visual outcomes and occasionally loss of sight, such as acute bacterial endophthalmitis or corneal ulceration.

Table 1. Biofilm-associated infections of the eye.

Disease	Main Causative Agents of Infection and/or Found in the Biofilms	Biofilm Localization
Endophthalmitis	Coagulase negative staphylococci and <i>Propionibacterium acnes</i>	Intraocular lens Posterior capsule
Keratitis	<i>Staphylococcus aureus</i> and other staphylococcal species, <i>Pseudomonas aeruginosa</i> and <i>Serratia</i> spp. Fungi and <i>Acanthamoeba</i> less frequently	Contact lens
	Viridans group streptococci. Gram negative bacilli and yeasts less frequently	Corneal stroma (crystalline keratopathy)
Scleral buckle infection	Gram positive cocci and nontuberculous <i>Mycobacterium</i> ¹	Scleral buckles
Lacrimal system infections	<i>Staphylococcus</i> spp., <i>P. aeruginosa</i> and <i>M. chelonae</i> <i>Staphylococcus</i> spp. ²	Lacrimal intubation devices Punctual plugs
Periorbital infections	<i>Staphylococcus</i> spp. and mixed species biofilms	Sockets and orbital plates

¹ Common causative agents of buckle-associated infections. Scleritis resulting of scleral extension of corneal infections are mainly caused by *P. aeruginosa* and other common agents of infectious keratitis;

² The presence of biofilms has not been demonstrated on plugs recovered from symptomatic eyes presenting with dacryocystitis and canaliculitis. However, clinical features of these infections are compatible with biofilm-associated infections such as the late onset, and difficulty to treat with antimicrobial therapy alone.

4. Biofilms in Endophthalmitis

Endophthalmitis is a rare but severe intraocular inflammation that results from the introduction of a microbial pathogen into the posterior segment of the eye. Organisms may gain access to the intraocular tissues exogenously after trauma caused to the ocular globe following intraocular surgery, intravitreal injections, penetrating open globe injury, and in cases of keratitis progressing to corneal perforation. Endogenous endophthalmitis may occur in patients with bacteremia by seeding the eye with bacteria from a distal site of infection [40].

Endophthalmitis Following Cataract Surgery

Postoperative endophthalmitis is the most common presentation and is frequently associated with cataract surgery [41,42]. It is estimated that 17.2% of the population older than 40 years in the United States suffers from cataracts. This prevalence increases with age, being more than 35% for patients between 70–74 and almost 50% for patients between 75–79 years of age [43]. As a result, cataract extraction with replacement of the crystalline lens by an artificial intraocular lens (IOL) represents the most frequent surgery procedure performed by ophthalmologists, with more than 1 million procedures performed each year in United States [44]. Postoperative endophthalmitis is the leading blinding complication of cataract surgery. Its overall incidence varies according to the technique and region of the world, ranging from 0.028% to 0.2% [45–47]. Despite the low overall incidence, given the large number of cataract surgeries performed annually, a substantial number of patients are affected by this sight-threatening infection.

Post-cataract endophthalmitis is caused predominantly by Gram positive organisms originating from the ocular surface microbiota. Coagulase-negative staphylococci (CoNS), especially *Staphylococcus epidermidis*, are the most frequently encountered microbes from culture-proven acute endophthalmitis [41,42,48]. Delayed-onset endophthalmitis is mainly caused by *Propionibacterium acnes*, which usually presents with a more indolent and persistent infection, with lower frequency of hypopyon and better final visual outcome compared to acute cases [49]. Both pathogens, *S. epidermidis* and *P. acnes*, are able to adhere to, and form biofilms on intraocular lenses. Some evidences suggest that they can also adhere to and form biofilms in the posterior capsular bag [50–58]. Commensal organisms colonizing the ocular surface and periocular tissues are the primary source of bacteria that cause postoperative endophthalmitis. In the large Endophthalmitis Vitrectomy Study, 67.7% of paired CoNS isolates from the eyelid and intraocular fluids were indistinguishable by pulsed field gel electrophoresis [59].

The ocular surface is often colonized by Gram positive organisms, with CoNS being most commonly associated with healthy conjunctiva, lids and tears, followed by *Propionibacterium acnes*, *Corynebacterium* spp. and *S. aureus* [60]. Rates of contamination of the anterior chamber after uneventful cataract surgery range from 2% to 46%, and are due to the most common Gram positive commensal organisms found on the ocular surface, most frequently *S. epidermidis* [61–64]. Rates of anterior chamber contamination are much higher than the incidence of postoperative intraocular infection. This suggests that in most cases, the anterior chamber is capable of clearing the bacterial inoculum without progressing to endophthalmitis, likely due in part to the rapid turnover of the aqueous humor [65]. However, most of the organisms found in the ocular microbiota are able to attach to the IOL and posterior lens capsule, and often become well established if they reach the posterior chamber. In comparison to aqueous humor, vitreous is relatively static and constitutes a good environment for establishment of an infection. Intraocular lenses, such as those constructed from polymethylmethacrylate (PMMA) may become contaminated with commensal conjunctival bacteria (mainly *S. epidermidis*) during insertion, and carry the organisms from the ocular surface to the posterior chamber [66,67]. The ability of commensal bacteria to form biofilms on the surface of IOLs prevents their clearance, and likely

represents an important mechanism in the pathogenesis of post-cataract endophthalmitis. The occurrence of microbes in biofilms is consistent with the low rates of culture positivity for aqueous and vitreous samples [68].

As described above, the *ica* locus and other genes play an integral part in staphylococcal biofilm formation. One report from Japan found a prevalence of 60% and 69.4% for *icaA* positive strains of *S. epidermidis* isolated from the conjunctiva of healthy volunteers and patients undergoing cataract surgery, respectively. Most of these isolates (approximately 40%) tested positive for slime production on Congo red agar [29]. Among a collection of *S. epidermidis* isolates from Mexico, 17% of commensal conjunctival isolates were able to form biofilms under conditions used *in vitro*, and 26.7% were positive for the *icaA* and/or *icaD* genes [27]. The frequency of *ica* genes was observed to be 36% for CoNS species other than *S. epidermidis* recovered from the normal conjunctiva of student and staff eyes at an institute in India [28]. For *S. epidermidis* recovered from intraocular fluids of patients with endophthalmitis, the distribution of *icaA* and *icaD* genes seemed to be group specific. Among strains isolated from endophthalmitis cases in South Florida, *icaAD* genes were present (86%) only among isolates that possess the accessory gene regulator locus (*agr*) type I. The frequency of *aap* and *bhp* genes among all isolates was 78.5% and 43.1%, respectively, with *bhp* being more prevalent among *agr* type II isolates [69].

Previous reports have demonstrated the ability of *S. epidermidis* to form biofilms on IOLs (Figure 1) using different *in vitro* conditions [53–55,57], and in a model that resembles the intraocular environment [51,52]. The degree of biofilm formation is affected by the material used to manufacture the IOL and also by the genomic content of each *S. epidermidis* lineage tested. Strains of *S. epidermidis* carrying the *ica* locus are able to form stronger biofilms on different IOL surfaces compared to strains lacking this locus [53,55,57]. In one study [55], using various hydrophobic IOLs, the ability of *S. epidermidis* strains ATCC 12228 (*ica* negative) and ATCC 35984 (*ica* positive) to form biofilms was significantly higher on acrylic lenses followed by PMMA and MPC (2-methacryloyloxyethyl phosphorylcholine) surface-modified acrylic. Weaker biofilms were found on silicone IOLs. Interestingly, modification of the acrylic IOL surface by treatment with MPC decreased biofilm formation [55] and this may be associated with an increase in the hydrophilicity [70]. The same effect has been demonstrated for MPC-modified silicone IOL [71]. Other reports [51,53,72,73], however, have found different results for each IOL material and that was also affected by the strains tested. Foldable IOLs made with silicone supported greater *S. epidermidis* biofilm formation compared to PMMA IOL for strain ATCC 35984, but the same was not seen for the strain ATCC 12228 [53]. In the same study, variations of up to two orders of magnitude in the degree of biofilm formation, as determined by CFU counting, were observed for the same IOL material depending on different models and manufacturers. Acrylic lenses were again the most prone to form stronger biofilms and fluorine-treated PMMA the least. The presence of polypropylene haptics in the PMMA IOL increased the biofilm quantity compared to single-piece PMMA IOL [53]. In agreement, it has been demonstrated that polypropylene haptics represents a significant risk factor for post-cataract surgery endophthalmitis [72], and increases *in vitro* adhesion of *S. epidermidis* compared to single- and three-piece PMMA IOL [73]. In a model using a bioreactor with flow conditions similar to the anterior chamber, *S. epidermidis* was able to form biofilms on

different IOL materials, which significantly increased as a function of time [52]. Silicone was more permissive to biofilm formation in this model, followed by hydrophobic acrylic and PMMA, with the fewest attached cells found on hydrophilic acrylic.

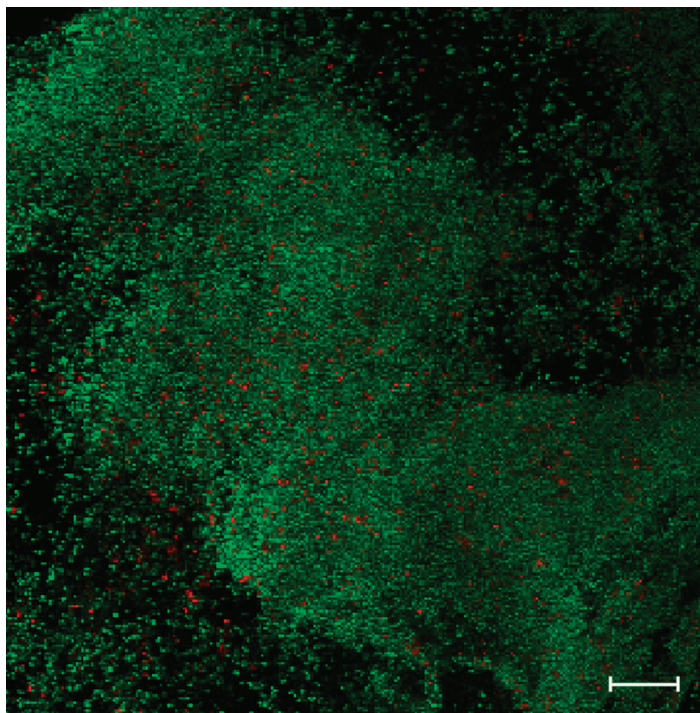


Figure 1. Confocal laser scanning micrograph of a 24 h biofilm. The biofilm of *Staphylococcus epidermidis* RP62A was grown *in vitro* on hydrophilic acrylic intraocular lens, and was visualized after staining using the live/dead viability stain, which contains SYTO9 (green fluorescence, live cells) and propidium iodide (red fluorescence, bacterial cells that have a defective cell membrane, which is indicative of dead cells). Magnification X 400, scale bar is 20 μm .

Despite the variations within each study, hydrophobicity is consistently observed to be an important determinant of biofilm formation. Modification of the surface to make it more hydrophilic may reduce initial binding and development of robust staphylococcal biofilms. The initial adherence of *S. epidermidis* to the IOL surface, an important initial step for subsequent colony expansion and biofilm maturation, has been demonstrated to be decreased by IOLs with hydrophilic surfaces [74,75]. Surface modifications that increase an IOL's water content have been made using different agents including MPC, fluorine and heparin. While MPC and fluorine have been demonstrated to decrease the density of biofilms formed on PMMA and silicone IOLs [53,55,71], data for heparin-modified lenses are mixed. Initial adhesion of *S. epidermidis*, *S. aureus* and *P. aeruginosa* to heparin-surface-modified (HSM) PMMA IOL has been shown to be reduced compared to non-treated PMMA IOLs [76,77]. However, biofilm formation on HSM PMMA IOLs

seems not to be affected, and in fact biomass measures were higher compared to non-treated PMMA [53]. In addition, although the anti-adhesive effect of soluble heparin in the media has been demonstrated *in vitro* using *S. epidermidis* and PMMA IOLs [78], this protective effect was not demonstrated *in vivo* by the addition of low molecular weight heparin to the infusion fluid used during the phacoemulsification procedure [79].

Although infrequent, enterococcal endophthalmitis may occur after cataract surgery and is associated with poor visual outcomes even after appropriate clinical and surgical management [80]. *In vitro* studies have demonstrated that *Enterococcus faecalis* is able to form robust biofilms on IOLs, especially on PMMA and hydrophobic acrylic IOLs after 48 h and 72 h of incubation, while less biomass was observed on silicone IOLs [81]. Recurrent cases of post-cataract endophthalmitis caused by *E. faecalis* have been associated with bacteria attached to the capsular bag and acrylic IOL [82,83].

While acrylic IOLs have demonstrated to be the most permissive material for biofilm formation of Gram positive pathogens, as described above, this material seems to be less susceptible to adherence and biofilm formation of *Pseudomonas aeruginosa*, compared to PMMA and silicone [84]. This is consistent with the finding that the surface of *P. aeruginosa* is highly hydrophobic, while that of staphylococci is highly hydrophilic [17]. Although *P. aeruginosa* does not represent an important organism associated with endophthalmitis after uneventful cataract surgery, it has been associated with multiple outbreaks of post-cataract surgery endophthalmitis, usually due to environmental contamination including the internal tubes of phacoemulsification machines and contaminated solutions used during the surgery [85]. In a recent outbreak of *P. aeruginosa* endophthalmitis following cataract surgery, a thorough investigation identified the hydrophilic acrylic IOL implanted in the patients and the preservative solution as the source of the contamination. The *P. aeruginosa* isolates from the IOL and the preservative solution had the same genetic profile as the isolates from the patients' aqueous and vitreous fluids, as demonstrated by ERIC-PCR [86]. In the context of *P. aeruginosa* endophthalmitis outbreaks after cataract surgery, biofilm formation has not been directly associated in the pathogenesis of these infections, but it likely plays a role as a reservoir for contamination. Biofilms found in the hospital environment have been demonstrated to be a common source of *P. aeruginosa* associated with outbreaks in intensive care units [87,88]. *P. aeruginosa* evolved to form biofilms on surfaces in contact with water, including sinks, water pipes and other natural interfaces [89], and this property is undoubtedly central to its ability to colonize the surgical equipment and irrigation fluids that have been linked to endophthalmitis outbreaks.

5. Biofilms in Keratitis

Microbial keratitis is an infection of the cornea that can lead to loss of vision if not carefully managed [90]. Decades ago, most cases were associated with ocular surface disease and trauma. However, the widespread of contact lenses has made them the most common predisposing risk factor for infectious keratitis [91,92]. The type of organisms causing keratitis varies by geography because of differences in climate, environment and occupational risk [93]. Bacterial keratitis, especially contact lens-associated infection, is caused by both Gram negative pathogens, such as

P. aeruginosa and *Serratia* spp., and Gram positive organisms, such as *S. aureus* [94,95] and other staphylococcal species [93,96,97]. Risk factors for fungal keratitis include tropical or subtropical climate, agricultural work, and corneal trauma [93,98]. In the United States, the prevalence of fungal keratitis is much lower than bacterial keratitis [99], and the primary predisposing factor is unambiguously contact lens wear [100,101]. When it does occur, *Fusarium* spp. usually accounts for the majority of fungal keratitis cases [101]. Additionally, *Acanthamoeba* are protozoa that cause a rare but aggressive form of infectious keratitis that is also frequently associated with contact lens wear [102].

5.1. Contact Lens-Associated Keratitis

Contact lens use represents the main risk factor for the development of microbial keratitis in developed countries, where it is associated with bacterial, fungal and amoebic keratitis [91,92,100–103]. In the United States, previous estimates of microbial keratitis suggested more than 30,000 cases per year [104]. Other estimates of ulcerative keratitis in northern California found an incidence of 27.6 cases per 100,000 person/year [103]. This incidence was higher than observed previously, and was thought to be associated with increasing contact lens wear, since the rate of keratitis was half of that for non-contact lens wearers [103].

The incidence of contact lens-associated microbial keratitis has been shown to be impacted by the contact lens material, and also by the wear schedule. Early epidemiological studies reported a higher risk for daily wear soft contact lenses compared to daily wear rigid gas permeable lenses. The risk was further increased for extended wear (overnight wear) soft contact lenses [95,105]. More recently introduced daily disposable and silicone hydrogel contact lenses have also been associated with a higher incidence of keratitis compared to rigid gas permeable contact lenses [106,107].

The increased risk for the development of microbial keratitis in contact lens wearers has been associated with the ability of the lens to induce modification of the corneal epithelium, to carry organisms to the ocular surface that otherwise would not be found in this niche, and to limit natural clearance mechanisms [108,109]. The close interaction between the lens and the corneal epithelium induces local alterations, including hypoxia and hypercapnia, which affect the ability of the epithelium to respond to damage. Tear fluid exchange may be compromised between the anterior and posterior sides of the lens, altering the composition of the tear fluid on the ocular surface and limiting its antimicrobial properties [108]. In addition, contact lenses provide a surface where microorganisms may attach and colonize the surface as a biofilm, which represents a source for microorganisms to spread to a previously damaged corneal epithelium [109]. It has been demonstrated that poor hygiene and infrequent replacement of the contact lens storage cases were independent risk factors for moderate and severe keratitis [110]. However, not all individuals with poor contact lens hygiene will experience keratitis, while others with good cleaning routines also suffered infections, suggesting that other factors play a role as well [111].

Corneal damage promotes colonization and infection by commensal and environmental organisms. The high prevalence of Gram negative organisms among contact lens-associated keratitis isolates, which are usually not found as commensals on the ocular surface, is likely due to their ubiquitous presence in the environment and their ability to adhere and subsequently form

biofilms on the surface of contact lenses and storage cases. *P. aeruginosa* has a repertoire of genes that allow its adaptation and survival under different stress conditions [112]. Its ability to adhere to different contact lens materials has been demonstrated *in vitro* [113] and is mainly driven by surface hydrophobicity [17,114]. Development of mature biofilms in the posterior surface of the contact lens has been associated with *P. aeruginosa* keratitis in humans [115] and in animal models [31].

In addition to bacteria, fungi and *Acanthamoeba spp.* are also able of causing contact lens associated keratitis. Some of these cases were linked to specific multipurpose contact lens cleaning solutions (MPS), which led to the removal of these products from the market. The first case involved the fungus *Fusarium solani*, which was associated with keratitis in patients that used ReNu with MoistureLoc. This finding came as a surprise because all MPS have to pass antimicrobial efficacy testing against several microorganisms, including *F. solani*. An investigation [116] found that planktonic *Fusarium* strains were susceptible to MoistureLoc as expected, but *Fusarium* biofilms showed reduced susceptibility. In addition, *F. solani* ATCC 36031, the reference strain recommended for antimicrobial efficacy testing, was shown to be incapable of forming biofilms under the conditions tested [116]. This case highlights the need to consider the physiological state of microbes and the strains used in establishing testing standards—standards largely developed using planktonic cells. The second case involves Complete MoisturePlus and protozoa of the genus *Acanthamoeba*. These amoebae are ubiquitous in water and are able to survive harsh conditions, including chemical treatment, by differentiating into dormant cysts that can resume growth once favorable conditions return. Bacterial biofilm formation on contact lenses is a risk factor for contact lens-associated keratitis by *Acanthamoeba* because these organisms graze on the biofilm [117–119]. Complete MoisturePlus was recalled because, rather than killing all cells, it resulted in the encystment of *Acanthamoeba*, which then went on to cause keratitis [120]. Prior to this recall, antimicrobial efficacy testing that included *Acanthamoeba* was not required [121].

5.2. Infectious Crystalline Keratopathy

Crystalline keratopathy is a disease associated with crystalline deposits in the corneal epithelium and stroma, which may be a result of multiple conditions that ultimately lead to the accumulation of metabolic products in the affected corneal tissue. Among the causes of such deposits are corneal infections and systemic diseases [122]. Infectious crystalline keratopathy (ICK) is a chronic and difficult to treat infection of the cornea characterized by the presence of branching crystalline opacities associated with minimum inflammatory response [123,124]. It may occur in normal or diseased corneas and is often associated with corneal surgery, especially penetrating keratoplasty, and the topical use of steroids [122,124,125]. Viridans streptococci are the main pathogens associated with ICK, but other bacterial and fungal species, as well as *Acanthamoeba*, may cause this infection [125–128].

Because of the indolent clinical evolution of this disease, and the relative lack of immune response and recalcitrance to antimicrobial therapy, it was first hypothesized that ICK resulted from organisms associated with a biofilm in the corneal tissue. This was supported by analysis of corneal samples collected by biopsy or penetrating keratoplasty from patients with ICK [129–131]. Transmission electron microscopy examination of corneal samples fixed with ruthenium red found

Candida albicans and also bacteria surrounded by an extracellular matrix consistent with a biofilm [129,130]. Microscopic evidence of bacterial biofilms growing on the corneal tissue was also found for 3 patients diagnosed with ICK, but for 5 other cases of chronic bacterial and fungal keratitis. This indicates that *in vivo* biofilm formation in the corneal stroma is fairly specific for ICK [130]. The intensity of the periodic acid-Schiff stain of the corneal samples from ICK [130] is indicative of high concentrations of polysaccharides, often a main factor associated with the ability of bacteria to form a strong and well organized multicellular structure. The presence of extracellular polysaccharides in abundant amounts also has been observed in a histologic analysis of corneal sections from rabbits with crystalline lesions induced by a *Streptococcus sanguis* type II strain, but were absent in the eyes that developed suppurative stromal lesion [132]. Interestingly, this study showed that *S. sanguis* grown in medium supplemented with sucrose produced exopolysaccharides, resulting in a mucoid phenotype and a crystalline lesion similar to ICK in 71% of the corneas inoculated. In contrast, strains grown without sucrose showed a rough phenotype, caused crystalline lesions in only 25% of the eyes, and were more frequently associated with suppurative infiltrates [132].

It is unclear what cues induce microbes to grow as planktonic cells or invade tissues and form a biofilm in ICK. Prolonged topical corticosteroid therapy and/or prior penetrating keratoplasty have been identified as risk factors for the development of ICK in the vast majority of patients [123–126,128–131]. Anatomical modification from the keratoplasty procedure, often resulting in inflammation and altered local immune activity, appears to predispose microbes to grow in a biofilm, but the underlying mechanism has not been thoroughly explored.

As for other biofilm-related infections, antimicrobial treatment of ICK is challenging in that it is usually prolonged and the disease is often unresponsive. Physical means have been explored for improving the success of ICK treatment, including the use of laser disruption of biofilms [133,134]. In this case, Nd:YAG (neodymium-doped yttrium aluminum garnet) laser photocoagulation was used to disrupt the crystalline deposits in the cornea of patients unsuccessfully treated with antibiotics. In all cases, laser disruption with further antimicrobial therapy resolved ICK within weeks, and no recurrence was observed.

6. Biofilms Associated with Other Implant-Related Ocular Infections

6.1. Scleral Buckles

The placement of permanent scleral buckles between the conjunctiva and sclera is a common surgical treatment for rhegmatogenous retinal detachment. The bands encircling the sclera are commonly made of silicone and may have a solid or sponge form. One of the main complications associated with this surgery is the extrusion of the bands, which is frequently associated with an infection. Scleral buckle-associated infections are frequently caused by Gram positive cocci, especially coagulase-negative staphylococci, and nontuberculous *Mycobacterium* [135,136]. These infections often have a delayed onset and are usually refractory to antimicrobial therapy, requiring removal of the bands for complete resolution [135]. Due to the chronic evolution of this infection, the presence of a biofilm in the explanted material has been assumed to play an important role in its

pathogenesis. Biofilms have been demonstrated by scanning electron microscopy for 65% of scleral buckles (solid and sponge forms) removed for infection and extrusion [137]. Gram positive bacteria, and less frequently *Mycobacterium chelonae* and *Proteus mirabilis*, were cultured from these buckle elements [137]. Buckle materials removed from patients because of conjunctival erosion and infection, or due to technical reasons at the time of revision surgery, contained demonstrable bacterial biofilms in 5 out of 28 cases examined by scanning electron microscopy following fixation with ruthenium red [138]. Of those five, one was removed due to extrusion, and one was associated with a diagnosed infection. The three remaining buckles were removed for other reasons from patients lacking signs of infection. It seems likely that at the time of surgery, bacteria attach to the buckle material and form biofilms that lead to indolent infections, or infections lacking any overt signs.

6.2. Conjunctival Plug

Punctual plugs are frequently used to treat ocular surface dryness unresponsive to topical medication, by occluding the lacrimal ducts and blocking tear drainage. Plugs are made of silicone, hydrophobic acrylic, collagen and hydrogel. However, secondary complications may occur following implantation, including canaliculitis, dacryocystitis and acute conjunctivitis [139,140]. Although the presence of biofilms has not been demonstrated on plugs recovered from patients with dacryocystitis and canaliculitis, these infections typically have a late onset and are usually not responsive to antimicrobial treatment alone, requiring additional intervention, as is typical for biofilm-related infections [141,142]. Examination of punctual plugs removed from patients without clinical signs of infection revealed the presence of bacterial biofilms in 53% of the samples assessed by electron microscopy [143]. However, since the patients were asymptomatic with respect to infection, the causal association between the biofilms growing on punctual plugs and progress to an eye infection remains speculative. A single case of conjunctivitis has been associated with biofilm formation on a punctual plug [140]. That patient presented with acute conjunctivitis in the same eye that had received a punctual plug five and half months earlier. A whitish material that was culture positive for *S. haemolyticus* and *Candida tropicalis* was present in the top of the plug. Analysis of this plug by scanning electron microscopy demonstrated the presence of a bacterial biofilm. The plug was removed and the patient resolved the infection after 1 month of topical antimicrobial treatment [140].

6.3. Lacrimal Intubation Devices and Orbital Implants

Lacrimal intubation devices including lacrimal stents and Jones tubes are commonly used during the dacryocystorhinostomy procedure to treat nasolacrimal duct obstruction, a common cause of epiphora. As for other biomaterials implanted in the eyes, both lacrimal stents and Jones tube may provide with a surface for biofilm formation [144–146]. Biofilm formation on polyurethane nasolacrimal stents has been associated with delayed failure of the device [145]. In a study undertaken to identify the rates of biofilm colonization on silicone stents inserted during dacryocystorhinostomy, 90% of stent fragments removed 8 weeks after surgery revealed the

presence of coccoid and/or rod-shaped bacteria encased in a biofilm matrix [144]. All patients included in this last study had received postoperative antibiotics for 1 week (oral) and 3 weeks (topical) and the silicone tubes were collected at the 8th week post-surgery. Most of these silicone stents were culture positive for *S. epidermidis* and *P. aeruginosa* [144].

A report of 2 cases of nasolacrimal infection following placement of a lacrimal silicone and a Jones tube described recalcitrant culture-negative infections associated with the presence of bacterial biofilms in the lacrimal intubation devices [147]. Evaluation of both silicone stent and Jones tube by scanning electron microscopy revealed the presence of a polymicrobial biofilm. Interestingly, the authors were able to identify a variety of cell morphologies in the silicone stent biofilms, including short rods, spirochetes, fusiforms and cocci. Analysis of the internal surfaces of the silicone stent by confocal laser scanning microscopy revealed the presence of viable biofilms along the tube [147].

In a series of cases with recalcitrant infections associated with silicone stents ($n = 10$) and Jones tube implants ($n = 2$), a high prevalence of *Mycobacterium chelonae* (90%) was found associated with the silicone stents, sometimes along with other bacterial organisms [146]. In this same study, the authors evaluated the culture results and presence of biofilms in other periorbital biomaterials, including orbital plates ($n = 5$) and anophthalmic socket sphere implants ($n = 4$) [146]. Cultures were positive for *S. aureus* in all orbital spheres, in addition to one isolate of *M. chelonae* and one *Pantoea agglomerans* that were found in polymicrobial cases. Culture results of orbital plates demonstrated more species diversity with the isolation of yeasts (*Candida* spp., and *Trichosporon* spp.), *Staphylococcus* spp., *M. chelonae* and Gram negative bacilli (*Achromobacter xylosoxidans* and *P. aeruginosa*). Scanning electron microscopy analysis of selected samples also demonstrated the presence of mixed species biofilms on porous polyethylene orbital floor implant, metal screws from orbital plate implant and on orbital sphere implants [146].

7. Perspectives on Agents for Prevention and Treatment of Biofilms

Since biofilms have been recognized for their great medical importance, efforts have been made to either prevent their formation, or to remove them once they have formed. The colonization of surfaces can be prevented by covalently attaching biocidal molecules, slowly releasing antibiotics, or modifying the surface topology to interfere with microbial adhesion. While the first two approaches would be practically easier to achieve as it depends basically on coating current ocular devices with available molecules, the last may be more challenging as modifications in the topology of the material may alter its optical clarity. However, both approaches have their advantages and disadvantages. As an example for prevention of surface colonization using biocides, in a previous study authors have demonstrated a 100-fold reduction in cell counts when *S. aureus*, *S. epidermidis*, *E. coli*, or *P. aeruginosa* were sprayed onto glass slides that were coated with covalently attached poly(4-vinyl-*N*-alkylpyridinium bromide) or *N*-hexylated poly(4-vinylpyridine) [148]. Surface coatings that slowly release antibiotics, such as rifampin, clarithromycin and doxycycline, were able to prevent biofilm formation for up to three weeks *in vitro* [149]. An IOL designed to release norfloxacin to prevent postoperative bacterial infection after cataract surgery has been tested *in vitro* and in a rabbit model, and might soon become

commercially available [150]. Antimicrobial peptides have also been used successfully to prevent biofilm formation and have the added advantage that they are active against antibiotic-resistant strains [151]. Other molecules that can prevent the formation of biofilms, such as gallium nitrate or silver, have also shown potential [151]. While molecules that are slowly released from a surface to prevent biofilm formation show great promise for some applications, questions remain regarding their use in medical devices. The concentration of the antimicrobial agent would have to remain sufficiently high as long as colonization and infection is a risk, and not select for resistant strains. In addition, lack of toxicity and long-term compatibility with surrounding tissues is important [152]. The advantages of using ocular devices that slowly release antimicrobial agents is that tissue toxicity, penetration and half-life is already known for a number of antibiotics routinely used in ophthalmology. On the other hand, long-term exposure to these drugs may favor selection of spontaneous resistant mutants and perturbs the ocular surface microbiome.

Modifying the surface structure of ocular devices to make it less adhesive for bacteria attempting to colonize is a tempting approach since it would potentially eliminate the need for coating with antimicrobial or biocide agents that could be reserved for treatment and perioperative prophylaxis. However, alterations in the material topology that results in material opacities may limit its use for optical correction. Polymers, such polyacrylamide, dextran, or polyethylene glycol, can form linear, star-shaped, or 'bottle brush' shaped surface nanostructures that interfere with the microbe's ability to adhere to the substrate [153,154]. Nanopores, nanotubes, and nanopillars made of anodized aluminum, titanium dioxide, or polymethylmethacrylate have also been investigated to reduce microbial adhesion to coated surfaces [152]. Even low-fouling substrates may eventually become colonized due to degradation or erosion of the anti-adhesive surface [154], so this will have to be explored. Furthermore, some medical devices require the firm binding of the implant to the surrounding tissue for optimal biointegration, which may limit the use of this strategy in some cases. Other devices, such as contact lenses, may be difficult to modify this way without negatively affecting critical properties, such as optical clarity.

An alternative strategy for the removal of microbial biofilms is to stimulate the reversion of microbes to planktonic physiology. While the enzymes that degrade the ECM or the substratum might be too large and costly to be of practical clinical value, small signaling molecules that induce expression of factors that stimulate the dissimulation of biofilms might be a viable alternative. Cell signaling molecules, such as C4HSL, PQS, AI-2, and AIP-I or their derivatives, may be of great therapeutic value [35,38].

While typical antibiotics work well against growing planktonic cells and are less active, or inactive, against the dormant cells in a biofilm, they may still have a use in combination therapies. The acyldepsipeptide antibiotic, ADEP4, was shown to bind to the ClpP protease of *S. aureus* and convert it into a nonspecific protease that degrades over 400 proteins, killing growing as well as dormant cells [155]. Treatment with ADEP4 resulted in the emergence of ADEP4-resistant *clpP* mutants, but those were highly susceptible to killing by various antibiotics. Using a deep-seated mouse biofilm infection model, the authors showed that ADEP4 in combination with rifampicin was able to reduce the number of *S. aureus* below detectable limits, while neither rifampicin, vancomycin, nor ADEP4 by themselves were able to do so [155]. The use of bacteriophage

endolysins as well as engineered phages expressing anti-biofilm enzymes may also be promising options for eradication of bacterial biofilms in the site of infection [156,157]. Evolutionary distinct bacteriophage endolysins have shown to be effective in killing planktonic cells as well biofilms of *S. aureus* and prevented death of 100% of mice inoculated intraperitoneally with lethal doses of MRSA [157]. A T7 bacteriophage engineered to express dispersin B (DspB), an enzyme that hydrolyzes β -1,6-*N*-acetyl-D-glucosamine, has been successfully used to simultaneously infect and kill the bacterial cells in the biofilm, in this case *E. coli*, and also attack the extracellular polymeric biofilm matrix [156].

The field of biofilm dispersal and eradication is an area of very active research and the next five years will see a substantial increase in our understanding of the physiological states associated with biofilms. It is hoped that this will lead to the development of new agents that will pass clinical trials and serve as new treatments for microbial infections caused by cells in a biofilm state.

8. Conclusions

Our understanding of biofilms has advanced substantially since early descriptions more than three decades ago. As medical interventions rely increasingly on medical devices and prosthesis, the need to prevent, reduce, or eliminate microbial biofilms is becoming an important constraint. In the eye care field, contact lenses and IOLs have had a great impact on restoring and improving vision, but their use is limited by ocular infection. Strategies, such as anti-biofilm surface coatings and developing biofilm-active therapeutics, are exciting avenues of future research to reduce the risk of biofilm-associated ocular infection.

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Conflicts of Interest

The authors declare no conflict of interest.

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Role of Daptomycin in the Induction and Persistence of the Viable but Non-Culturable State of *Staphylococcus Aureus* Biofilms

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Abstract: We have recently demonstrated that antibiotic pressure can induce the viable but non-culturable (VBNC) state in *Staphylococcus aureus* biofilms. Since dormant bacterial cells can undermine anti-infective therapy, a greater understanding of the role of antibiotics of last resort, including daptomycin, is crucial. Methicillin-resistant *S. aureus* 10850 biofilms were maintained on non-nutrient (NN) agar in the presence or absence of the MIC of daptomycin until loss of culturability. Viable cells were monitored by epifluorescence microscopy and flow cytometry for 150 days. All biofilms reached non-culturability at 40 days and showed a similar amount of viable cells; however, in biofilms exposed to daptomycin, their number remained unchanged throughout the experiment, whereas in those maintained on NN agar alone, no viable cells were detected after 150 days. Gene expression assays showed that after achievement of non-culturability, 16S rDNA and *mecA* were expressed by all biofilms, whereas *glt* expression was found only in daptomycin-exposed biofilms. Our findings suggest that low daptomycin concentrations, such as those that are likely to obtain within biofilms, can influence the viability and gene expression of non-culturable *S. aureus* cells. Resuscitation experiments are needed to establish the VBNC state of daptomycin-exposed biofilms.

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1. Introduction

Biofilm production protects bacteria from a number of stress conditions [1,2], it promotes antibiotic resistance [2,3] and is often related to the onset of persistent infections [1,2], especially those associated with indwelling medical devices [3,4].

The continuous increase in antimicrobial resistance hampers the treatment of infections. The success of multidrug-resistant Gram-positive pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA) [5–7], vancomycin-resistant enterococci (VRE) [8] and coagulase-negative staphylococci [9], emphasizes the need for new antimicrobials with alternative mechanisms of action. Daptomycin is a cyclic anionic lipopeptide antibiotic produced by *Streptomyces roseosporus* that, in the EU, has been approved to treat skin and soft-tissue infections since 2006 [10]. Daptomycin has bactericidal activity against Gram-positive bacteria, including MRSA [11,12] and VRE [13], and is currently the last line of defense against severe Gram-positive infections. It has a unique, but not completely elucidated, mechanism of action, where a calcium-dependent dissipation of membrane potential leads to the release of intracellular ions from the cell and,

ultimately, to death [14]. Daptomycin is effective in treating skin infections, endocarditis and bacteremia [15] and in counteracting biofilm-based infections associated with medical devices [16,17], which frequently require combination therapy [18]. The combination with rifampicin or beta-lactams has been reported to be effective in treating biofilm-related enterococcal [18] and staphylococcal [12] infections.

The viable but non-culturable (VBNC) state is a survival strategy characterized by low-level metabolic activity and bacterial growth failure on standard media [19]. It protects bacterial cells from environmental stress, such as nutrient depletion, changes in temperature, pH or salinity [20], and presence of antibiotics [21]. The VBNC state has been reported for several human pathogens [19], including biofilm-producing staphylococci [21]. The critical feature of cells in the VBNC state is their ability to regain culturability in the presence of resuscitation-promoting factors [22]. In a recent *in vitro* study by our group, vancomycin and quinupristin-dalfopristin, which are often used to treat biofilm-associated chronic infections [23], have been demonstrated to promote the emergence of persistent VBNC forms in *S. aureus* biofilms [22]. These findings prompted us to establish whether daptomycin, which is considered as a last line of defense antibiotic, also induces the VBNC state.

2. Results and Discussion

2.1. Biofilm Production, Stress Exposure and Non-Culturability

The strong biofilm producer, *S. aureus* 10850 [21,24], was analyzed for susceptibility to daptomycin by MIC determination and showed low-level resistance with a MIC value of 2 $\mu\text{g/mL}$ (European Committee on Antimicrobial Susceptibility Testing (EUCAST) susceptibility breakpoint $\leq 1 \mu\text{g/mL}$).

To induce the VBNC state, *S. aureus* 10850 biofilms developed on membrane filters were placed on non-nutrient (NN) agar plates without or with daptomycin (at a concentration equal to the MIC) to induce the largest possible amount of VBNC cells [21] and incubated at 37 °C. Culturability was tested every two days.

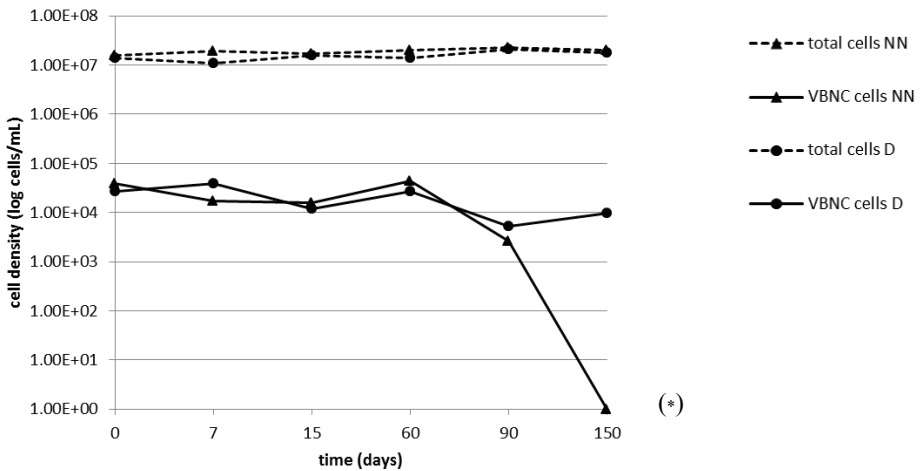
All biofilms reached non culturability in 40 days. They were then detached from the filters, stained by the live/dead method, and examined for viable cells by epifluorescence microscopy. All filters were culture-negative and contained green coccoid cells whose average counts were $3.8 \times 10^4/\text{mL}$ (starved biofilms) and $2.7 \times 10^4/\text{mL}$ (starved + daptomycin-exposed biofilms).

2.2. Persistence of the VBNC State

After achievement of non-culturability on the 40th day, the persistence of the VBNC state was monitored by epifluorescence microscopy for 150 days in biofilms maintained on NN agar plates without or with daptomycin (Figure 1). Live/dead staining of a non-culturable daptomycin-exposed biofilm on the 15th day followed by flow cytometry demonstrated a viable subpopulation (Table 1), whose abundance was comparable to that documented by epifluorescence counts (differences $\leq 0.5 \log$).

The number of viable cells remained substantially unchanged throughout the experiment in starved and daptomycin-exposed biofilms; in those maintained on NN agar alone, it did not change significantly over the first 90 days, but no viable cells were left on the 150th day (Figure 1, Table 1).

Figure 1. Epifluorescence counts after live-dead staining of viable and total cells in detached non-culturable *S. aureus* 10850 biofilms exposed to nutrient depletion with (D) or without (NN) daptomycin. Counts were performed at various intervals (0–150 days) from the loss of culturability. The results are the means of two counts. VBNC, viable but non-culturable.



* No viable cells detected by epifluorescence microscopy (< 60 cells/mL).

Table 1. Total and viable cells counts in biofilms exposed to nutrient depletion or nutrient depletion with daptomycin.

Stress Condition	Days Since Achievement of Non-Culturability	Cells/mL	
		Total	Viable
Nutrient depletion	0	1.6×10 ⁷	3.8 × 10 ⁴
	7	1.9 × 10 ⁷	1.7 × 10 ⁴
	15	1.7 × 10 ⁷ /1.5 × 10 ⁸ *	1.6 × 10 ⁴ /8.2 × 10 ⁴ *
	60	2.0 × 10 ⁷	4.4 × 10 ⁴
	90	2.3 × 10 ⁷	2.6 × 10 ³
	150	2.0 × 10 ⁷	<60
Nutrient depletion + daptomycin	0	1.4 × 10 ⁷	2.7 × 10 ⁴
	7	1.1 × 10 ⁷	3.9 × 10 ⁴
	15	1.6 × 10 ⁷ /1.4 × 10 ⁸ *	1.2 × 10 ⁴ /7.6 × 10 ⁴ *
	60	1.4 × 10 ⁷	2.7 × 10 ⁴
	90	2.1 × 10 ⁷	5.3 × 10 ³
	150	1.8 × 10 ⁷	9.8 × 10 ³

* Flow cytometric analysis.

These findings indicate that daptomycin exposure extended the viability of non-culturable cells compared with starvation alone, whereas nutrient depletion rather seemed to give rise to premortem VBNC forms, supporting previous data by our group [21].

2.3. Gene Expression of VBNC Cells

The presence of viable subpopulations after loss of culturability was tested by gene expression experiments. Non-culturable biofilms, either starved and starved and daptomycin-exposed, were tested for the expression of two *S. aureus* housekeeping genes, 16S rDNA and *glt* (coding for glutamate synthase). Aliquots of non-culturable biofilms detached 0, 7, 15 and 60 days from the loss of culturability were analyzed by real-time RT-PCR. The expression of 16S rRNA was detected in all biofilms tested, whereas *glt* was expressed exclusively in daptomycin-exposed biofilms at 0, 7 and 15 days (Table 2). Since 16S rRNA quantification is considered a reliable assay of viability [25], these findings support the epifluorescence and cytofluorimetric evidence of the presence of VBNC cells, in line with data from an earlier study by our group [21]. Given that glutamate synthase is involved in the incorporation of ammonium ions into organic compounds, which is a key step in amino acid production [26], it could have a role in the persistence of a true viable state in non-culturable biofilms via continued amino acid uptake and incorporation [27].

The ability of non-culturable biofilms to express virulence and antibiotic resistance genes was explored by further real-time RT-PCR assays targeting the thermonuclease (*nuc*) and the methicillin resistance penicillin binding protein 2a (PBP2a) (*mecA*) genes. *nuc* expression was detected in none of the biofilms analyzed and *mecA* expression in all of them (Table 2). These findings suggest that thermonuclease may not be essential for biofilm survival *in vitro*. Moreover, the paucity of metabolically active cells suggests that its expression could be inhibited by quorum sensing [28]. Conversely, the *mecA* gene encodes a PBP involved in cell wall formation [5] that could play an important role in maintaining the wall of VBNC cells intact and functional.

Table 2. Expression of key genes in non-culturable *S. aureus* 10850 biofilms exposed to nutrient depletion with or without daptomycin.

Stress Condition	Time from non Culturability	Gene Analysis			
		<i>16SrDNA</i>	<i>glt</i>	<i>nuc</i>	<i>mecA</i>
Nutrient depletion	T0	+	ND	ND	ND
	T7	+	-	-	+
	T15	+	-	-	+
	T60	+	-	-	+
Nutrient depletion + daptomycin	T0	+	ND	ND	ND
	T7	+	+	-	+
	T15	+	+	-	+
	T60	+	-	-	+

ND: not determined.

Taken together, the findings of our experiments seem to indicate that daptomycin acts as an inducer of the VBNC state of *S. aureus* by modulating gene expression, activating or repressing the

transcription of specific genes. This effect may be explained by a poor penetration of daptomycin and the achievement of subinhibitory concentrations in the deep layers of the *S. aureus* biofilm matrix.

Indeed, low concentrations of a number of antibiotics exert biological activities other than inhibition, with major effects on transcription patterns [29]. The phenomenon, known as hormesis, involves biological responses to environmental signals or stress conditions that are characterized by biphasic dose-response relationships exhibiting low-dose stimulation and high-dose inhibition [30]. Subinhibitory concentrations of antimicrobial peptides, such as the cyclic lipopeptide daptomycin, which causes rapid membrane depolarization and potassium ion efflux, can thus increase the transcription levels of osmoprotectants, countering the osmotic stress, and downregulate the ribose transport system [30]. Given that ribose is a key element for ATP and RNA synthesis, this condition could be a stress factor for bacterial cells, contributing to VBNC state induction and persistence.

Since increased cell wall thickness is typical of the VBNC state [19], it may be hypothesized that low daptomycin concentrations stimulate the synthesis of peptidoglycan genes, as reported for imipenem in studies of the *Pseudomonas aeruginosa* transcriptome [30]. On the other hand, an involvement of daptomycin in the regulation of cell wall synthesis is quite likely, given that PBPs are membrane proteins. A role for daptomycin may also be inferred based on its reported synergism with beta-lactams against staphylococcal biofilms [12].

3. Experimental Section

3.1. Bacterial Strains, Media, Antibiotics and Enzymes

The strong biofilm producer, *S. aureus* 10850 [24], was routinely grown in tryptic soy broth (TSB) or agar (TSA) (Oxoid, Basingstoke, U.K.), supplemented with 1% (v/v) glucose (TSBG or TSAG) to promote biofilm production. M9 minimal medium without glucose was used as NN agar in VBNC induction assays, as described by Pasquaroli *et al.* [21]. The following antibiotics and enzymes were used: daptomycin (Cubicin, Novartis Pharma SpA, Italy) and lysozyme and lysostaphin (Sigma-Aldrich St Louis, MO, USA).

3.2. MIC Determination

The MIC of daptomycin was determined by a broth microdilution method, and *S. aureus* susceptibility was defined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [31]. The test medium was MHII broth (Becton-Dickinson, Milan, Italy) supplemented with CaCl₂ (calcium chloride; Merck KGaA, Darmstadt, Germany) to a final Ca²⁺ concentration of 50 µg/mL. *S. aureus* ATCC 29213 was used as the control strain.

3.3. Biofilm Production, Stress Exposure and Culturability Assays

In vitro biofilm production, stress exposure and culturability assays were performed as described by Pasquaroli *et al.* [21]. Briefly, 100 µL of a late-log culture of *S. aureus* 10850 grown in TSBG was spotted on 0.22-µm sterile nitrocellulose filters (Millipore Corporation, Billerica,

MA, USA); the filters were placed onto TSAG plates for 48 h at 37 °C to allow biofilm development at the filter-air interface. They were then moved to NN agar plates, unsupplemented or supplemented with daptomycin (2 µg/mL), and incubated at 37 °C until loss of culturability. Filter cultures were transferred weekly to fresh agar plates without washing. Culturability was assessed every 2 days by placing a loop of filter cultures in TSB and onto TSA, followed by incubation for 48 h at 37 °C. Filter cultures testing negative on culturability assays were placed in 5 mL saline, detached by 3 cycles of sonication and vortexing, washed and resuspended in the same volume of saline.

3.4. Epifluorescence Microscopy and Flow Cytometry

Epifluorescence microscopy and flow cytometry counts were performed as described previously [21]; each count was carried out in duplicate. The limit of detection of epifluorescence counts was 60 cells/mL of detached biofilm.

3.5. Real-Time RT-PCR Assays

Total RNA was extracted from 5 mL of detached biofilm using the RNeasy Mini Kit (Qiagen, Hilden, Germany), as described previously [21]. Total RNA was retro-transcribed using Qiagen's QuantiTect Reserve Transcription kit.

Real-time PCRs were carried out in a total volume of 20 µL containing 0.25 µM of each primer (Table 3), 10 µL of 2× Supermix (Qiagen) and 2 µL of reverse transcription mixture. Cycling conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, different annealing temperatures (Table 3) for 20 s and 72 °C for 20 s. Amplification reactions and melt-curve analysis were performed using the Rotor-Gene Q MDx (Qiagen). cDNA obtained starting from a broth culture of *S. aureus* 10850 was used as a positive control.

Table 3. Target genes and primer pairs used in gene expression assays.

Target Gene	Gene Function	Primer Pair (5'-3')	Annealing Temperature (°C)	Product Size (bp)	Reference
<i>16S rDNA</i>	Housekeeping	F-TGGAGCATGTGGTTTAATTCGA R-TGCGGGACTTAACCCAACA	60	159	[32]
<i>glt</i>	Species specific, coding for glutamate synthase	F-AATCTTTGTCGGTACA CGATATTCTTCACG R-CGTAATGAGATTCA GTAGATAATACAACA	58	108	[33]
<i>nuc</i>	Virulence factor, coding for thermonuclease	F- GACTATTATTGGTTGATCCACCTG R- GCCTTGACGAACTAAAGCTTCG	60	218	[34]
<i>mecA</i>	Methicillin resistance	F-TCCAGATTACAACCTCACCAGG R-CCACTTCATATCTTGTAACG	57	162	[35]

4. Conclusions

The present findings provide evidence that daptomycin may play a role in the induction and persistence of VBNC *S. aureus* biofilms by showing cell viability and gene expression for months after achievement of non-culturability. Infections caused by staphylococcal biofilms should be treated by carefully selected and dosed antibiotics, to maintain drug concentrations capable of exerting full inhibitory activity. The ability of non-culturable daptomycin-exposed staphylococcal biofilms to resuscitate requires additional testing. Further experiments are under way in our laboratory.

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Author Contributions

Sonia Pasquaroli designed the experiments, cultured the biofilms, performed RNA extractions, epifluorescence and cytofluorimetric counts and wrote the paper draft. Barbara Citterio contributed to monitoring non-culturability, to extracting RNA from non-culturable biofilms and to performing retro-transcription assays. Andrea Di Cesare and Mehdi Amiri performed the RT-PCR assays and analyzed the data. Anita Manti performed the cytofluorimetric analyses. Claudia Vuotto analyzed the biofilm production by SEM. Francesca Biavasco conceived of the experiments, analyzed the data and wrote the paper.

Conflicts of Interest

The authors have no conflicts of interest.

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Evolution of Antimicrobial Peptides to Self-Assembled Peptides for Biomaterial Applications

Alice P. McCloskey, Brendan F. Gilmore and Garry Laverty

Abstract: Biomaterial-related infections are a persistent burden on patient health, recovery, mortality and healthcare budgets. Self-assembled antimicrobial peptides have evolved from the area of antimicrobial peptides. Peptides serve as important weapons in nature, and increasingly medicine, for combating microbial infection and biofilms. Self-assembled peptides harness a “bottom-up” approach, whereby the primary peptide sequence may be modified with natural and unnatural amino acids to produce an inherently antimicrobial hydrogel. Gelation may be tailored to occur in the presence of physiological and infective indicators (e.g. pH, enzymes) and therefore allow local, targeted antimicrobial therapy at the site of infection. Peptides demonstrate inherent biocompatibility, antimicrobial activity, biodegradability and numerous functional groups. They are therefore prime candidates for the production of polymeric molecules that have the potential to be conjugated to biomaterials with precision. Non-native chemistries and functional groups are easily incorporated into the peptide backbone allowing peptide hydrogels to be tailored to specific functional requirements. This article reviews an area of increasing interest, namely self-assembled peptides and their potential therapeutic applications as innovative hydrogels and biomaterials in the prevention of biofilm-related infection.

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1. Introduction

Biomaterials have an increasingly important role in patient care. Approximately five million medical devices and implants are used in the US each year [1]. They are beneficial not only as temporary interventions but also as permanent features to replace or facilitate normal bodily functions. There is a diverse array of such materials available including: intravascular and urinary catheters, heart valve prostheses, artificial hip joints, dental implants, and intraocular lenses [2]. Their use is particularly prominent in modern medicine as population demographics demonstrate an increasing trend toward an ageing population, in tandem with increased life expectancy and improved healthcare [3].

Grainger and colleagues researched the contribution medical devices have to improving patients' quality of life [4]. They concluded that 50% of patients with aortic heart valve disease could potentially die within three years of diagnosis. Valve replacements resulted in an increase in ten year survival to approximately 70% of patients. Despite this improvement, 60% of these patients were shown to have device-associated complications thus affecting patient quality of life. Implantation of a medical device is an invasive surgical procedure that may result in irritation, wounding and infection of the surgical site. A macrophage-mediated collection of immune responses, collectively

referred to as the host or foreign body response, is triggered leading to compromised device function and scarring of surrounding tissue [5]. Anderson and colleagues provide a comprehensive outline of the sequence of events following surgical implantation [6]. The initial response follows the normal wound-healing cascade of: injury, blood-material interactions, provisional matrix formation, acute inflammation, chronic inflammation, granulation tissue development, foreign body reaction, and fibrosis. The resulting host or foreign body response to a device is estimated to affect approximately 5% of patients and can have a significant impact on patient recovery and long-term health. Surgical trauma can result in a compromised immune response leading to unchallenged accumulation and adherence of pathogens at the site of implantation, resulting in biofilm formation. Biomaterials provide an optimal surface for biofilm formation [1,7]. The long-term effects are considerable. Economically, biomaterial associated infections have costly implications on health-care budgets. Bacterial accumulation and the subsequent formation of a biofilm contributes to an infectious profile 10–1000 times more resistant to standard therapeutic regimens, leading to compromised implant function and/or failure [8]. Symptomatic infection often occurs within 2 weeks of implantation [4,6,7,9].

Biofilms are phenotypically heterogeneous, sessile microbial communities that form at biotic and abiotic sites. They may be composed of a single or multiple species (poly-microbial) of bacteria and fungi [10–12]. Biofilm formation can be divided into three significant stages: (1) initial non-specific, reversible primary adhesion; (2) specific irreversible adhesion (coating of the device, transport of cells to the interface, adhesion of cells; accumulation at the surface involving biomolecular processes including quorum sensing, up-regulation of virulence factors and secretion of extracellular polymers; (3) biofilm detachment/dispersal and recolonization of alternative sites. Following implantation the surface chemistry of a biomaterial is modified by the laying down of a host-derived conditioning film. Primary adhesion is dependent on two key factors—hydrophilicity and charge at the cell-device interface. Primary adhesion is a non-specific process mediated by hydrophobic and van der Waal's interactions between the microbial cell and the generally hydrophobic surface, as is the case for silicone [1,10,11]. Binding sites such as fibrinogen, fibronectin, vitronectin, binding autolysin, albumin and extracellular matrix binding protein become exposed on the biomaterial surface. Microbial adhesins, for example teichoic acid expressed in *Staphylococcus epidermidis*, specifically recognize binding sites and mediate adherence to the surface via covalent attachment.

The potential for microbial spoilage is not confined to hydrophobic surfaces. More hydrophilic materials, for example Teflon[®], also suffer from microbial contamination due to mechanical rather than chemical factors [13]. MacKintosh and co-workers investigated how the modification of poly(ethylene terephthalate) surface chemistry affected bacterial-biomaterial interaction, attachment and subsequent biofilm formation of *Staphylococcus epidermidis* [14]. They demonstrated that hydrophobic interactions do influence the adhesion of bacteria to the material surface. Of greater significance is the presence of serum proteins within the conditioning film. These tend to have a greater effect on adhesion and biofilm formation *in vivo*. Similar research performed by the Gottenbos group suggested that biofilm formation is a complex relationship involving not only the biomaterial surface properties and the ability of the bacteria to bind to a given surface, but also the availability of nutrients at the site of adherence [15].

Accumulation on a surface occurs when bacterial cells multiply and form multi-layered cell clusters resulting in a complex three dimensional architecture- the classic biofilm [16]. The biofilm is maintained through specific quorum sensing pathways. Quorum sensing is a communicatory and regulatory process controlling the up- and down-regulation of genes that govern virulence and adhesion factors, relative to environmental conditions and population density. It is an essential process for biofilm survival ensuring that biofilms act as a mutualistic community rather than individual cells [17]. As the microbial population increases the supply of nutrients and oxygen become limited within the confines of the biofilm. Therefore biofilm bacteria generally grow more slowly than planktonic bacteria due to a reduced rate of respiration/metabolism. The protective environment, provided by the polymeric exopolysaccharide matrix, means that bacteria are much more resistant to the host's immune response and therapeutic antimicrobials than their planktonic counterparts [1,18,19]. Biofilm mediated infections are thus extremely difficult to treat and the extent and rate of eradication is largely determined by the most resistant phenotype within the biofilm. Chemotherapeutic failure often results in surgical removal of the biomaterial, particularly those involving *Staphylococcus aureus* and *Candida* species [7]. Removal, combined with potential chemotherapeutic failure, is not an ideal scenario as concerns grow regarding increased antimicrobial resistance, and the relative lack of new antimicrobials in development [20–22].

Typical biofilm related medical device infections include: Catheter associated urinary tract infections; peristomal skin infections following insertion of percutaneous endoscopic gastrostomy feeding tubes; and pneumonia or tracheobronchitis with tracheostomy devices. The most commonly implicated pathogens are: staphylococci, enterococci, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas*, and *Candida* [19,23,24]. The prognosis for such infections depend on the patient's initial health, which in many cases is poor due to age and co-morbidities, and the duration of implantation. Medical implants are commonly required in immunocompromised patients. Therefore, insertion of an implant and the resulting trauma further compromises the immune response increasing patient recovery time and morbidity.

Numerous strategies to reduce biomaterial-associated infections have been developed but few have translated to clinical practice [9]. Hospital stays can be up to two and a half times longer than for uninfected patients, with a total of 3.6 million extra days being spent in hospital per year in England. Nosocomial infections cost the health sector in England almost £1 billion per year [25]. In the United States medical device related infections contribute to over 50,000 deaths per year [4]. As for the majority of disease states prevention is the key aim. Poor hygiene practice within the healthcare setting has been shown to increase the risk of infection. Simple measures such as correct hand washing technique, by both staff and patients, can have a dramatic decrease in infections [26]. There is an increasing demand globally for medical devices to replace normal physiological function. Therefore managing and preventing implant associated infections is a huge challenge [1]. These problems have to be addressed on a global-scale and require the development of biomaterials that are both biocompatible and anti-infective. This review examines the current strategies employed to reduce the occurrence of biofilm mediated device related infections and investigates the potential of future innovative strategies, namely peptide based biomaterials.

2. Current Research Based Strategies for the Prevention of Medical Device Related Infection

Biomaterials cover a diverse range of pharmaceutical applications from drug delivery to tissue engineering [27]. Every device is prone to infection. Surfaces are particularly vulnerable to biofilm formation. Therefore antimicrobial coatings are a plausible solution for the development of devices with anti-infective properties. Implantation of medical devices may be classified as temporary or permanent/long-term. Temporary devices, for example contact lenses, are not fully integrated into the host tissue. Other internally-based devices, for example heart valves, tend to be more permanent. Prevention of temporary device-related infections can be managed with non-adhesive, antimicrobial impregnated or releasing coatings, which kill bacteria that come into contact with the device [9]. Permanent device coatings must be multi-functioning, facilitating incorporation of the device into the host tissue whilst simultaneously preventing microbial adhesion over an extended period within the lifetime of the device. Examples of such coatings include those investigated by the Saldarriaga group [28]. They produced multi-component cross-linked poly(ethylene-glycol) based polymers and demonstrated that the degree of hydration and steric hindrance contributed to the efficacy of these multi-functioning coatings. Hydrogel coatings display great promise as they can incorporate and/or release antimicrobial agents. They allow for improved tissue integration and a reduction in biofilm formation [29]. Hydrogels comprise a group of insoluble, swellable, hydrophilic polymers. When fully swollen they are composed of a significant amount of water (up to ~99%) but also display solid-like properties which provide desirable characteristics such as increased mechanical strength. Hydrogel classification is dependent upon the nature of the crosslinks which bind the hydrogel structure, influencing its swelling ability. Chemical hydrogels are also influenced by the structure of the primary monomer chains and the density of the crosslinks within the polymer system. Hydrogel architecture is also determined by secondary non-covalent molecular interactions and entangled molecules. The presence of a porous three-dimensional network means hydrogels are ideal biomaterials as they structurally similar to the extracellular matrix and tissue. The presence of defined functional groups, for example carboxylic acids, allow for the production of bioactive biomaterials that respond to environmental, chemical and physical stimuli. These so-called 'smart' polymer systems display significant potential as future drug delivery and biomaterial platforms [30–32].

Current research has seen an increased focus on the antimicrobial properties of silver. Hydrogel/silver coated urinary catheters have been investigated and promising results obtained. A reduction in the primary bacterial adherence was observed in comparison to a standard silicone catheter indicating that hydrogel/silver coatings have the potential to delay the onset of catheter associated infections [33]. Synergistic combinations of antibiotic(s) and/or antiseptics are also utilized clinically to reduce the incidence of infection. ARROWgard™ central venous catheters are coated by a combination of silver sulphadiazine and chlorhexidine, whilst the commercially available alternatives BioGuard Spectrum™ and Cook Spectrum® are both coated by a combination of minocycline and rifampicin [34]. Silver sulphadiazine and chlorhexidine coated catheters displayed significant activity against a range of microorganisms including *Candida albicans* and *Escherichia coli* for up to seven days [35]. Minocycline-rifampicin catheters were shown to display only bacteriostatic activity against slime producing forms of *Staphylococcus epidermidis* (ATCC 35984)

and *Staphylococcus aureus* (ATCC 29213) but for an extended period of up to 21 days [36]. A range of other anti-infective biomaterials are available commercially, aiming to deliver antimicrobials locally at the device surface for the prevention of biofilm formation. Many challenges exist with this strategy. It is difficult to ensure the dose of antimicrobial delivered is uniform within the vicinity of the device surface. Areas of the implant may be exposed to sub-therapeutic concentrations of antimicrobials [37]. Sub-inhibitory concentrations of antimicrobial agents may lead to increased microbial resistance. Research by Rachid highlighted this effect whereby an increase in *ica* operon expression [38], linked with staphylococcal polysaccharide intercellular adhesin accumulation [39,40], occurred in response to sub-optimal levels of the antibiotic tetracycline and the semi-synthetic molecule quinuprisin- dalfopristin.

One of the major limitations regarding the delivery of antimicrobials in a biomaterial model is the effect of burst-release. Burst-release is consistent with an initial high and rapid release of the antimicrobial from the biomaterial. It is one of the major challenges of modern drug delivery. The antimicrobial reservoir depletes to sub-inhibitory levels within days allowing infection to develop unchallenged. Covalent attachment of antimicrobial, as outlined by the examples of ARROWgard™, BioGuard Spectrum™ and Cook Spectrum®, suffer from a reduction in activity due to masking of antimicrobials by the host's conditioning film [41]. A significant profile of prolonged release over weeks or an infection responsive system is more desirable [42]. A multitude of studies exist in antimicrobial drug delivery to resolve this issue. Examples include sustained release systems such as calcium-mediated delivery of the broad-spectrum antibiotic minocycline, as demonstrated by Zhang and colleagues [29]. The authors utilized layer-by-layer assembly and calcium binding to create a sustained release platform. Delivery of minocycline is due to a local change in pH in the vicinity of the device. Acidosis present in the tissues as a result of medical device induced inflammation or infection, weakens the chelation reaction between minocycline and calcium ions, resulting in subsequent release of minocycline. This so-called 'smart' approach to drug delivery shows promise for future biomedical application.

There has also been increasing interest in using peptides for biomaterial applications [43]. As antimicrobials, peptides serve as barriers to infection throughout nature as part of the innate immune response [44]. There are a multitude of examples whereby antimicrobial peptides have been synthesized to disrupt microbial biofilms [45–47]. Peptides also display a diverse array of properties making them suitable for biomaterial applications including: increased biocompatibility and minimal immunogenicity; the availability of moieties for functionalization; chemical versatility and biodegradability [48]. Peptides have also demonstrated an ability to self-assemble into supramolecular hydrogels in response to environmental stimuli for example pH, the presence of salts, heat and enzymatic cleavage [49,50]. These characteristics may allow such compounds to be utilized to form inherently antimicrobial peptide hydrogel structures in response to infection.

3. Current Approaches to Self-Assembling Biomaterials

The process of self-assembly is an important parameter for the development of novel biomaterials. It is particularly relevant with regards to nanotechnology. These principles have been adopted from nature. Ribosomes and the quaternary haemoglobin structures are examples of naturally occurring

self-assembled architectures [51]. Assembly involves the spontaneous arrangement of pre-existing, disordered molecules of similar properties, to form higher ordered structures. It is mediated by non-covalent, local interactions: van der Waals forces, hydrogen bonding, π - π stacking, and electrostatic interactions [52,53]. Movement at a molecular level facilitates assembly. Environmental factors such as volume and binding influence assembly. Equilibrium between the aggregate and non-aggregate states is essential to maintain a higher ordered structure [54,55]. Self-assembly has recently attracted heavy investment from both the private and public sectors. In 2010 global public investment was estimated at approximately \$8.2 billion and private sector funding slightly higher at \$9.6 billion [56].

Current polymer technologies include the area of Pluronics or poloxamers. Composed of three polymers polyoxyethylene (PEO), polyoxypropylene (PPO) and polyoxyethylene (PEO), Pluronics is an area that has been extensively studied with regard to micellization and the formation of structures for drug delivery purposes. Pluronics have the ability to assemble at physiological temperatures, in a variety of solvents, and over a range of concentrations as opposed to a single critical concentration [57]. There is a great deal of interest within the drug delivery field in exploiting gels that have the ability to flow at ambient temperatures but form a gel upon exposure to physiological temperatures. These gels have great potential in terms of targeted or localized drug delivery of anticancer and antimicrobial drugs in particular [58]. Self-assembling Pluronics are examples of non-ionic and non-toxic gels [59]. F127 is an example of a Pluronic polymer whereby assembly is triggered due to temperature change. Its ability to form micelles at human body temperature means that F127 has potential in the delivery of poorly soluble, hydrophobic drugs [57]. Sustained release of drugs from Pluronics has been widely investigated. Barichello's group used F127 alone and in addition to poly-co-glycolic acid (PLGA) nanoparticles for protein delivery, using insulin as a model drug [60]. They demonstrated F127 incorporated insulin has the potential to provide a controlled release system. The short-acting, opioid analgesic fentanyl has also been delivered using gels formed by PEO-PPO-PEO. Investigations have shown to demonstrate a similar release flux to the commercially available Durogesic[®] patch [59]. The possibility exists that this type of delivery could be adopted for sustained delivery of antimicrobials to wounds or burns, removing the need for frequent changes of dressings. The antibiofilm activity of Pluronics has been studied due to its non-ionic surfactant-like properties [61]. Wesenberg-Ward and colleagues discovered that Pluronic F127 conditioned polystyrene reduced *Candida albicans* biofilm formation relative to untreated polystyrene controls [62]. Reduction in *Staphylococcus aureus* and *Staphylococcus epidermidis* adherence to polymethylmethacrylate and increased susceptibility to vancomycin and gentamicin was also observed in the presence of poloxamer 407 [63]. These studies highlight the potential use of Pluronics in the biomaterials field to produce surfaces that are resistant to microbial adhesion. Recent research by Leszczyńska outlined the use of Pluronic F127 topical antimicrobial in combination with the synthetic cationic antibacterial peptide Ceragenin CSA-13 [64]. In this instance, Pluronic F127 acted as the drug delivery vehicle with broad spectrum activity demonstrated against methicillin-resistant *Staphylococcus aureus* and a *Pseudomonas aeruginosa* strain isolated from cystic fibrosis patients. Pluronics have also been linked with increased wound-healing in animal models validating continuous research into their applications as effective biomaterials [65].

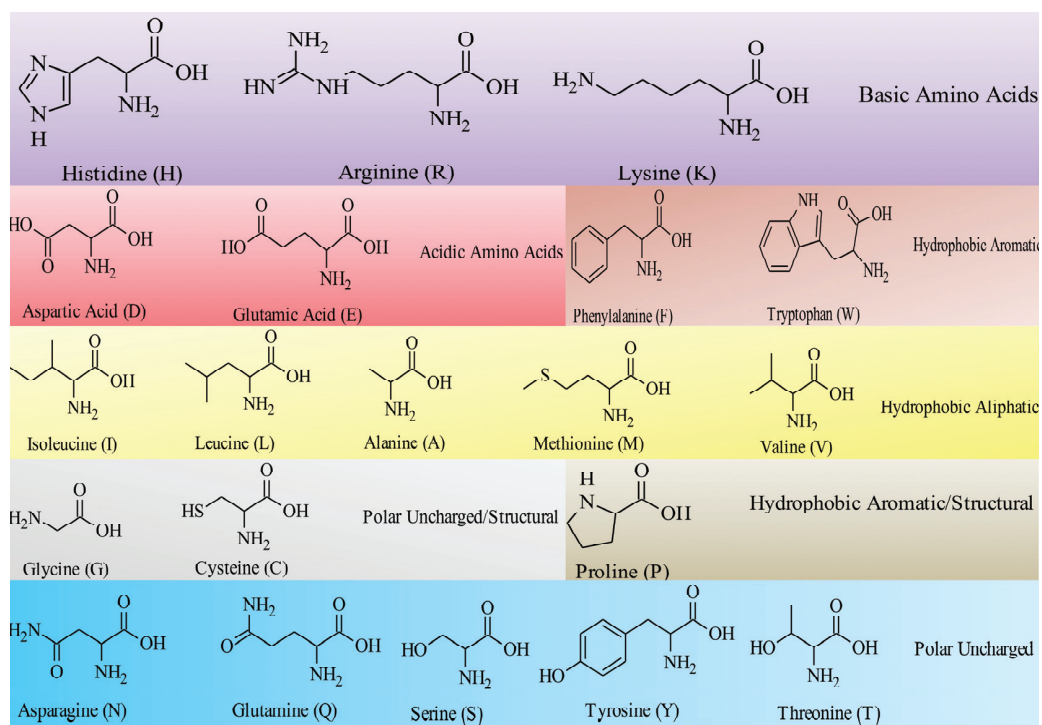
Related research combines innovative approaches to stimulate antimicrobial release with self-assembled hydrogel polymers. Norris and colleagues synthesized a self-assembled poly(2-hydroxyethyl methacrylate) coating containing ordered methylene chains [66]. This polymer released ciprofloxacin in response to low intensity ultrasound and displayed significantly reduced accumulation of established 24 h *Pseudomonas aeruginosa* biofilm over a three day period. Self-assembled monolayers have been created to modify the surface properties of biomaterials with the aim of reducing bacterial attachment. Recently Kruszewski *et al.* produced stainless steel, commonly employed in orthopaedic implants, modified by a self-assembled monolayer of long alkyl chains terminated with hydrophobic ($-\text{CH}_3$) or hydrophilic (oligoethylene glycol) tail groups [67]. These groups facilitated the attachment of gentamicin or vancomycin and reduced *Staphylococcus aureus* biofilm formation for up to 24 and 48 h respectively. Protein deposition by the host conditioning film remains a problem due to masking of covalently attached molecules. Studies exist for self-assembled monolayer materials that display resistance to biofilm formation and host protein adsorption. Self-assembled monolayers of alkanethiols, presenting a tri(ethylene glycol) functional group, displayed a profile of reduced protein adsorption and mammalian cell adhesion and resisted *Escherichia coli* biofilm formation [68]. The authors concluded this may be due to the ability of tri(ethylene glycol) to repel cells and inhibit bacterial cell motility, a key factor in biofilm formation. Silicon coated with a self-assembled micro-gel consisting of poly(ethylene glycol) and poly(ethylene glycol)-*co*-acrylic acid proved resistant to adhesion by *Staphylococcus epidermidis* for up to 10 h [69]. Loading the micro-gel with an antimicrobial peptide (L5) resulted in significant anti-adherent properties at the 10 h time-point due to the localized release and inhibitory action of the L5 peptide. After 10 h, colonization was observed due to depletion of the peptide reservoir.

Such approaches have been significant in advancing biofilm-resistant self-assembled polymer research. Limitations exist affecting translation into clinical practice, namely sufficient antimicrobial action over a sustained period of time. Self-assembled polymers have contributed to a multitude of innovative investigations and applications within biomedical engineering. It is now a widely accepted method of material development. The theory has been expanded to investigate the potential of peptide self-assembly and peptide-based nanomaterials. Self-assembled peptides display great promise as antimicrobial coatings. Alteration of the peptide backbone allows materials to be tailored to their application. The production of an inherently antimicrobial peptide hydrogel, which self-assembles in response to environmental or specific pathogenic stimuli, may allow for significant reduction of biofilm formation over a prolonged period.

Uses for self-assembled peptides range from medical to engineering applications. They have recently come to the fore of bioscience contributing significantly to the nanotech revolution [70]. Work by Zhang in 1993 on ionic self-complementary peptides is regarded as particularly noteworthy in terms of evolving this area [71]. Other experts of note include: Aggeli, who investigated the hierarchical structures of peptides [72]; Tirrell, who patented secondary structure forming peptide amphiphiles [27,73]; and Ghadiri, who was responsible for the development of cyclic nanopeptides [74]. To fully understand the potential of self-assembling peptides in biomaterial applications an appreciation of the chemical composition governing their hierarchical properties is required.

Peptides are ideal building blocks for the formation of nanostructures. Utilizing a “bottom up” approach, individual amino acid residues are used to build higher ordered structures [75]. The twenty naturally occurring amino acids alone allow a huge variety of potential combinations. Their structures and corresponding single letter abbreviation are detailed in Figure 1. An immense range of primary peptide sequences and higher ordered structures can be created for biomedical applications. Variation of the R-group at the alpha (α) carbon provides unique characteristics (hydrophobic, hydrophilic, aliphatic, aromatic, positive or negative charge) to each amino acid. These influence the extent to which the molecules can participate in non-covalent interactions and therefore self-assemble to form defined nanostructures. The hydrophobic: hydrophilic balance and ability of these molecules to hydrogen bond are particularly influential factors [55]. Ulijn and Smith provide detail regarding the effect of various amino acids on the process of assembly [76]. Their review refers to the relationship between an increase in methylene chains on the R-group side chain; the degree of hydrophobicity; and extent of steric hindrance in affecting assembly of monomer units. In particular, the degree of hydrophobicity is key in determining assembly. Peptide assembly is also influenced by the concentration of peptide present. Increasing the number of amino acid repeats results in the formation of higher assemblies. The number of amino acids present also affects the critical concentration of assembly. The critical concentration increases relative to a rise in the number of amino acid residues due to a shift in the entropic-enthalpic balance. For assembly to occur entropic loss must be balanced with an enthalpic gain within the system [77]. Environmental factors such as pH, temperature, light, and the presence of proteolytic enzymes influence assembly. Peptide systems commonly follow those found in nature. The greater the number of hydrophobic residues present, the lower the critical concentration for assembly to occur. Crucially the degree of charge and hydrophilicity must be optimal to ensure efficient assembly. An increase in hydrophobicity above optimal levels may result in precipitation of the peptide molecule in solution. Hydrophilic groups, including those present within the peptide bond ($-\text{NHCO}$), carboxylic acids ($-\text{COOH}$) and amines ($-\text{NH}_2$) can also contribute to assembly via hydrogen bond intermolecular interactions with surrounding solvent and neighboring peptide molecules. Charged amino acids, for example lysine, result in charge-charge electrostatic interactions. These have potential to drive or prevent assembly depending on the degree of polarity and charge density. Salt concentration is also a contributing factor, as demonstrated through work on the peptide EAK16-II by Hong and colleagues [78]. A low salt concentration resulted in a desired level of intermolecular interaction, driving assembly. Ionisation, which is strongly linked to pH and pK_a , has a large influence on the self-assembly process. The natural amino acid tyrosine allows further functionalization of peptide molecules. Its R-group consists of an amphiphilic phenol grouping which allows, for example, formation of imines under relatively mild conditions (pH \sim 6.5) [79]. The use of microbial enzymes to facilitate self-assembly of antimicrobial peptides is covered in detail in Section 5.4. The phenol grouping of tyrosine provides an alternative functional group for conjugation of possible microbial enzyme targets, for example phosphate groupings [80].

Figure 1. The structures and single amino acid code for the twenty naturally occurring amino acids. Each amino acid shares a carboxylic acid ($-\text{COOH}$) and a primary amine group ($-\text{NH}_2$). The properties of the individual amino acids are governed by the nature and functionality of the R-group attached to the α -carbon. Researchers exploit the differences in individual amino acid units to develop peptide-based therapeutics. Of particular importance to antimicrobial peptides and peptide self-assembly is the hydrophobic: hydrophilic balance of the primary peptide structure.

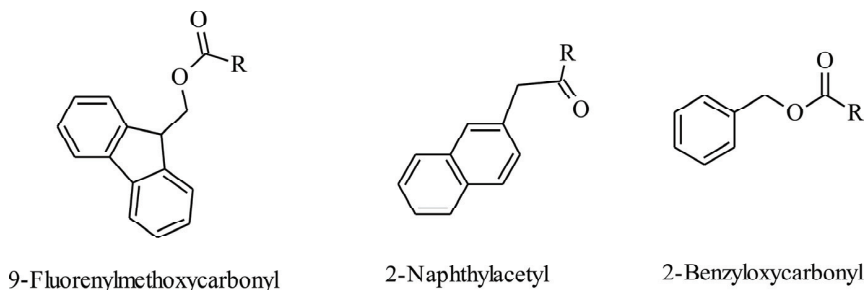


Peptide derivatives as detailed include amphiphiles and π -stacked aromatic peptides. Amphiphiles have a similar structure to membrane phospholipids, possessing a hydrophobic alkyl tail conjugated to a charged moiety. They are ideal molecules for interacting with and disrupting bacterial membranes [81]. Assembly results in a variety of higher structures, mainly rods, which possess a hydrophobic core and a hydrophilic exterior. The process of assembly is modified by altering the charge of the residues involved or by changing the position of the alkyl chain on the alternative termini (amine or carboxylic acid functional groups) [82]. Conjugated amphiphilic peptides with assembling properties for example IKVAV, have been employed in tissue engineering where the assembled nanofibrous network created scaffolds facilitating differentiation of cells into neurons [83].

Reviews such as those by Gazit and Martinez [84,85] provide more detailed information regarding π -stacked peptides. In brief, π -stacked peptides are composed of residues with aromatic groups such as phenylalanine, tryptophan, tyrosine and histidine. Burley and Petsko [86] initially investigated the

aforementioned, naturally occurring aromatic amino acids, and their interactions with neighboring aromatic moieties. They highlighted the influence of a residue's environment on the availability of free energy to interact with other residues, and the contribution of this to the resulting structural stability. More recently π - π interactions particularly diphenylalanine interactions, are attracting a great deal of interest. They have a key role in β -amyloid polypeptide/ fibril formation and subsequent formation of fibrous plaques, which are implicated in disease states such as Alzheimer's [87]. π - π stacking may also be attributed to peptides that have a 9-fluorenylmethoxycarbonyl (Fmoc), naphthalene (Nap), or benzyloxycarbonyl (Cbz) group on the amine terminal (Figure 2). π -stacking occurs due to the presence of a conjugated system, allowing the aromatic π electrons within this system to overlap and interact [88]. The restricted geometries within the aromatic systems provide direction to the assembled structure, with interactions between the stacks acting as a glue and contributing to the overall rigidity of such assembled structures [84].

Figure 2. Examples of aromatic moieties utilized to provide π - π electrostatic interactions. The polycyclic aromatic hydrocarbon residues of fluorene (in 9-fluorenylmethoxycarbonyl groups), naphthalene (as in 2-naphthylacetyl) and benzene (as in 2-benzyloxycarbonyl) facilitates π -stacking due to the presence of a conjugated system of delocalized π -electrons. Such intermolecular interactions are enhanced by the presence of hydrophobic aromatic amino acid molecules, for example phenylalanine.



Although the aim of assembly is to achieve higher-ordered structures, these must be uniform, homogenous and reproducible with defined and useful properties. Peptides provide an ideal basis for such structures for many reasons. They are biocompatible and biodegradable [89]. Their synthesis is relatively uncomplicated [90]. They can assemble (forming gels) *in situ* [71]. Peptide bioactivity, such as antimicrobial potency, can also be modified according to the amino acid residues selected and structure activity relationships can often be determined with high levels of precision. Aromatic groups contribute to π - π intermolecular van der Waal's interactions. Molecules that contain such groups act as hydrogelators by supplying increased hydrophobic bulk to the molecule. Fmoc, naphthalene, and Cbz may facilitate assembly at a reduced peptide length and cost. Naphthalene is preferred due to an established safety profile as it is used in many licensed drug molecules including propranolol. The cytotoxicity of naphthalene-based peptides was previously investigated by Yang and co-workers [89]. They determined a cell survival of almost 100% when HeLa cells were exposed to 200 micromolar of naphthalene dipeptides. Supramolecular properties of these structures including

electrical, mechanical and bioactivity are also determined by the process and kinetics of self-assembly. Therefore the nature of the route and rate of assembly must also be carefully considered when designing self-assembling peptide systems.

4. Antimicrobial Peptides

The chemical, physical and structural properties that govern peptide self-assembly are closely associated with factors that determine the antimicrobial potency and spectrum of activity of antimicrobial peptides. Antimicrobial peptides occur throughout nature and are involved in the innate immune response. This response is mounted a short time following exposure to an infective agent and stimulation of cells known as Toll-like receptors [91]. Unlike conventional antimicrobials, most naturally occurring antimicrobial peptides act synergistically in great numbers and at micromolar concentrations. The combined effect of this is a relatively potent antimicrobial response due to action at multiple sites, in comparison to a single target as is the case with most conventional antibiotics [92].

The amphiphilicity of antimicrobial peptides allows them to target microbial cell membranes, particularly those of bacterial cells. Bacterial membranes possess an overall negative charge, compared with neutrally charged eukaryotic membranes, due to the presence of acidic hydroxylated phospholipids. These include cardiolipin, phosphatidylglycerol and phosphatidylserine [93]. The presence of phosphate groups on membrane-bound lipopolysaccharides in Gram-negative bacteria and acidic polymers, such as teichoic acids, in Gram-positive bacteria, allow areas of dense anionic charge to develop on bacterial membranes [44]. Cationic peptides target these areas to exert their effect. Antimicrobial peptides act directly on the cell membrane by disrupting the integrity or function of the phospholipid bilayer via four recognized mechanisms: the aggregate, toroidal pore, barrel-stave and carpet models [94]. Bacterial membranes are an excellent antimicrobial target as bacteria would be required to alter the overall properties of their membrane to confer resistance characteristics, rather than modifying individual receptors. The likelihood for bacteria developing resistance is significantly decreased as antimicrobial peptides also possess intracellular targets. Peptides have been proven to inhibit ATP-dependent enzymes and disrupt processes within the cell, for example RNA and protein synthesis, DNA replication and protein folding [95–99].

Cationic peptides are the most studied form of antimicrobial peptides. Possessing a net positive charge, they are most commonly composed of 12–50 amino acids, approximately 50% of which are hydrophobic [100]. Cationic antimicrobial peptides were first derived from insect haemolymph in the 1970s. They are amphiphilic in nature enabling them to interact with the dense phospholipid exterior of bacterial cell membranes [95]. Cationic peptides have been referred to as ‘nature’s antibiotics,’ however the term “defence proteins” may be more appropriate due to their role in modulating the innate immune response [101]. Anionic peptides are also found throughout nature and have a role in providing innate immunity in a variety of animals and plants. Typically, composed of 7–50 residues, they are also amphiphilic in nature but have an overall net negative charge due to the presence of aspartic acid residues [102,103]. Anionic peptides often require cationic co-factors, for example zinc ions, to produce an antimicrobial effect. Brogden hypothesized zinc ions may form a cationic salt bridge between anionic antimicrobial peptides and the anionic microbial cell surface [104]. These interactions facilitate movement of such peptides across the bacterial membrane, into the cell

cytoplasm, allowing intracellular attack. Epithelial cells are prime locations for host defense anionic peptides, not only are they key sites for ion exchange but they are also commonly exposed to pathogenic microbes [105].

Antimicrobial activity and self-assembly of peptides are influenced by similar factors namely: charge, bulk and lipophilicity. Strøm and co-workers investigated the contribution of these factors to antimicrobial activity [106]. Their findings showed that an optimal balance exists between degree of hydrophobicity or bulk and charge governing antimicrobial potency and spectrum of activity. Modification of the peptide sequence with unnatural molecules possessing amino and/or carboxylic acid groups may be of benefit in increasing the cost-effectiveness and activity of the peptide. For example, the addition of cinnamic acid has been shown to increase antimicrobial activity by providing hydrophobic bulk to the primary peptide sequence, at a reduced cost compared with commercially available hydrophobic amino acids [107]. Hydrophobicity and antimicrobial activity can be optimized sequentially via the addition of fatty acids to the peptide motif, creating the promising antimicrobial lipopeptides [45,108]. The type and nature of amino acids employed to generate peptide motifs are also important. Utilizing unnatural amino acids, for example ornithine, may provide the peptide with stability against proteases which are unable to be recognized at a molecular level for incorporation into enzyme active sites [107]. Most amino acids in nature are present as the L-stereoisomer form. Stereoisomerism is based on the ability of molecules to rotate plane-polarized light. L-amino acids are easily recognized by host and bacterial enzymes. They are ideal substrates for proteolysis by peptide specific enzymes termed proteases. In theory this limitation can be reduced, to increase both therapeutic efficacy and retention within the host, by replacement with D-amino acids. Amino acids that are in the D-enantiomeric form do not fit in the active site of proteases and therefore cannot be broken down. D-amino acids do have limitations. They are typically expensive, and for short peptides the realistic enzyme stability they offer is debatable [109].

Lipopeptides are a form of antimicrobial peptides present in nature among bacteria and provide selective advantage against competing microbial strains. Their success is reflected in the pharmaceutical industry, with antimicrobial lipopeptides already successfully utilized clinically. These include anionic daptomycin [110], which forms micelles in the presence of calcium ions facilitating membrane interaction, and cationic polymyxins which interact with membrane-bound lipopolysaccharides inserting into bacterial membranes causing membrane disruption [111]. Cost and toxicity has rendered their use restricted to mainly topical application and second or even third line treatments for pathogens resistant to other antimicrobials. A research paper by Levine presented the findings of patients registered on the Cubicin® Outcomes Registry and Experience (CORE) 2004 database. This covered patients administered with the injectable form of daptomycin (brand name: Cubicin®) for the treatment of infective endocarditis [112]. Of those treated, 83% of *Staphylococcus aureus* infections were methicillin resistant, and 43% enterococci infections were vancomycin resistant. The findings showed that daptomycin has a potential role in treating these types of infections, with 63% of treatments proving successful overall. Similarly a review by Li highlights in great detail the effective role of polymyxin E (colistin) via inhalation and intravenous administration to treat multi-resistant pseudomonal infections in cystic fibrosis patients [113]. There is a clinical need to improve toxicity, bioavailability, and selectivity profiles. Activity is influenced by the amino

acid chain and hydrophobicity, with the length of the conjugated fatty acid chain an influential factor [91].

Research is increasingly focused on the action of antimicrobial peptides against resistant biofilm phenotypes [114]. Evidence shows that peptides interact with polysaccharide components of the biofilm causing break down within the polymeric matrix [115]. Peptides also act synergistically with standardly employed antimicrobials, eradicating the biofilm and membrane barriers, allowing clinically employed antibiotics access to intracellular targets. The antimicrobial peptides magainin-II (amphibian-derived) and cecropin-A (insect-derived) were proven by Cirioni *et al.* to act synergistically with rifampicin against multi-drug resistant *Pseudomonas aeruginosa* in both *in vivo* and *in vitro* models [116]. The lipopeptide polymyxin E demonstrated synergistic efficacy with ticarcillin/clavulanate against *Stenotrophomonas maltophilia* isolated from cystic fibrosis patients [117]. Infectious diseases and their causative pathogens continue to display a profile of increased resistance to standardly employed antimicrobials. A recent investigation in a Spanish hospital urology department showed that approximately 52% of isolated *Escherichia coli* and 36% *Pseudomonas aeruginosa* displayed resistance to fluoroquinolones [118]. There is a pressing need not only to develop new antimicrobials but also to refine the properties of these potentially novel chemotherapeutics so that stability, size, immunogenicity, and cost-effectiveness can be improved [119]. The area of antimicrobial peptides hold much promise in the development of antimicrobial therapeutics and their potential is covered in more detail in reviews by Bahar [47] and our own research group [44].

Ultrashort refers to peptides up to 7 amino acids in length. They have become increasingly attractive in the development of peptide therapeutics due to reduction in associated cost and ease of synthesis. Amino acids are rationally selected in line with a minimum pharmacophore required to permit antimicrobial activity [120]. Residues are selected to achieve an optimal lipophilic-charge balance. Typically, more active short peptides are the result of greater lipophilic bulk provided by non-proteinogenic groups. Strøm and co-workers investigated the effect of ultrashort antimicrobial peptides on a variety of nosocomial bacteria including methicillin resistant and sensitive *Staphylococcus aureus*, methicillin resistant *Staphylococcus epidermidis* and *Escherichia coli* and defined the minimum motif required for activity [106]. They hypothesized that activity in staphylococci required a combination of no more than two units of hydrophobic bulk and two units of charge. The Gram-negative rod required three units of bulk and at least two charged species for antibacterial activity to be observed. Such specificity allows ultrashort antimicrobial peptide therapy to be potentially tailored to causative microorganisms in infectious diseases. Harnessing this theory allowed the development of ultrashort lipopeptide variants. Peptides composed of four amino acids and a general sequence of KXXX (K = lysine, X = one of leucine, alanine, glycine, lysine or glutamic acid) were characterized for activity by the Shai group [108]. Aliphatic chains of various lengths were conjugated to these molecules. The resulting ultrashort lipopeptides were tested against a variety of bacteria, fungi, and yeast: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, gentamicin-resistant *Acinetobacter baumannii*, *Aspergillus fumigatus*, *Aspergillus flavus*, and *Candida albicans*. This study resulted in three key findings. (1) The aliphatic side chain compensated for a reduced peptide chain length and contributed to hydrophobicity of the structure and resulting

antimicrobial properties; (2) Substrate specificity is linked to the amino acid sequence or aliphatic chain length; (3) Many conventional antimicrobial peptides display activity against specific microbes. Some of the lipopeptides tested displayed activity against fungi as well as bacteria. Our own group used these findings together with the work of Bisht *et al.* to produce a series of ultrashort cationic lipopeptides [107]. Based on an ornithine-ornithine-tryptophan-tryptophan tetrapeptide amide motif, our group sequentially increased the lipophilic tail via conjugation of fatty acids to the ornithine terminus. C₁₂-ornithine-ornithine-tryptophan-tryptophan displayed the most potent activity particularly against Gram-positive bacteria with complete eradication obtained within 24 h exposure against mature biofilms of *Staphylococcus epidermidis* (ATCC 35984) at concentrations as low as 15 µg/mL [45]. The group demonstrated it was possible to incorporate the peptide and the amphibian peptide maximin-4 into a poly(2-hydroxyethyl methacrylate) hydrogel in order to prevent short-term (24 h) *Staphylococcus epidermidis* biofilm adherence [46].

An alternative approach is to tag the end of the peptide residues with hydrophobic, bulky aromatics such as phenylalanine and tryptophan. Work on this area has involved ultrashort peptides composed of 4–7 amino acid residues. As with lipopeptides, these compounds are amphiphilic in nature and readily interact with target cell membranes. Of the tagged peptides investigated by Pasupuleti and colleagues [121], the most potent molecule, KNK10-WWWWW, displayed similar antimicrobial activity to human cathelicidin LL-37. Findings were similar for the phenylalanine-tagged peptides. Tagging also gave increased protection against proteolysis, providing an advantage for such compounds over the enzymatically degradable LL-37. This protection may be attributed to steric hindrance resulting from the bulky nature of the tags employed, preventing incorporation of the peptide into the protease active site.

Oligo-acyl-lysines (OAKs) are peptidomimetic molecules that mimic the primary structure and function of naturally occurring peptides. They are composed of acyl-lysines, with the acyl chain length determining hydrophobicity of the molecule and lysine conferring cationic charge for targeting of bacterial membranes or inhibition of cellular processes linked to intracellular DNA [122]. They can be linked to an inert resin to enhance the ability of OAKs to bind and capture bacteria for pathogen detection. A study conducted by Rotem and colleagues demonstrated that resin-bound OAKs have the potential to capture both Gram-positive and Gram-negative bacteria under continuous flow and stationary settings [123]. These molecules may be useful in terms of filtration of contaminated samples including drinking water in a hospital or community setting. Antimicrobial activity of OAKs has also been investigated *in vivo* highlighting their clinical potential. Livne demonstrated OAKs incorporating synergistic erythromycin had improved survival rates in neutropenic mice infected with multi-drug resistant *Escherichia coli* compared to erythromycin or OAK monotherapy [124]. Sarig determined the optimal lipid mixtures in the preparation of OAK-erythromycin cochleates, utilized for drug delivery [125]. Housing OAK molecules in a cochleate drug-delivery vehicle produced molecules with enhanced *in vivo* erythromycin efficacy. Cochleates are a drug delivery vehicle consisting of liposome-like molecules formed by a lipid-based supramolecular assembly of natural products (phosphatidylserine), negatively charged phospholipid and a divalent cation (calcium). The work of Sarig underlines the importance of the drug delivery vehicle in ensuring promising *in vitro* results for antimicrobial peptide-like molecules are translated

efficiently to clinical practice. This is the greatest challenge in drug development and is particularly difficult with regard to peptides and proteins which suffer from poor solubility, pharmacokinetic properties, stability and antigenicity issues [126]. Hence antimicrobial peptides are often limited to topical use. The short peptides structures outlined possess many pharmaceutical advantages compared with larger peptide/protein molecules as their macromolecular structure allows for decreased recognition by proteases and antigens, with potential extension of their pharmacokinetic profile [127].

5. Self-Assembled Antimicrobial Peptides

The development of peptides that self-assemble upon exposure to environmental stimuli is of increasing interest in biomedicine. The ability to modify a peptide to assemble on cue provides the peptide with a range of desirable properties and potential applications including targeted drug delivery in the area of antimicrobials. For biomaterials, preventing the formation of a highly resistant biofilm phenotype is pivotal in preventing infection throughout the life of the material [128]. Harnessing environmental changes is a significant strategy to deliver activity when most required. Potential triggers for the formation of an inherently antimicrobial self-assembling hydrogel include: pH and ionic strength [50], temperature [129], light [130] and microbial enzymes [131].

5.1. Self-Assembly via Changes in pH and Ionic Strength

pH and ionic strength are the most commonly utilized methods to achieve assembly and disassembly of peptides on cue. Charge interactions are crucial to the assembly process and are influenced by the pKa of the peptide, the amino acid backbone and substituent functional groups. The effect of salt on pH-triggered assembly is also an important factor in determining the degree of ionic interactions [78]. The Schneider group is responsible for a large body of research in this area. They developed a synthetic peptide MAX-1, which adopted a beta(β)-hairpin structure due to the presence of a central V^DPPT peptide motif (V = D-enantiomer of valine, ^DP = D-enantiomer of proline, T = threonine) [132]. Lysine and valine amino acid residues flank the type-II β -turn structure in alternating sequences. Basic pH, above the pKa of lysine's primary amine R-group (pH > 9) resulted in self-assembly as intramolecular folding resulted in a hydrophobic valine core surrounded by a hydrophilic lysine surface. Lowering the pH, below the pKa of lysine favored charge repulsion and disassembly. Addition of buffered saline (150 millimolar sodium chloride) or Dulbecco's Modified Eagle's Medium (165 millimolar sodium chloride) to 2% w/v of MAX-1 in water at pH 7.4 directed the formation of a biocompatible hydrogel. Formation of a hydrogel was due to screening of positive charged lysine moieties by chloride ions and alteration of the hydrophobic: charge balance allowing a decrease in solubility, therefore promoting assembly [133].

Replacing lysine with aspartic acid at amino acid position 15 (MAX-8) lowered the overall charge of the molecule by +2 and was sufficient to decrease gelation time from 30 min (for MAX-1) to 1 min [134]. Encapsulation of antimicrobial drugs and/or cells into this matrix could conceivably allow the targeted delivery of a hydrogel dressing for wound application via a syringe. This is especially true as MAX-1 was proven to display inherent antimicrobial properties against a broad

spectrum of Gram-positive (*Staphylococcus epidermidis*, *Staphylococcus aureus* and *Streptococcus pyogenes*) and Gram-negative (*Klebsiella pneumoniae* and *Escherichia coli*) bacteria [132]. The results obtained highlight the role of the polycationic lysine surface in compromising negatively charged bacterial membranes resulting in bacterial cell death (Figure 3). The Schneider group altered the MAX1 template further to produce second generation cationic self-assembled antimicrobial hydrogels containing both cationic arginine and lysine residues [132]. MARG1, consisting of two arginine residues, was highly potent against methicillin resistant *Staphylococcus aureus*. A second peptide molecule, PEP6R, consisting of six arginines within its peptide primary structure, demonstrated broad spectrum activity against *Staphylococcus aureus*, *Escherichia coli* and multi-drug resistant *Pseudomonas aeruginosa*. Both were able to form hydrogels at physiological pH.

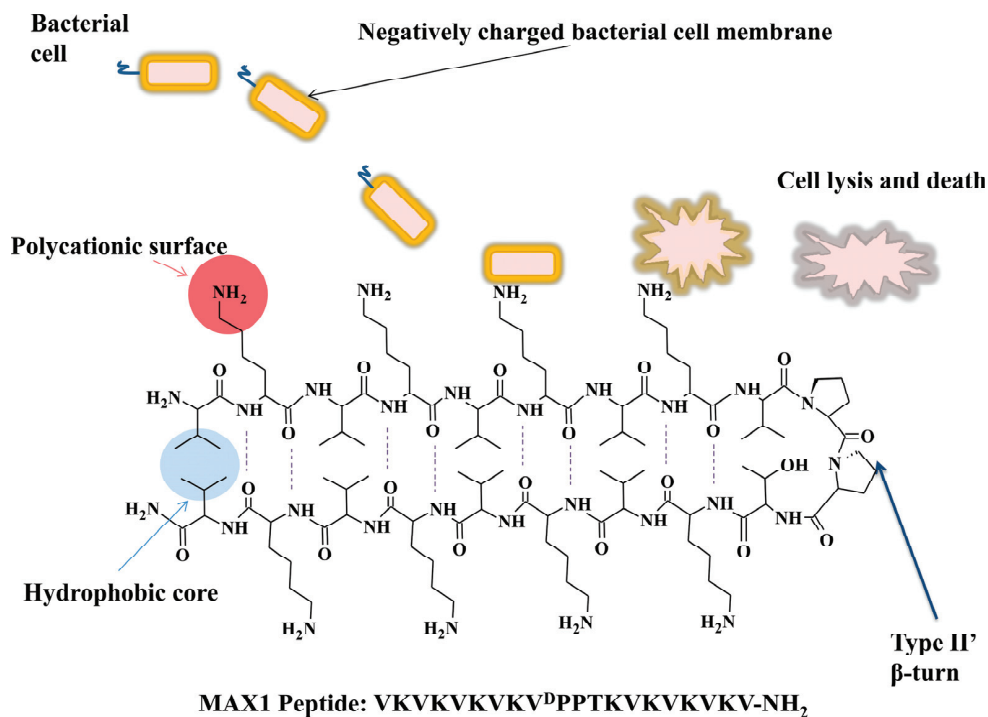
A related arginine rich peptide PEP8R (containing 8 arginine residues) was also created [135]. Variations of PEP8R were prepared, with arginine residues replaced by lysines to determine the optimal number of arginines required to achieve an antimicrobial effect. Decreasing the number of arginine residues formed PEP6R, a peptide that formed more rigid gels. The variants at all percentages tested were active against *Escherichia coli* and *Staphylococcus aureus*. Gels containing only two arginine residues demonstrated limited activity against Gram-negative *Escherichia coli*. Peptide derivatives were more active against Gram-positive bacteria. The presence of divalent ions such as calcium (Ca^{2+}) reduced the peptides antimicrobial activity. The group concluded that in order to achieve optimal antimicrobial activity a minimum of four arginine residues were required.

Recent research by Liu and colleagues demonstrated the rational design of a pH dependent self-assembled antimicrobial peptide (ASCP1) consisting of two peripheral (KIGAKI)₃-NH₂ species conjugated to a central tetrapeptide linker (T^DPPG) [50]. Similarly to the MAX peptides, the central motif allowed for the formation of a predominantly β -sheet structure with a central β -hairpin at pH greater than 10. The presence of twelve lysine primary amine side chains created sufficient electrostatic repulsion to prevent self-assembly. Elimination of these net charges via an increase in pH or addition of greater than 40 millimolar sodium chloride permits self-assembly. ASCP1 displayed inherent antibacterial properties against cultures of *Escherichia coli* after 36 h incubation. High bacterial loads (greater than 10^7 colony forming units per mL) resulted in loss of inhibitory capacity. Again membrane disruption due to the presence of polycationic lysine residues was the most plausible mechanism for antibacterial activity.

RADA16 is one of the most comprehensively studied self-assembled peptides due to its ability to self-assemble at physiological pH [136]. Marketed commercially as PuraMatrix™ it has the ability to support cell growth and attachment leading to research into its use as a nanofiber scaffold to support wound healing. It has been hypothesized that functionalisation with antimicrobials and wound healing stimulants, such as epidermal growth factor, provide wound protection while encouraging wound closure [137]. Debnath and co-workers developed antimicrobial peptide amphiphiles which self-assemble at physiological pH [138]. These Fmoc pyridinium functionalised peptides were tested against a variety of Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria, with minimum inhibitory concentrations determined. Compounds with the most broad-spectrum antimicrobial effect had hydrophobic phenylalanine closely attached to the Fmoc moiety. The close proximity of fluorenyl

and phenyl moieties allowed an optimum hydrophobicity, described by the authors as the “threshold hydrophobicity”, to be obtained thus allowing significant activity against bacteria via membrane attack.

Figure 3. The antibacterial mechanism of action of self-assembling β -sheet cationic peptides using the example of MAX1 peptide developed by the Schneider group [132]. Basic pH, above the pK_a of lysine’s primary amine R-group ($pH > 9$), results in self-assembly of the primary peptide motif into a β -sheet secondary structure. The central V^DPPT peptide forms a type II β -turn resulting in the formation of a hydrophobic valine core (blue) and a hydrophilic cationic lysine face (red). The primary amine ($-\text{NH}_2$) R-groups of lysine protrude from the β -sheet structure forming a surface of polycationic character that is selective for negatively charged bacterial membranes. Adhesion and biofilm formation is prevented as bacterial membranes are compromised resulting in leakage of cell contents and bacterial cell death. In the hydrogel form the cationic groups may also displace divalent metal ions from the bacterial cell wall causing membrane disruption in biofilm cells, leading to cell death in both Gram-positive and –negative pathogens.



pH triggered self-assembly has potential to be utilized in antimicrobial therapies. Urinary catheter associated infections are synonymous with an increase in alkaline conditions due to the presence of the enzyme urease synthesized by Gram-negative *Proteus mirabilis*. Urease catalyses the breakdown of urea to ammonia via hydrolysis leading to increased pH at the urine-catheter interface and the precipitation of mineral salts such as calcium phosphate (hydroxyapatite) and magnesium ammonium

phosphate (struvite). A combination of encrustation and biofilm formation leads to blockage of the catheter, with removal of the device necessary to resolve infection [139]. We hypothesize a role whereby an antimicrobial peptide may self-assemble at the device surface in response to an increase in pH (or the presence of urease) forming a protective barrier, reducing biofilm formation and encrustation thus preventing the need for catheter removal. This strategy would require the peptide molecule to be attached to the catheter surface, physicochemically stable at pH 7.4 and active against a broad spectrum of urinary pathogens, especially biofilm forms of *Proteus mirabilis*.

5.2. Photo-Activated Self-Assembly

Light may be used as an environmental trigger for the formation of hydrogels. These are typically formed when a water-soluble polymer and photo-initiator are exposed to a specific wavelength of light and crosslinking occurs. The resulting hydrogels display temporal and spatial resolution. Macromolecules are the preferred starting material and these are chosen to achieve the desired crosslinking and mechanical properties for the final hydrogel. The photo-initiator must be sufficiently reactive yet cytocompatible. Exposure to ultraviolet light triggers arrangement of a peptide into β -hairpins and subsequent self-assembly to a hydrogel occurs provided that the side chains permit this. Cysteine is hydrophobic in nature and easily functionalized. Therefore it is a useful side chain component for facilitating assembly. An example of this type of assembly is the 20-residue MAX7CNB peptide [130]. This caged (α -carboxy-2-nitrobenzyl) peptide is unfolded in ambient conditions but exposure to UV light triggers decaging and peptide folding to form a supramolecular hydrogel. There is no specific investigation in the literature for a peptide that induces self-assembly and antimicrobial activity in response to light. The use of light to stimulate antimicrobial delivery has been evaluated previously and therefore may serve as a valid mechanism for future research. McCoy and colleagues, developed an antimicrobial biomaterial containing tetracationic porphyrin, (tetrakis(4-*N*-methylpyridyl)porphyrin), which binds electrostatically with methacrylate groups of a methacrylic acid or a methyl methacrylate copolymer [140]. Visible light allowed the porphyrin to catalytically generate short-lived singlet oxygen at the device surface, with antimicrobial activity displayed against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

5.3. Thermo-Responsive Self-Assembly

Temperature can be used as a trigger to form higher assemblies (hydrogel structures) either through cooling or heating of molecules. The degree of hydrophobicity governs the temperature at which folding occurs. Typically, assembly of more hydrophobic molecules takes place at a lower temperature [141]. Schneider and colleagues modified their MAX-1 peptide, which forms a hydrogel at 25 °C and pH 9, by the replacement of two valine residues (at amino acid positions 7 and 16) with threonine [141]. This resulted in a peptide that formed hydrogel structures at higher temperatures (~60 °C). A replacement at position 16 only resulted in a gelation temperature of 40 °C, only slightly above that of normal body temperature, suggesting possible use as a thermo-responsive hydrogel in drug delivery. Liu's ASCP1 peptide displayed similar self-supporting hydrogel structures at higher temperatures (~60 °C) [50]. Temperature change alters the solubility of the hydrophobic amino acid

residues in these peptides, thus altering the hydrophobic: hydrophilic balance to favor decreased solubility. The process is thermally-reversible. Temperature-responsive gelation is not only limited to large peptide structures. Tang discovered replacing the terminal phenylalanine residue in Fmoc-FF with glycine (forming FmocFG) created a peptide that also gelled at increased temperatures [142]. Rheological analysis below its pK_a demonstrated self-assembly had occurred at 25 °C but only sufficient to produce a viscous solution. Gelation occurred between 55 and 80 °C which the authors attributed to dissolution of precipitate, formed due to a low pH, allowing homogenous hydrogels to be formed in solution. Further work by Tang studied glycine and leucine substituted Fmoc dipeptides [143]. In addition, employing pH and temperature to facilitate the formation of Fmoc hydrogels, FmocFG and FmocLG gelation proved also to be temperature-dependent. Both FmocFG and FmocLG hydrogels formed upon heating to 80 °C. Mechanical properties were retained upon subsequent cooling of heated peptides to 25 °C and 4 °C. FmocLG appeared to gel at 25 °C, with further heating and cooling of this gel to 4 °C forming stiffer hydrogel structures. This confirmed that a heating step was shown to improve the homogeneity of the samples without altering the topography of the self-assembled structures. Therefore, hydrophobicity of the peptide molecule, pH and homogeneity in aqueous solutions have to be taken into account in the design of a temperature triggered antimicrobial self-assembly peptide structure.

5.4. Bacterial Enzymatic Self-Assembly

Enzyme mediated self-assembly occurs as a result of catalysis or removal of a blocking group within the peptide primary sequence. It occurs under standard physiological conditions (pH and temperature) [144]. Enzymes such as proteases [145], phosphatases [49] and esterases [146], serve as viable molecules to drive peptide self-assembly. Catalysis occurs due to a thermodynamic shift resulting from condensation or hydrolysis of the peptide bond. In some cases both of these processes may occur in order to form peptide structures that are more stable. Research by Toledano demonstrated environmentally dependent protease triggered reverse hydrolysis of Fmoc amino acids [147]. Enzymes are selected, and the concentrations modified, in order to achieve optimum assembly under a defined set of conditions [148]. Enzymatic approaches to self-assembly are becoming more popular in research due to the abundance of bacterial enzymes, allowing tailored specificity for selected pathogens.

As outlined previously (Section 3), selection of specific amino acid residues and their corresponding properties is crucial in developing ultrashort peptides. Investigations into self-assembling ultrashort antimicrobial peptides are relatively novel but are increasing in the literature, especially in the area of enzyme triggered self-assembly. Removal or addition of one or two amino acid units has a larger influence on the overall properties of short peptide structures, including its solubility and therefore ability to self-assemble. One of the first examples of such research was conducted by the Xu group [49]. They were able to demonstrate that a strain of *Escherichia coli*, that overexpressed a phosphatase enzyme, was able to be selectively inhibited by a short naphthalene containing peptide. Dephosphorylation of soluble phosphorylated NapFFY(p), Nap^{D^FD^F}Y(p) and Nap-β³-HPhg-β³-HPhgY(p) (β³-HPhg: a β-amino acid named β³-homophenylglycine, p: phosphorylated) occurred in the cell cytoplasm causing inhibition of multiple intracellular processes resulting in a reduction in

bacterial viability. Further work by the Xu group demonstrated how peptide self-assembly can be governed by a tyrosine-linked kinase/phosphatase switch [80]. Phosphorylation (via kinase) and dephosphorylation (via phosphatase) of the hydrogelator at the tyrosine terminus regulated the formation of supramolecular hydrogels. Gelation occurred *in vivo* in the presence of phosphatase enzyme, as removal of hydrophilic phosphate groupings increased the hydrophobicity of the molecule, driving self-assembly. The presence of phosphatases have been attributed to medical device related pathogens such as *Escherichia coli*, where alkaline phosphatase is present in the periplasmic space and therefore serves as a valid microbial target [149,150]. The Xu group [151] also produced a NapFF precursor conjugated to a β -lactam ring, capable of hydrogelation in response to the addition of β -lactamase enzymes from cell lysates of *Escherichia coli*. This mechanism has potential to be exploited for the detection of extended spectrum β -lactamases in a clinical setting and allowing screening of potential inhibitors.

Enzymatic assembly of ultrashort peptides, utilizing alkaline phosphatase, has been conducted more recently by the Ulijn group [131]. The Fmoc dipeptides (FY, YT, YS, YN and YQ in a FmocYpX-OH motif, where X = any amino acid) were central to the study, with tyrosine providing the hydroxyl grouping for phosphorylation. Phosphorous nuclear magnetic resonance (^{31}P NMR) allowed determination of the rate of dephosphorylation and inhibition within defined areas of the bacterial cell and enabled the location of formed fibers to be identified. Treatment with alkaline phosphatase facilitates protonation of phosphate groups and the formation of higher assemblies. Peptide hydrogels formed over 24 h. Assembly was evident at a molecular level and there was evidence of β sheets, hydrogen bonding and π -stacking. Ability to assemble *in vivo* was investigated using media with and without the nucleoside inosine, which increases alkaline phosphatase synthesis two-fold. Bacterial cells were treated with a FmocFYp-OH precursor and HPLC analysis showed that the peptide moved into the hydrophobic environment of the bacterial cells. This demonstrated that assembly could occur *in vivo*. Treatment of *Escherichia coli* cells with other precursors showed similar results (self-assembly) however the effect of hydrophobicity on assembly and the resulting location of the formed peptides varied. More hydrophilic peptides were more likely to partition into the surrounding media rather than remaining within the hydrophobic environment of the bacterial cell. The findings indicated that formation of the peptides followed by movement out of the bacterial cells was sufficient to significantly reduce the number of viable bacterial cells. Therefore retention of the formed nanostructure within the cells was not essential to exert an antimicrobial effect. The intracellular and extracellular modes of action of these peptides reduce the ability of bacteria to develop resistance and bode well for their future development as therapeutics. The Ulijn group also employed the esterase subtilisin, obtained from the soil derived bacterium *Bacillus licheniformis*, as a mediator for assembly of Fmoc dipeptide methyl esters proving the versatility of the enzymatic approach to allow self-assembly [146].

6. Future Perspectives and Translation of Peptide Self-Assembly to Antimicrobial Therapeutics

Challenges exist to the use of peptides as antimicrobial therapeutics. There is currently only one licensed self-assembled peptide therapeutic; the injectable β -sheet forming octapeptide lanreotide

administered for the relief of neuroendocrine symptoms [152]. The licensing of lanreotide by the Food and Drug administration in 2007 provides great hope that similar molecules can be translated into therapeutics. In antimicrobial drug delivery there is increasing research into the utilization of self-assembled peptides as practical therapeutics. It is becoming increasingly relevant in the innovative production of antimicrobials and their delivery platforms. Peptide self-assembly has been utilized in combination with standardly employed antibiotics. Marchesan *et al.*, produced a macroscopic tripeptide hydrogel (^PLFF) at physiological pH which incorporated the sparingly soluble antibiotic ciprofloxacin [153]. The hydrophobic tripeptide acted as a suitable drug delivery vehicle for the release of ciprofloxacin *in vitro*. Mild antibiofilm activity was demonstrated for the tripeptide alone against *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*, with significant reduction in viability obtained via inclusion of 30% w/w ciprofloxacin.

Paladini and co-workers investigated the antimicrobial effects of silver incorporated into ultrashort Fmoc diphenylalanine (FmocFF) hydrogels on *Staphylococcus aureus*. [154]. Hydrogels (as detailed in Section 2) are commonly used in wound management and can be impregnated with antimicrobial agents, including silver. The nature of the hydrogels themselves creates a hydrated environment and protects the wound, creating an ideal environment for wound healing. Self-assembling peptide hydrogels such as those formed from assembly of FmocFF create a similar environment, making them ideal candidates for wound dressings. Paladini's 'silver-doped' FmocFF hydrogels are composed of varying concentrations of silver nitrate (0.01%, 0.1%, 2% weight). The hydrogels produced were used to coat flax textiles and exposed to *Staphylococcus aureus* overnight. The work demonstrated the ability of FmocFF to disassemble and reassemble when added to the flax substrates. Scanning electron microscopy suggested that the higher the concentration of silver present, the more uniform the nature of the flax coverage. Precipitates formed with the 2% w/v gels thus homogeneity was not achieved. Antimicrobial studies demonstrated a reduction in bacterial numbers with an increased silver concentration. Bacterial adhesion and biofilm formation was also investigated. Incorporation of silver was necessary to prevent biofilm formation and the minimum concentration of silver needed to achieve this effect was 0.1% weight.

The amphipathic peptide, FmocFFECG, contains both hydrophobic and hydrophilic moieties. The presence of carboxylic acid functionalities allowed adsorption of silver nanoparticles into the peptide motif [155]. Activity was proven against Gram-positive *Bacillus subtilis* and Gram-negative *Escherichia coli* for up to 30 days. Stability was also increased compared with silver nanoparticles alone suggesting the peptide hydrogel protects silver nanoparticles from oxidation. The hydrophobic character of ultrashort self-assembling peptides may also allow the incorporation of other highly lipophilic antimicrobial drugs.

Peptides also provide access to infections in the brain. Research by Liu demonstrated how the peptide CG₃R₆TAT (C = hydrophobic cholesterol, G = glycine spacers, R = cationic arginine residues, TAT = minimal amino acid sequence required for membrane translocation) assembles to form micelles with a hydrophobic cholesterol core and hydrophilic peptide exterior [156]. This peptide displays antimicrobial activity against a range of Gram-positive and Gram-negative bacteria. Ability to cross the blood brain barrier and exert an antimicrobial effect was also demonstrated *in vivo* in an infected *Staphylococcus aureus* induced meningitis rabbit model. This work highlights

the ability of assembled peptides as tailored molecules to achieve targeted antimicrobial effects in challenging areas of drug delivery.

Key problems associated with peptide design namely, high manufacturing costs and susceptibility to enzymatic degradation, mean there is still a need to develop compounds that have similar properties to conventional antimicrobial peptides in physiological conditions namely: cationic charge, hydrophobicity and amphiphilic nature to enable antimicrobial action and self-assembly. Peptidomimetics and other synthetic oligomers are thus coming to the forefront of current and future research plans. The design of ultrashort peptides has the potential to create functional peptide molecules with a relatively reasonable manufacturing cost. Our own group has moved research forward in this area with the synthesis of a variety of ultrashort and more cost-effective cationic naphthalene containing peptide hydrogels, which are highly active against biofilm forms of biomaterial related pathogens [157]. The very nature of peptides and their proven activity against pathogenic biofilms renders them useful compounds for prevention and management of nosocomial infections and wound healing. Further development of peptidomimetics will provide novel biomaterial platforms and the development of new molecules with tunable chemical and mechanical properties.

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Conflicts of Interest

The authors declare no conflict of interest.

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Antimicrobial Activity of Selected Phytochemicals against *Escherichia coli* and *Staphylococcus aureus* and Their Biofilms

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Abstract: Bacteria can be resistant to multiple antibiotics and we are fast approaching a time when antibiotics will not work on some bacterial infections. New antimicrobial compounds are urgently necessary. Plants are considered the greatest source to obtain new antimicrobials. This study aimed to assess the antimicrobial activity of four phytochemicals—7-hydroxycoumarin (7-HC), indole-3-carbinol (I3C), salicylic acid (SA) and saponin (SP)—against *Escherichia coli* and *Staphylococcus aureus*, either as planktonic cells or as biofilms. These bacteria are commonly found in hospital-acquired infections. Some aspects on the phytochemicals mode of action, including surface charge, hydrophobicity, motility and quorum-sensing inhibition (QSI) were investigated. In addition, the phytochemicals were combined with three antibiotics in order to assess any synergistic effect. 7-HC and I3C were the most effective phytochemicals against *E. coli* and *S. aureus*. Both phytochemicals affected the motility and quorum-sensing (QS) activity, which means that they can play an important role in the interference of cell-cell interactions and in biofilm formation and control. However, total biofilm removal was not achieved with any of the selected phytochemicals. Dual combinations between tetracycline (TET), erythromycin (ERY) and ciprofloxacin (CIP) and I3C produced synergistic effects against *S. aureus* resistant strains. The overall results demonstrates the potential of phytochemicals to control the growth of *E. coli* and *S. aureus* in both planktonic and biofilm states. In addition, the phytochemicals demonstrated the potential to act synergistically with antibiotics, contributing to the recycling of old antibiotics that were once considered ineffective due to resistance problems.

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1. Introduction

Resistance to antibiotics is one of the biggest problems that faces public health [1,2]. This problem is a natural consequence of the adaption of infectious pathogens to antimicrobials used in several areas, including medicine, food animals, crop production and disinfectants in farms, hospital and households [3–6]. Bacteria have developed resistance to all known antibiotics and, as so, the economic burden associated with these multidrug-resistant bacteria is high.

In order to find novel antimicrobial agents with new modes of action, plants have been explored as sources for the identification of new and effective antimicrobials. Plants are an important source of antimicrobial products, most of them with efficiency against diverse organisms including fungi, yeasts and bacteria, insects, nematodes and other plants [7]. Phytochemicals are able to inhibit peptidoglycan synthesis, damage microbial membrane structures, modify bacterial membrane surface hydrophobicity and also modulate quorum-sensing (QS) [8].

While planktonic bacteria are already resistant to many antimicrobials, in biofilms this resistance can increase several times. Biofilm may be formed on a variety of surfaces including living tissues, indwelling medical devices, contact lenses, *etc.* [9]. Biofilms constitute a threat in the clinical environment by acting as pools of multidrug resistant pathogenic bacteria. Consequently, in order to prevent biofilm formation, several studies have been performed to find antimicrobial agents that affect the viability of bacteria in biofilms. Natural products from plants have been shown to influence microbial biofilm formation [8,10–13].

Diverse mechanisms allow microorganisms to come into closer contact with a surface, attach to it, promote cell interactions and grow as a 3-D structure [14]. Maximum attachment depends upon high surface free energy or wettability of surfaces. Surfaces with high surface free energies are more hydrophilic and generally show greater bacterial attachment than hydrophobic surfaces [9]. The properties of cells, including cell surface hydrophobicity, presence of fimbriae and flagella, and production of extracellular polymeric substances (EPS) also influence the attachment of microbial cell [15]. QS, related with cell-cell signaling, play a role in cell attachment and detachment from biofilms [16]. QS regulates a wide number of physiological activities, such as motility, conjugation, competence, sporulation, virulence and biofilm formation. The QS signal molecules may alter distribution of bacterial species in the biofilm, alter protein expression, introduce new genetic trait and incorporate bacteria in biofilm [15].

The purpose of this study was to assess the antimicrobial efficacy of selected phytochemicals (7-hydroxycoumarin (7-HC), indole-3-carbinol (I3C), salicylic acid (SA) and saponin (SP)) against *E. coli* and *S. aureus* planktonic cells. These phytochemicals belong to two different chemical classes: phenolics and glucosinolates. These chemicals are characterized for their antimicrobial, antifungal, antioxidant and anticancer activities [7,17–21]. Moreover, the effects of these phytochemicals were assessed on biofilm control. The phytochemicals were placed in contact with 24 h old biofilms of *E. coli* and *S. aureus* to verify if they were able to promote biomass removal and/or metabolic inactivation. The mode of action of the selected phytochemicals on planktonic cells and in the early stages of biofilm formation was also evaluated by analyzing the effects on the surface charge, free energy of adhesion, bacterial motility and quorum-sensing inhibition (QSI). Additionally, since co-therapies between antimicrobials are being extensively applied in the clinical setting in order to increase the pharmacological activity or decrease the resistance behavior of bacteria, the phytochemicals were combined with three antibiotics (ciprofloxacin (CIP), tetracycline (TET) and erythromycin (ERY)) against several resistant *S. aureus* strains (*S. aureus* RN4220, SA1199B and XU 212) owning different efflux pumps.

2. Results and Discussion

2.1. Evaluation of Antimicrobial Activity of Phytochemicals

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 7-HC, I3C, SA and SP were assessed for *E. coli* and *S. aureus* (Table 1). SP was the unique compound that had neither detectable MIC nor MBC for concentrations lower than 3200 µg/mL. The 7-HC and I3C were the most effective compounds against both bacteria, since they presented

the lowest values of MIC (800 µg/mL for both phytochemicals against *E. coli*; 200 and 400 µg/mL for 7-HC and I3C, respectively, against *S. aureus*). Considering that most reports about natural products and extracts describe MIC values over 1000 µg/mL, which has little relevance for clinical application [22], the results obtained with 7-HC and I3C are considered relevant. Therefore, in this sense and considering that phytochemicals are routinely classified as antimicrobials on the basis of susceptibility tests that produce inhibitory concentration in the range of 100–1000 µg/mL, these compounds could be classified as antimicrobial [23,24]. Concerning the MBC, I3C seems to be the most effective phytochemical against both bacteria. The MBC for 7-HC was not detected for both bacteria, until the maximum value tested (10,000 µg/mL). The MIC/MBC values obtained are in the range of those described in other studies. In comparison with previous studies with phytochemical products, essential oils of *Laportea aestuans* in that a methyl ester of SA, methyl salicylate, as the main compound, showed inhibitory potential especially at 200 mg/mL, against various microorganisms, including *E. coli* and *S. aureus* [25]. In a study performed by Hassan and collaborators [26], saponin-rich extracts from guar meal and quillaja exhibited antibacterial activity against *S. aureus*. Another study showed antibacterial activity of saponin from leaves and bark of *Acacia Arabica* against diarrheagenic *E. coli* [27]. Antimicrobial activity of coumarin and several derivatives against *E. coli* and *S. aureus*, was demonstrated by Souza *et al.* [28]. The MIC and MBC values ranging between 500 to 2000 µg/mL, and 1000 to 2000 µg/mL, respectively. Another study reported that 7-HC derivatives had antimicrobial activity against a panel of Gram-negative and -positive bacteria, including *E. coli* and *S. aureus* [29]. Moreover, these authors found that the long-chain of fatty acids esters are related with the efficacy of the 7-HC. An amino-coumarin, 7-amino-4-methylcoumarin, from *Ginkgo biloba*, had broad-spectrum antibacterial activities against *S. aureus*, *E. coli*, *Salmonella enterica* serovar Typhimurium, *Salmonella enteritidis*, *Aeromonas hydrophila*, *Yersinia* sp., *Shigella* sp. and *Vibrio parahaemolyticus* [30]. Strong activity was also obtained by Kuete *et al.* [31], with 7-HC isolated from *Treulia obovoidea* (Moraceae) against some Gram-positive and -negative bacteria.

Table 1. MIC and MBC of the selected phytochemicals against *E. coli* and *S. aureus*.

Strains	Phytochemicals (µg/mL)			
	7-HC	I3C	SA	SP
MIC				
<i>E. coli</i> CECT 434	800	800	3200	ND
<i>S. aureus</i> CECT 976	200	400	1600	ND
MBC				
<i>E. coli</i> CECT 434	ND	1600	5000	ND
<i>S. aureus</i> CECT 976	ND	800	3200	ND

ND—Not detected.

In a study performed by Borges *et al.* [32], gallic (hydroxybenzoic acid) and ferulic acids (hydroxycinnamic acid) demonstrated antimicrobial activity against *E. coli* and *S. aureus*. In addition, these authors showed that hydroxycinnamic acid was more effective than hydroxybenzoic acid, considering the MIC and MBC values. These results are in accordance with those obtaining in

the present study for 7-HC (hydroxycinnamic acid) and SA (hydroxybenzoic acid), relatively to the MIC values. Hydroxycinnamic acids are, generally, antibacterial and less polar than the corresponding hydroxybenzoic acids, due to their propenoid side chain, and this property might facilitate the transport of these molecules through the cell membrane [33,34]. Nonetheless, the values of MBC of SA for the two strains tested were lower than those of 7-HC.

Other authors tested the antimicrobial activity of some glucosinolates and their enzymatic hydrolysis products including I3C, against several Gram-negative (*Acinetobacter baumannii*, *Citrobacter freundii*, *Enterobacter asburiae*, *Enterobacter cloacae*, *Enterobacter hormaechei*, *Escherichia coli*, *Hafnia alvei*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *S. typhimurium* and *Stenotrophomonas maltophilia*) and –positive bacteria (*Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus saprophyticus*) isolated from the human intestinal tract [35]. The authors found that intact glucosinolates had no effect on any of the Gram-positive or -negative bacteria tested, isothiocyanates were the most effective glucosinolate hydrolysis products (GHP) against both Gram-negative and –positive bacteria, and I3C had only some inhibitory activity against the Gram-positive bacteria.

In our study, the values of MIC and MBC for *E. coli* were higher than those for *S. aureus*. This result was expected because *E. coli* is a Gram-negative bacterium and the presence of an outer membrane (OM) can make it less susceptible to antimicrobials than Gram-positive bacteria [36,37]. Indeed, the Gram-negative bacteria have lipopolysaccharide (LPS) in their OM, which functions as a barrier that slows the penetration of antimicrobials. In these bacteria, the passage through the OM is regulated by the presence of hydrophilic channels (porins) that usually exclude the entry of hydrophobic compounds. Moreover, the OM of these bacteria lacks phosphoglycerides and, hence, the effective channels for hydrophobic diffusion [38,39].

2.2. Aspects Underlying the Antibacterial Action and Biofilm Control of Phytochemicals

2.2.1. Surface Charge and Hydrophobicity

It is important to remember that microorganisms have different mechanisms of adhesion and retention, influenced by the substrata, nutrients, ionic strength, pH values and temperatures, and also their phenotype and genotype [40]. The ability of microorganisms to attach to the surfaces is crucial for the beginning of colonization. The process of adhesion of microorganisms to surfaces is very complex, and is affected by many variables. The hydrophobicity and the charge of the cell surface, the presence of bacterial adhesins (e.g., fimbriae, flagella and pili), and particularly the quantity and composition of generated EPS, are the main factors that influence both the rate and degree of microbial adhesion [41]. Indeed, the surface properties of microbial cells have a major impact on adhesion to a substratum. The surface charge of the cells is often determined as its zeta potential, which is measured from the mobility of cells in the presence of an electrical field under well-known conditions (pH and salt concentrations) [42,43]. At physiologic conditions, most of the microorganisms are negatively charged due to the presence of anionic groups, such as carboxyl and

phosphate, in their membranes [42]. Table 2 shows the several variations of zeta potential of cells in the presence of the different phytochemicals.

Table 2. Zeta potential (mV) results of suspensions of *E. coli* and *S. aureus* exposed to the phytochemicals at their MIC.

	Zeta Potencial (mV)	
	<i>E. coli</i> CECT 434	<i>S. aureus</i> CECT 976
Control	-13.0 ± 1.4	-29.8 ± 1.3
7-HC	-13.5 ± 1.8	-26.0 ± 6.2
I3C	-21.0 ± 5.7	-27.3 ± 6.3
SA	-1.80 ± 0.3	1.80 ± 0.5
SP	-12.7 ± 1.6	-19.4 ± 2.2

Both bacterial strains present a negative surface charge: -13.0 mV for *E. coli* and -29.8 mV for *S. aureus*. 7-HC and SP have no significant effect in the charge of *E. coli* ($p > 0.05$). In contrast, I3C and SA seem to change the *E. coli* surface charge ($p < 0.05$) for more and less negative values, respectively. The charge of *S. aureus* surface become less negative when exposed to all compounds, but significant alteration was observed when in contact with SA and SP ($p < 0.05$). The first one was able to make the membrane surface positive, while the exposure to SP changed the surface charge of cells to less negative values. Other authors have also studied the effects of phenolic compounds in cell surface charge with the same bacteria [32]. In accordance with our results, they showed that *E. coli* and *S. aureus* has a negative surface charge. The interaction between bacteria and phenolics (gallic and ferulic acids) change the surface charge of cells to less negative values especially for *E. coli*. In the case of *S. aureus*, the zeta potential values were similar to the control. In a study performed by Abreu *et al.* [7], the zeta potential was studied for *E. coli* and *S. aureus* when in contact with a glucosinolate (phenyl isothiocyanate). It was verified that the values of surface charge of the cells become less negative for both bacteria. In our study, this was particularly verified in the case of *S. aureus*.

As mentioned above, the hydrophobicity has been characterized as one important aspect in bacterial adhesion [44]. The hydrophobicity can be calculated through the energy of hydrophobic attraction (ΔG^{TOT}). If $\Delta G^{\text{TOT}} < 0$ mJ/cm², the interaction between the surface molecules is attractive, which means that molecules have less affinity for water than among themselves, and the surface is considered hydrophobic. If $\Delta G^{\text{TOT}} > 0$, the surface is considered hydrophilic, and the interaction between the surface molecules is repulsive. Therefore, the more negative the value of ΔG^{TOT} , more hydrophobic is the surface; and the more positive the value of ΔG^{TOT} , more hydrophilic is the surface [44,45].

Table 3 presents the results of hydrophobicity obtained for *E. coli* and *S. aureus* cells in the presence of the four phytochemicals. Regarding the several parameters, the apolar component (γ^{LW}) was particularly changed when *E. coli* is treated with I3C and SP ($p < 0.05$), making their surface less apolar. The same phytochemicals were also able to change the γ^{AB} component making the cells more polar ($p < 0.05$). However, in the presence of 7-HC and mainly SA, *E. coli* become less polar. The electron donor component increased with the application of I3C and SP and decreased with

7-HC and SP. In the case of *S. aureus*, the treatment with the several phytochemicals did not permit variations on the polarity of surface molecules ($p > 0.05$). Except in the case of SA, the value of γ^{AB} was lower than the observed in the control, which means that the surface of molecules was less hydrophilic. Regarding their electron donor capacity, 7-HC and SA varied significantly ($p < 0.05$), compared with the values obtained when *S. aureus* was not treated with phytochemicals. These phytochemicals decreased the capacity to donate electrons, *i.e.*, when *S. aureus* was in contact with 7-HC and SA the cell lost its ability to give electrons (γ^- decreased).

The ΔG^{TOT} values obtained for *E. coli* and *S. aureus*, before exposure to the phytochemicals, show that they have hydrophilic character ($\Delta G^{TOT} > 0$ mJ/m²). It is possible to observe changes in the bacterial membrane physicochemical character of *E. coli* with the application of all compounds particularly with SA and SP ($p < 0.05$). The application of 7-HC, I3C and SP promoted the decrease of hydrophilic properties of *E. coli*. However, with SA the cell surface of this bacterium become more hydrophilic. The opposite effect was observed for *S. aureus* with SA. This phytochemical induced a cell surface hydrophobic character ($p < 0.05$). Similar result was obtained with 7-HC. However, I3C increased bacterial hydrophilic character ($p < 0.05$). In general, the results obtained demonstrated that the selected phytochemicals significantly interact with bacterial surface constituents, modifying their physicochemical properties. In other work [7], the interaction of a GHP, phenyl isothiocyanate, with bacterial cells was assessed. The results demonstrated that the interaction caused an alteration of cell surface hydrophobicity. The interaction with the phytochemical promoted the increase of their hydrophilic character. Even if the cell membrane is the first contact point between the microorganism and the phytochemical, to our knowledge there are no relevant studies on the effects of phytochemicals on microbial surface properties.

Table 3. Hydrophobicity (ΔG^{TOT}), and apolar (γ^{LW}) and polar (γ^{AB}) components of the surface tension of untreated (control) and phytochemical treated cells. The means \pm SDs are illustrated.

Bacteria	Phytochemical	Surface Tension Parameters (mJ/m ²)				Hydrophobicity (mJ/m ²)
		γ^{LW}	γ^{AB}	γ^+	γ^-	ΔG^{TOT}
<i>E. coli</i> CECT 434	Control	33.6 \pm 5.0	22.4 \pm 5.4	2.6 \pm 0.5	52.0 \pm 4.8	28.9 \pm 7.1
	7-HC	30.7 \pm 4.8	20.8 \pm 4.7	1.50 \pm 0.3	44.9 \pm 7.1	21.0 \pm 5.1
	I3C	20.2 \pm 4.8	37.1 \pm 7.3	7.80 \pm 1.9	55.8 \pm 6.7	20.6 \pm 3.8
	SA	31.0 \pm 5.2	2.07 \pm 6.0	1.50 \pm 0.3	59.7 \pm 11	37.6 \pm 18
	SP	21.0 \pm 1.8	40.1 \pm 3.1	7.80 \pm 1.3	52.1 \pm 2.8	19.7 \pm 3.3
<i>S. aureus</i> CECT 976	Control	35.4 \pm 5.4	19.7 \pm 4.6	2.00 \pm 0.4	53.5 \pm 3.8	30.2 \pm 3.2
	7-HC	36.2 \pm 3.4	21.1 \pm 3.8	2.70 \pm 0.3	47.8 \pm 4.2	22.4 \pm 4.8
	I3C	34.5 \pm 4.2	20.4 \pm 4.8	2.20 \pm 0.5	55.4 \pm 5.1	32.2 \pm 7.3
	SA	37.4 \pm 3.0	15.2 \pm 3.5	1.50 \pm 0.3	44.8 \pm 7.7	22.6 \pm 5.6
	SP	36.1 \pm 4.4	18.3 \pm 4.4	2.1 \pm 0.5	54.4 \pm 1.5	30.4 \pm 2.9

$\Delta G^{TOT} > 0$ mJ/m²—Hydrophilic; $\Delta G^{TOT} < 0$ mJ/m²—Hydrophobic.

Bacterial adhesion to a surface is a complex process that can be influenced by several factors: physicochemical properties of the cell (hydrophobicity and surface charge), material surface

properties and environmental factors (temperature, pH, exposure time, concentration of bacteria, chemical treatment or the presence of antimicrobials and fluid flow conditions) [40]. Behind these conditions, biological properties of bacteria also influence the attachment to surface, such as: presence of fimbriae and flagella, and the production of EPS [40].

The polystyrene (PS) microtiter plates are usually used as the standard bioreactor system for adhesion and bacterial biofilm formation. According to the PS physicochemical surface properties, this material was characterized for being hydrophobic and present negative surface charge ($\Delta G^{\text{TOT}} = -44 \text{ mJ/m}^2$; zeta potential = $-32 \pm 2 \text{ mV}$) [40]. Therefore, in order to predict the ability of microorganisms to adhere to PS surfaces, the free energy of interaction between the bacterial surface and the PS surface was assessed according to a thermodynamic approach (Table 4). The thermodynamic theory of adhesion permits the quantification of the free energy of adhesion and predicts the possibility of establishment of an interface between a surface and the microorganism. Analyzing the free energy of adhesion, it is possible to conclude that both *S. aureus* and *E. coli* have no theoretical thermodynamic capacity to adhere to PS ($\Delta G_{\text{bws}}^{\text{Tot}} > 0 \text{ mJ/m}^2$). This was significantly reversed when *S. aureus* was treated with SA ($\Delta G_{\text{bws}}^{\text{Tot}} < 0 \text{ mJ/m}^2$). Besides, a decrease in the free energy of adhesion was found for *S. aureus* and *E. coli* when exposed to 7-HC, and for *S. aureus* exposed to SP. An increase in the $\Delta G_{\text{bws}}^{\text{Tot}}$ value (less favorable adhesion) was found for *E. coli* when exposed to I3C, SA and SP. A similar effect was observed for *S. aureus* with I3C, where there was an increase in the free energy of adhesion. These distinct values of $\Delta G_{\text{bws}}^{\text{Tot}}$ found after exposure to phytochemicals is apparently related to the distinct physicochemical cell surface properties (Table 3). The interaction of the different compounds tested with bacterial cells, appears to depend on the bacteria tested and on the molecule used. Several authors explained that increasing the hydrophobicity can cause an increasing extension of adhesion [46–48]. Although it is known that hydrophobicity plays an important role in the adhesion phenomena, the results obtained in this work suggest that this phenomenon is not only influenced by physicochemical surface properties and is governed by other factors. Indeed, the thermodynamic theory of adhesion not do consider the electrostatic interactions and biological aspects of adhesion [44].

Table 4. Free energy of adhesion ($\Delta G_{\text{bws}}^{\text{TOT}}$) of *E. coli* and *S. aureus* to polystyrene (PS), with and without exposure to the phytochemicals.

Strain	Phytochemical	Free Energy of Adhesion— $\Delta G_{\text{bws}}^{\text{TOT}}$ (mJ/m ²)
<i>E. coli</i> CECT 434	Control	4.4 ± 1.2
	7-HC	3.5 ± 0.5
	I3C	15.3 ± 3.3
	SA	10.3 ± 2.1
	SP	13.5 ± 2.1
<i>S. aureus</i> CECT 976	Control	5.7 ± 1.2
	7-HC	1.4 ± 0.2
	I3C	6.4 ± 1.2
	SA	-3.2 ± 0.4
	SP	5.4 ± 0.5

$\Delta G_{\text{bws}}^{\text{TOT}} < 0 \text{ mJ/m}^2$ —thermodynamic favorable adhesion; $\Delta G_{\text{bws}}^{\text{TOT}} > 0 \text{ mJ/m}^2$ —thermodynamic unfavorable adhesion.

Opposite effects were obtained, when comparing the $\Delta G_{\text{bws}}^{\text{Tot}}$ for the Gram-negative (the free energy of adhesion increased), except with 7-HC, and the Gram-positive bacterium (the free energy of adhesion decreased), except with I3C. Therefore, it is expected that I3C, SA and SP can hinder the adhesion of *E. coli* to PS. In addition, I3C may also impair the adhesion of *S. aureus*. In previous studies [49–52] the anti-adhesive properties of some polyphenolics was described. In these studies, the anti-adhesive tests were performed mainly with *Streptococcus mutans* using glass [52] and saliva-coated hydroxyapatite [49–52] as adhesion surfaces. Moreover, Borges *et al.* [18,23], demonstrated that some phenolic acids (gallic and ferulic acids) and GHP (allylisothiocyanate and 2-phenylethylisothiocyanate) reduced the potential of adhesion to PS of some pathogenic bacteria, including *E. coli* and *S. aureus*. In other work performed by Luis and coworkers [53], gallic acid was also able to influence the adhesion properties of methicillin-resistant *S. aureus* (MRSA) to PS. Similar results were obtained by Lemos *et al.* [54], with SA against *Bacillus cereus*.

2.2.2. Cell Motility

Motility is one of the most important features in microbial physiology. Bacteria show different ways of motility. Swimming and swarming motilities are two forms of surface flagella-dependent motility existing in *E. coli* [23,54,55]. These types of motility contribute to the virulence of pathogens through adhesion and biofilm formation on biotic and abiotic surfaces [23]. *S. aureus* is a non-flagellated bacterium with a kind of motility defined as sliding or colony spreading [23]. This sliding motility is produced by the expansive forces of a growing colony in combination with reduced surface tension [23].

The phytochemicals at their MIC were tested for their ability to interfere with swimming and swarming motilities of *E. coli*, and sliding motility of *S. aureus*. The results obtained are presented in Table 5. *E. coli* showed an increasing in swimming and swarming motilities over time. Swimming and sliding motility were mostly affected when I3C was added ($p < 0.05$). However, I3C did not influence swarming motility (24 to 72 h). SA was also able to promote a decrease in swimming and sliding motilities but not swarming motility, despite being very low in the first 24 h, increased in the last 48 h. Probably, *E. coli* was able to adapt to SA after a long period of exposure. The application of SP caused an increase in swarming and sliding motilities in the first hours, but after a long period of exposure, both motilities decreased. Finally, 7-HC influenced swarming and swimming motilities but it was not able to change sliding motility. These results may be important because changes in motility can be associated with a reduced ability of bacteria to form biofilms. Likewise, other reports shown that, many natural compounds (extracts or pure products) have capability to interfere with bacterial motility of several microorganisms. Methanolic extracts of *Cuminum cyminum*, which contain methyl eugenol, inhibited swimming and swarming motility of *P. aeruginosa*, *P. mirabilis* and *Serratia marcescens* [56]. Cinnamaldehyde and eugenol from *Cinnamomum cassia* affected the biofilm formation of *E. coli*, through interference with their swimming motility [57].

Table 5. Motility results for bacteria with and without phytochemicals. The drop baseline was 6 mm, which was subtracted from the results presented.

Time/Phytochemical	<i>E. coli</i> CECT 434		<i>S. aureus</i> CECT 976
	Swimming (mm)	Swarming (mm)	Sliding (mm)
24 h			
Control	79.0 ± 1.2	8.70 ± 0.6	7.00 ± 0.0
7-HC	7.00 ± 1.0	7.70 ± 1.5	5.00 ± 0.0
I3C	4.70 ± 0.6	7.70 ± 2.9	0.0 ± 0.0
SA	3.30 ± 0.9	2.00 ± 0.9	7.70 ± 0.6
SP	80.0 ± 0.0	56.0 ± 2.0	84.0 ± 0.0
48 h			
Control	84.7 ± 0.6	13.7 ± 3.8	8.00 ± 1.0
7-HC	43.3 ± 2.9	8.70 ± 1.2	8.30 ± 0.6
I3C	0.0 ± 0.0	10.0 ± 7.8	0.0 ± 0.0
SA	0.0 ± 0.0	55.0 ± 8.7	0.0 ± 0.0
SP	84.0 ± 0.0	61.7 ± 9.1	56.7 ± 5.8
72 h			
Control	84.0 ± 0.0	64.3 ± 7.6	7.70 ± 0.6
7-HC	51.3 ± 2.3	8.30 ± 0.6	8.30 ± 0.6
I3C	0.0 ± 0.0	8.70 ± 5.5	0.0 ± 0.0
SA	0.0 ± 0.0	54.3 ± 6.4	2.00 ± 0.6
SP	84.0 ± 0.0	13.3 ± 3.2	55.0 ± 8.7

In a recent study performed by Borges *et al.* [23], two phenolic acids (gallic acid and ferulic acid), demonstrated potential to inhibit bacterial motility of four pathogenic bacteria (*E. coli*, *P. aeruginosa*, *S. aureus* and *L. monocytogenes*). These authors found similar results with two isothiocyanates (allylisothiocyanate and 2-phenylethylisothiocyanate). In another study, it was found that ferulic acid and SA can inhibit the swimming motility of *Bacillus cereus* and *Pseudomonas fluorescens* [23,54].

A relationship between cells surface motility and biofilm formation has been reported, especially in the case of swarming motility. Both processes, biofilm formation and swarming, require production of flagella and surface polysaccharides [23]. The major role of swarming motility in biofilm development is to promote initial attachment, probably because the force-generating motion helps to overcome electrostatic repulsive forces between bacterium and the substratum, improving the initial interactions between the two surfaces [58]. Therefore, this demonstrates their important function on the early stages of biofilm formation [59,60]. Several authors have reported mutants with altered swarming motility that made it difficult to form biofilm, concluding that they can play a crucial role in biofilm development [58,61].

2.2.3. Quorum-Sensing

QS is a mechanism by which a bacterial population senses its cell density [62]. This mechanism influences bacterial biofilm growth and development and it is related to cell-cell interactions [63]. This cell-cell communication system is dependent on several factors: synthesis, exchange and

perception of small signal molecules between bacteria [62]. A disc diffusion assay was performed for QSI screening using the biosensor strain *Chromobacterium violaceum* (CV12472). *C. violaceum* synthesizes the violet pigment as a result of their autoinducers *N*-acyl homoserine lactones (AHLs) based QS systems CviI/CviR (homologs of LuxI/LuxR systems), which sense and responds to autoinducers C6-AHL and C4-AHL [64]. The phytochemicals were tested as QS inhibitors, at several concentrations. Table 6 shows the results obtained.

Table 6. Quorum-sensing results for several phytochemicals at different concentrations.

Conc. ($\mu\text{g/mL}$)	7-HC			I3C			SA			SP		
	QSI pigm.	Inhibition halo (mm)	QS halo (mm)	QSI pigm.	Inhibition halo (mm)	QS halo (mm)	QSI pigm.	Inhibition halo (mm)	QS halo (mm)	QSI pigm.	Inhibition halo (mm)	QS halo (mm)
250	+/-	12	n.d.	+	11	n.d.	+	14	n.d.	+	10	n.d.
500	+/-	11	n.d.	+/-	11	5	+	14	n.d.	+	11	n.d.
750	+/-	10	n.d.	+/-	11	6	+	19	n.d.	+	12	n.d.
1000	+/-	11	7	+/-	11	14	+	16	n.d.	+	14	n.d.
1500	+/-	11	7	+/-	12	15	+/-	14	6	+	9	n.d.
2000	+/-	10	5	-	16	9	-	16	8	+	10	n.d.
3000	+/-	10	8	-	20	5	-	16	8	+	10	n.d.
4000	+/-	12	10	-	20	11	-	15	12	+	10	n.d.
5000	+/-	11	5	-	25	9	-	18	9	+	11	n.d.

(+)—There was formation of purple pigment in the plate; (-)—Purple pigment was not formed in the plate; (+/-)—The pigment formed was clearer. n.d.—halo not detected.

The MIC of the four phytochemicals tested against *C. violaceum* CV12472 ranged from 25 to 3200 $\mu\text{g/mL}$. This means that phytochemicals were able to inhibit bacterial growth. QS results showed the effects of the phytochemicals in bacterial growth (inhibition halo) and also the effect of phytochemical in QS, through the detection of pigment inhibition (QS halo). Inhibition of pigment production was detected with some phytochemicals at different concentrations. Of all phytochemicals tested, inhibition of pigment production was detected with 7-HC, I3C and SA with zones of pigment inhibition ranging between 5 to 19 mm. No effect on pigment production was observed with SP at the concentrations tested. The I3C was the most effective to inhibit pigment production. At 500 $\mu\text{g/mL}$ of I3C, inhibition was low, but increasing the concentration, the zone of pigment inhibition also increased. Regarding 7-HC and SA at low concentrations, there was no inhibition of pigment production; but for 1000 and 1500 $\mu\text{g/mL}$ of 7-HC and SA, respectively, QSI halos were detected. Although, SP showed antimicrobial activity, it was not possible to observe an effect on pigment inhibition at the concentrations tested. Regarding QS halos, the concentration of I3C, 7-HC and SA influenced significantly the quantity of pigment production. Therefore, inhibition of QS activity by phytochemicals is concentration dependent, as reported by other authors [62,64,65]. The same authors identified one compound (clove oil) able to inhibit pigment production with 19 mm of pigment inhibition zone against *C. violaceum* (CV12472). In addition, cinnamon, peppermint and lavender caused pigment inhibition against the same bacterium [62]. It has been reported that *Tecoma capensis*, *Sonchus oleraceus*, *Pityriasis alba*, *Pinus nigra*, *Jasminum sambac*, *Rosmarinus officinalis*, *Lavandula angustifolia* and *Laurus nobilis* are great

sources of antimicrobial compounds and QS inhibitors [66]. Other study demonstrated that isothiocyanates, like allylisothiocyanate, benzylisothiocyanate and 2-phenylethylisothiocyanate, have capacity for QSI by modulation of the activity and synthesis of autoinducers, particularly *N*-acyl homoserine lactones (AHLs), interfering with the QS systems of *C. violaceum* (CV12472) [32]. SA has been described as QS inhibitor for diverse bacteria, including *P. aeruginosa* [54,67].

As QS is an important event that is related with the different steps of bacterial biofilm formation and differentiation, QS inhibitors can be useful in biofilm eradication [16,64]. Moreover, QS inhibitors can help to overcome the selective pressure created by antibiotic use [64]. Therefore, the results obtained in this simple screening assay suggest that the selected phytochemicals can inhibit QS phenomena. In addition, these results reinforce the potential of phytochemical products as QS inhibitors.

The phytochemicals and other compounds that affect QS can act at different levels: inhibition of signal biosynthesis or inhibition of activity of AHL-producing enzymes, enzymatic signal degradation and inhibition of reception signal molecules [62]. More tests would be necessary in order to be conclusive about the specific mechanisms causing this QSI.

2.3. Biofilm Control

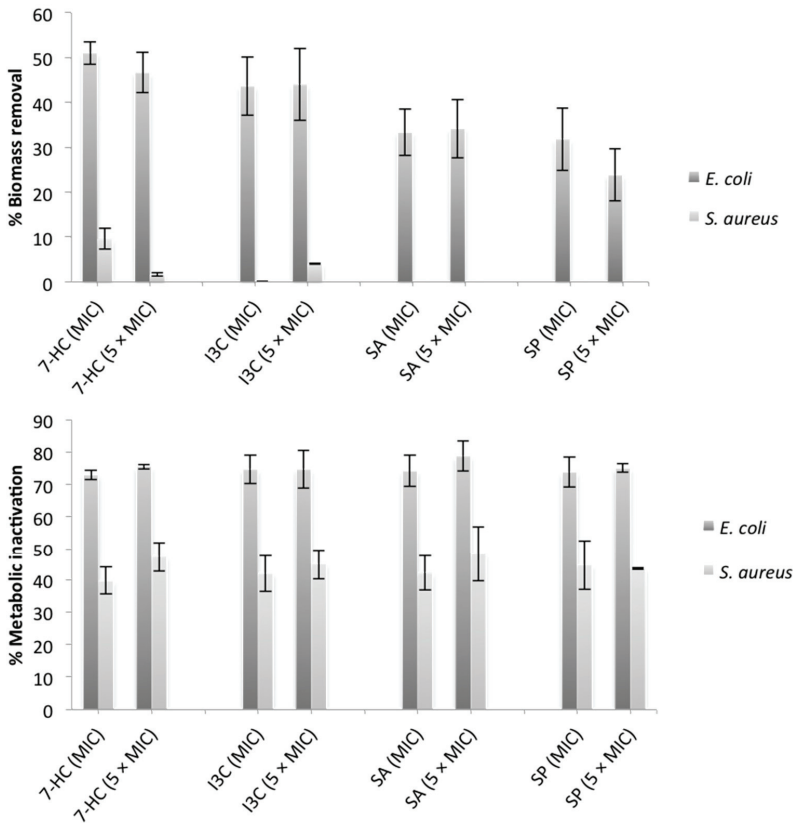
The effects of the phytochemicals were also tested on *E. coli* and *S. aureus* biofilms. Biofilm eradication is difficult to achieve due to their inherent resistance to antibiotics, biocides and host defenses. There are several mechanisms explaining the resistance of biofilms to antimicrobials [68], which makes difficult to predict the behavior of the biofilm cells. In this study, biofilm formation was performed in sterile 96-well polystyrene microtiter plates for 24 h. After that, biofilms were incubated with phytochemicals at MIC and $5 \times \text{MIC}$ for 1 h. The ability of phytochemicals to control (remove and inactivate) 24 h old biofilms was analyzed, based on their effects on biomass and metabolic activity.

Figure 1 presents the percentages of biofilm removal and inactivation with the selected phytochemicals at both concentrations. Comparing the values of obtained, it is perceptible that the phytochemical concentration (MIC and $5 \times \text{MIC}$) did not influence the removal and inactivation of the biofilms ($p > 0.05$), for both bacteria.

The same phytochemical, at the same concentration, caused different behavior in the Gram-negative and -positive bacterium ($p < 0.05$). The percentages of biomass removal and inactivation were always higher for *E. coli* than for *S. aureus* for all the phytochemicals and concentrations tested. Total biofilm removal was not achieved with any of the selected phytochemicals. The highest reduction in biomass was found for *E. coli* with 7-HC.

Despite the greater effect of phytochemicals on biofilms of *E. coli* than on those of *S. aureus*, this result is contradictory to the results obtained with planktonic tests where *E. coli* was less susceptible. In fact, the number of resistance mechanisms in biofilms increase significantly from the planktonic state. Therefore, one cannot infer on the biofilm susceptibility based on the results of planktonic cells. The morphology of the tested biofilms is known to be different. Generally, *S. aureus* biofilms are denser than those of *E. coli* [69].

Figure 1. Percentages of removal and inactivation by the selected phytochemicals against *E. coli* and *S. aureus* biofilms.



Other authors studied the control of *S. aureus* and *E. coli* biofilms on stainless steel, concluding that the last ones are more susceptible to removal by five types of cleaning agents, food additives and other compounds [69]. In other studies, phytochemicals showed higher potential to prevent and control biofilms formed by Gram-negative bacteria compared to Gram-positive [18,32]. In a recent study of Abreu *et al.* [70], the effects of a glucosinolate at different concentrations was used against biofilms of *E. coli* and *S. aureus*. The percentages of biofilm removal 1 h after treatment with phenyl isothiocyanate were considerable for both biofilms. However, *E. coli* biofilms were inactivated at a higher extent than those from *S. aureus* [70]. This result proposes that the mechanisms of antimicrobial resistance known for planktonic cells cannot be used to infer on the antimicrobial resistance of biofilms. In fact, planktonic Gram-negative bacteria are known to be more resistance to antimicrobial agents acting on multiple targets of the cell, such as phytochemicals [63], than Gram-positive bacteria. This is attributed to the presence of an outer membrane in the Gram-negative bacteria that provides a protective barrier to the entry of antimicrobials.

2.4. Combination of the Selected Phytochemicals with Antibiotics

The use of dual combinations of antimicrobial drugs with positive *in vitro* interactions has become an important parameter to potentiate the therapeutic action of antibiotics. These combinations are expected to exert a synergistic effect or to reduce possible adverse side effects. The use of active compounds, such as phytochemicals, in conjunction with antibiotics could avoid the emergence of resistant variants that might otherwise arise during treatment [71]. Several phytochemicals have been analyzed for their action as resistance-modifying agents (RMAs), *i.e.*, compounds able to modify or inhibit bacterial resistance, increasing the efficacy of antibiotics to kill resistant bacteria [71]. Efflux pumps contribute to the resistance of bacteria by pumping out a wide variety of products: dyes, detergents and antibiotics [16]. The role of efflux pumps in bacteria has been related to the elimination of metabolites that are poisonous to the cell and in cell stress responses [72]. The association of antibiotics with phytochemicals can create a synergistic effect against resistant bacteria, creating new choices for the treatment of infectious diseases.

S. aureus genome reveals high potential multidrug efflux-pump-encoding genes [73]. Several efflux resistance mechanisms have been described for *S. aureus* such as QacA and NorA, which are multidrug transporters, and the more specific MsrA and TetK transport proteins [74].

In this study, the antimicrobial activity of several phytochemicals was tested in combination with three antibiotics. For these experiments, besides *S. aureus* CECT976, three different strains of *S. aureus* were tested: *S. aureus* RN4220, SA1199B, XU212. The strains are characterized by the presence of different efflux pumps: MsrA macrolide efflux protein, NorA MDR efflux protein and TetK efflux pump. Table 7 shows the diameter of the inhibition halos (mm) obtained with the antibiotics (ciprofloxacin, tetracycline and erythromycin) and the phytochemicals alone against the different *S. aureus* strains.

Table 7. Antimicrobial activity of antibiotics. The means (mm) \pm standard deviation for at least three replicates are illustrated.

	Diameter of inhibition zone (mm)			
	<i>S. aureus</i> CECT 976	<i>S. aureus</i> XU212	<i>S. aureus</i> RN4220	<i>S. aureus</i> SA1199B
TET	41.5 \pm 9.2	16.0 \pm 4.2	42.5 \pm 0.7	46.5 \pm 2.1
ERY	37.5 \pm 3.5	24.5 \pm 4.9	22.0 \pm 4.2	35.5 \pm 3.5
CIP	40.5 \pm 0.7	26.0 \pm 2.8	31.5 \pm 2.1	18.0 \pm 1.4

According to Clinical Laboratory Standard Institute (CLSI) guidelines [75], *S. aureus* CECT 976 is considered susceptible to all antibiotics tested, *S. aureus* XU212, *S. aureus* RN4220 and *S. aureus* SA1199B are resistant to TET, ERY and CIP, respectively. The negative control performed with dimethyl sulfoxide (DMSO) in the preparation of phytochemical solutions presented no effects on bacterial growth (data not shown).

Tetracycline was the most effective against all the bacteria (except for *S. aureus* XU212), while erythromycin and ciprofloxacin had the lowest antimicrobial activity against *S. aureus* CECT 976/RN4220 and *S. aureus* SA1199B, respectively ($p < 0.05$).

Dual combinations of antibiotic-phytochemicals were performed. Antibiotic synergism occurs when the effects of combination of antimicrobials is greater than the sum of the effects of individual antimicrobials [76]. An additive effect of phytochemical combined with antibiotic may occur due to a double attack of both agents at different target sites of the cell [77]. Table 8 shows the classification of the combined application of tetracycline, erythromycin and ciprofloxacin with the phytochemicals according to Saavedra *et al.* [76].

Table 8. Classification of the effect of dual combinations of phytochemicals and antibiotics.

		7-HC	I3C	SA	SP
<i>S. aureus</i> CECT 976	TET	+	+++	+	-
	ERY	-	+++	+	++
	CIP	+	+++	+	-
<i>S. aureus</i> XU212	TET	++	+++	+++	+++
<i>S. aureus</i> RN4220	ERY	-	+++	+++	+++
<i>S. aureus</i> SA1199B	CIP	+	+++	+++	+++

(-)—Antagonist; (+)—Indifference; (++)—Additive; (+++)—Synergistic.

The combination of I3C with all the antibiotics showed synergistic effects against the four *S. aureus* strains tested. Synergistic activities were also verified when combining SA or SP with TET, ERY and CIP against *S. aureus* XU212, *S. aureus* RN4220 and *S. aureus* SA1199B. This reinforces the advantageous antimicrobial effect of phytochemical-antibiotic combinations. Only two combinations presented additive results: 7-HC-TET against *S. aureus* XU 212 and SP-ERY against *S. aureus* CECT 976. However, the results of combination of antibiotics with the phytochemicals also presented negative effects: the association of 7-HC-ERY was antagonist against *S. aureus* CECT 976 and *S. aureus* RN4220. Also, the combination of SP-TET or SP-CIP presented an antagonistic activity against *S. aureus* CECT 976. The other combinations presented indifferent effects.

Previous studies already demonstrated the synergistic potential of phytochemicals when combined with antibiotics against pathogenic bacteria [7,27,37,76,78]. The alkaloid piperine, in combination with ciprofloxacin markedly reduced the inhibitory concentration and the mutation concentration of ciprofloxacin for several *S. aureus* strains, including MRSA [78]. The combination of four antibiotics (ciprofloxacin, erythromycin, gentamicin and vancomycin) with some sesquiterpenoid increased their antimicrobial activity against *E. coli* and *S. aureus*, comparatively to the antibiotic/sesquiterpenoids single application [37]. The application of dual combination demonstrated synergy between the aminoglycoside streptomycin with gallic, ferulic and chlorogenic acids, allylisothiocyanate and 2-phenylethylisothiocyanate against *E. coli* and *P. aeruginosa* [76]. In other work [7], another isothiocyanate, phenyl isothiocyanate, showed a good efficacy against *S. aureus* strains when combined with CIP and ERY due to an additive effect. Synergistic interaction was observed by Biswas and Roymon [27], on combined administration of saponin with chloramphenicol to inhibit *E. coli* strains.

3. Experimental Section

3.1. Bacterial Strains

Two strains from the Spanish Type Culture Collection (CECT) were used in this study: *E. coli* CECT 434 and *S. aureus* CECT 976. *S. aureus* RN4220 containing plasmid pUL5054, which carries the gene encoding the MsrA macrolide efflux protein; *S. aureus* SA1199B, which over expresses the NorA MDR efflux protein and *S. aureus* XU212, which possesses the TetK efflux pump and is also a methicillin-resistant *Staphylococcus aureus* (MRSA) strain, were kindly provided by S. Gibbons (University College London, UCL) [74,79–81]. Prior to use, these strains at $-80\text{ }^{\circ}\text{C}$ were transferred onto Mueller-Hinton (MH, Merck Germany) agar plate, grown overnight, and inoculated into MH broth at $30\text{ }^{\circ}\text{C}$ and under agitation (150 rpm). Also, *Chromobacterium violaceum* ATCC 12472 were distributed over the surface of Luria-Bertani (LB, Merck Germany) agar and incubated for 24 h at $30 \pm 3\text{ }^{\circ}\text{C}$.

3.2. Phytochemicals and Antibiotics

The phytochemicals used were: 7-HC, I3C, SA and SP. The concentration of phytochemical used for the several experiments corresponds to MIC and $5 \times \text{MIC}$. Regarding the phytochemicals, 7-HC, SA and SP belong to phenolic group and I3C belongs to glucosinolate group. These compounds were obtained from Sigma (Sintra, Portugal) and prepared in DMSO (Sigma, Portugal). CIP, ERY and TET were obtained from Sigma (Portugal) and prepared in DMSO. The antibiotics were applied in different concentrations according to CLSI [75]. Ciprofloxacin was tested at $5\text{ }\mu\text{g}/\text{disc}$, erythromycin was evaluated at $15\text{ }\mu\text{g}/\text{disc}$ and finally, tetracycline was assessed at $30\text{ }\mu\text{g}/\text{disc}$. The three antibiotics tested belong to three different antibiotics classes: quinolone, macrolides, tetracycline. After preparation, antibiotic stock solutions were stored at $-4\text{ }^{\circ}\text{C}$.

3.3. Determination of Minimal Inhibitory Concentration and Minimal Bactericidal Concentration

The MIC of phytochemicals was determined by microdilution method in sterile 96-well microtiter plates according to CLSI [75]. Overnight cell cultures (14 h incubation) of *S. aureus* and *E. coli*, in the exponential phase of growth, were adjusted to a cell density of 1×10^6 cells/mL and added to sterile 96-well polystyrene microtiter plates (Orange Scientific, Belgium) with the different phytochemicals in a final volume of $200\text{ }\mu\text{L}$. The antimicrobial solutions did not exceed 10% (v/v) of the well. DMSO was used as negative control. No antimicrobial activity was detected by DMSO (data not shown). Plates were incubated for 24 h at $30 \pm 3\text{ }^{\circ}\text{C}$. MIC corresponds to the concentration in which the final optical density (OD) was inferior or equal to the initial OD.

MBC of phytochemicals was determined by the drop method [9,18]. After measuring the MIC, the wells corresponding to the phytochemicals concentrations equal and above the MIC were added ($10\text{ }\mu\text{L}$) to plate count agar (PCA, Sigma, Portugal) plates. The maximum concentration tested for the phytochemicals was $50,000\text{ }\mu\text{g}/\text{mL}$. The drops were drained along the plate. After 24 h at $30 \pm 3\text{ }^{\circ}\text{C}$, the plates were analyzed and the MBC of each phytochemical, corresponding to the

concentration which inhibited the growth of the bacteria, was recorded. All tests were performed in triplicate.

3.4. Determination of Zeta Potential

Zeta potential experiments were performed according to Simões *et al.* [40]. The overnight cultures of *E. coli* and *S. aureus* were centrifuged at 3777 g for 10 min and washed twice with sterile water. Cells suspensions (at a final concentration of 10^9 cells/mL), prepared in sterile tap water, of *E. coli* and *S. aureus* were incubated with phytochemicals (at MIC) for 30 min at 30 ± 3 °C. Cells suspensions without phytochemicals were used as control. The zeta potential experiments were performed using a Malvern Zetasizer instrument (Nano Zetasizer, Malvern instruments, Worcestershire, UK). All experiments were carried out in triplicate at room temperature and were repeated at least at three different occasions.

3.5. Physicochemical Characterization of Bacterial Surface

The physicochemical properties were measured using the sessile drop contact angle method [82]. After overnight growth, the cells suspensions were washed with NaCl (8.5 g/L) and centrifuged (10 min at 3777 g) twice. Cells suspensions prepared with sterile tap water ($OD_{640nm} = 0.2 \pm 0.02$) were incubated with phytochemicals (at MIC) during 30 min. The solutions were filtrated (0.45 μ m, Whatman, United Kingdom) and placed in microscope slides. The contact angle was measured with 3 different liquids: water, formamide (polar) and α -bromonaphtalene (nonpolar) (Sigma, Portugal).

The measurement of contact angles was performed using a model OCA 15 Plus (Dataphysics, Filderstadt, Germany) video based optical contact angle measuring instrument, allowing image acquisition and data analysis. The degree of hydrophobicity of a surface is expressed as the free energy of interaction between entities of that surface (s), when immersed in water (w)— ΔG_{sws} . ΔG_{sws} (mJ/m²) can be positive or negative according of the interaction between the surfaces. In the case of $\Delta G_{sws} > 0$, the material is considered hydrophilic, because the interaction between the two surfaces is weaker than the interaction of each entity with water. In contrast, when $\Delta G_{sws} < 0$, the interaction between the surfaces is stronger than the interaction of each entity with water and the material is hydrophobic. Hydrophobicity was evaluated after contact angles measurements, following the van Oss approach [83–85].

The degree of hydrophobicity can be calculated through the surface tension components of interacting entities, according to Equation (1).

$$\Delta G_{sws} = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_s^+ \gamma_w^-} + \sqrt{\gamma_s^- \gamma_w^+} - \sqrt{\gamma_s^+ \gamma_s^-} - \sqrt{\gamma_w^+ \gamma_w^-}\right) \quad (1)$$

Where, γ^{LW} is the Lifshitz-van der Waals component of the surface free energy and γ^+ and γ^- are the electron acceptor and donor, respectively, of the Lewis acid-base parameter (γ^{AB}), being $\gamma^{AB} = \sqrt{\gamma^+ \gamma^-}$.

The analysis was performed at room temperature using the three liquids referred before. The surface tension components of liquids were obtained from literature [86]. Subsequently, three equations can be solved:

$$(1 + \cos \theta) \gamma_1^{\text{Tot}} = 2 \left(\sqrt{\gamma_s^{\text{LW}} \gamma_w^{\text{LW}}} + \sqrt{\gamma_s^+ \gamma_w^-} + \sqrt{\gamma_s^- \gamma_w^+} \right) \quad (2)$$

where θ is the contact angle and $\gamma^{\text{TOT}} = \gamma^{\text{LW}} + \gamma^{\text{AB}}$.

These measurements were performed at least with 12 determinations for each liquid and microorganism.

Free Energy of Adhesion

The free energy of adhesion between the bacterial cells and polystyrene surfaces was calculated through the surface tension components of the entities involved in the process using the Dupré equation and the procedure described by Simões *et al.* [40]. The total interaction energy ($\Delta G_{\text{bws}}^{\text{TOT}}$) is studied by the interaction between one bacteria (b) and a substratum (s) that are immersed or dissolved in water (w) and is expressed by the interfacial tension components:

$$\Delta G_{\text{bws}}^{\text{TOT}} = \gamma_{\text{bs}} - \gamma_{\text{bw}} - \gamma_{\text{sw}} \quad (3)$$

The thermodynamic theory of the interfacial tension of one system of interaction (for example, bacteria/surface— γ_{bs}) can be defined by the following equations:

$$\gamma_{\text{bs}} = \gamma_{\text{bs}}^{\text{LW}} + \gamma_{\text{bs}}^{\text{AB}} \quad (4)$$

$$\gamma_{\text{bs}}^{\text{LW}} = \gamma_{\text{b}}^{\text{LW}} + \gamma_{\text{s}}^{\text{LW}} - 2 \times \sqrt{\gamma_{\text{b}}^{\text{LW}} \times \gamma_{\text{s}}^{\text{LW}}} \quad (5)$$

$$\gamma_{\text{bs}}^{\text{AB}} = 2 \times \left(\sqrt{\gamma_{\text{b}}^+ \times \gamma_{\text{b}}^-} + \sqrt{\gamma_{\text{s}}^+ \times \gamma_{\text{s}}^-} - \sqrt{\gamma_{\text{b}}^+ \times \gamma_{\text{s}}^-} - \sqrt{\gamma_{\text{b}}^- \times \gamma_{\text{s}}^+} \right) \quad (6)$$

The two other interfacial tension components, γ_{bw} and γ_{sw} , were calculated in the same way, which permits the assessment of thermodynamic energy of adhesion. The bacterial adhesion to the substratum can be favorable or is not expected to occur, according to the values of $\Delta G_{\text{bws}}^{\text{TOT}}$ if are negative or positive, respectively [40].

3.6. Motility Assay

The motility assays were performed according to Borges *et al.* and Simões *et al.* [23,87]. Plates containing 1% tryptone, 0.25% NaCl and 0.25% or 0.7% (w/v) agar (Merck, Portugal) were prepared for swimming (for *E. coli*)/sliding (for *S. aureus*) or swarming motilities, respectively [88,89]. Phytochemicals at MIC concentration were incorporated in the growth medium after sterilize and cooling the medium, to avoid the deterioration. Overnight cultures of *E. coli* and *S. aureus* grown on LB broth (Merck, Germany) were adjusted to 1×10^8 cell/mL and 15 μL of cell suspension were placed in the center of the plates. Then, plates were incubated at 30 °C and the diameter (mm) of the bacterial motility halos was measured at 24, 48 and 72 h [23]. All experiments were carried out in triplicate. The negative control was performed with DMSO.

3.7. Detection of Quorum-Sensing Inhibition

The biosensor strain *C. violaceum* CV12472 was grown overnight in LB broth at 30 ± 3 °C. MIC values were determined using the microdilution method, explained above. All the further experiments were performed at sub-MIC concentrations of phytochemicals [56].

Disc diffusion method was used to detect the inhibition of QS activity of the phytochemicals [90,91]. LB agar plates were spread with 100 μ L of overnight culture of *C. violaceum* CV12472 (approximately 1.4×10^8 CFU/mL). Sterile paper disks (6 mm diameter) (Oxoid, Spain) were placed in the plates and impregnated with various concentrations of each phytochemical (15 μ L). DMSO and LB broth were used as controls. The plates were incubated at 30 ± 3 °C for 24 h, and then the inhibition of the pigment production around the disc (a ring of colorless but viable cells) was checked. Antimicrobial activity was indicated by the lack of microbial growth. Bacterial growth inhibition was measured as diameter 1 (d1) in mm while both bacterial growth and pigment inhibition were measured as total diameter 2 (d2) in mm. Thus, QSI, assessed by pigment inhibition, was determined by subtracting the diameter of bacterial growth inhibition (d1) from the total diameter (d2) ($QSI = d2 - d1$) [32,64].

3.8. Antibiotic-Phytochemical Dual Combinations Assay—Efflux Pumps Inhibition

This procedure was a modification from the Kirby-Bauer method and it has been applied in other studies [7,76]. Each phytochemical was added to MH agar (after autoclaved and cooled) yielding the final concentration desired and the medium was poured into 90 mm Petri dishes. Colonies of *S. aureus* strains were picked from overnight cultures (log phase cultures) and adjusted with NaCl (8.5 g/L) to match to 0.5 McFarland turbidity standards. The suspension was spread with a sterile cotton swap into Petri dishes (90 mm of diameter) containing 20 mL of MH agar. Sterile filter paper discs (6 mm in diameter) (Oxoid, Spain), impregnated with 15 μ L of antibiotics, were placed on the agar plate seeded with the respective bacteria. Discs of ciprofloxacin, erythromycin and tetracycline (Sigma, Portugal) were used as positive controls and discs impregnated with DMSO were used as negative controls. The concentration of antibiotics used was according to CLSI [75]: ciprofloxacin—5 μ g/disc; erythromycin—15 μ g/disc; and tetracycline—30 μ g/disc. The plates were incubated at 30 ± 3 °C for 24 h. After incubation, each inhibition zone diameter (IZD) was measured and analyzed according to CLSI guidelines [75]. All tests were performed in triplicate and the antibacterial activity was expressed as the mean of IZD (mm).

Classification of Dual Combinations

For the phytochemicals with antimicrobial activity, the effect of dual combinations of antibiotics and phytochemicals can be classified according [76]: Antagonism (–) – $[\text{inhibition halo} - (\text{antibiotic inhibition halo} + \text{phytochemical inhibition halo})/2] < 0$; Indifference (+) – $0 \leq [\text{inhibition halo} - (\text{antibiotic inhibition halo} + \text{phytochemical inhibition halo})/2] < \text{antibiotic inhibition halo}$ or $\text{phytochemical inhibition halo}$; Additive (++)— $\text{antibiotic inhibition halo} < [\text{inhibition halo} - (\text{antibiotic inhibition halo} + \text{phytochemical inhibition halo})/2] < 2 \times \text{antibiotic inhibition halo}$ or $\text{phytochemical inhibition halo}$; Synergy (+++)— $\text{inhibition halo} > 3 \times \text{antibiotic inhibition halo}$ or $\text{phytochemical inhibition halo}$.

inhibition halo. For this classification the highest inhibition halos caused by the antibiotic or phytochemical were used.

In the case of no antimicrobial effect of phytochemicals, the classification of the dual combination is different [71]: additive (++)—4 mm \leq inhibition zone diameter combination—inhibition zone diameter most active agent) < 6 mm; synergistic (+++)—inhibition zone diameter combination—inhibition zone diameter most active agent \geq 6 mm.

3.9. Biofilm Formation and Control in Sterile 96-well Polystyrene Microtiter Plates

Biofilms were developed according to the modified microtiter plate test proposed by Stepanović *et al.* [92]. For both bacteria, at least 8 wells of a 96-well polystyrene microtiter plate were filled with 200 μ L of overnight batch cultures in MH broth (OD_{620nm} = 0.04 \pm 0.02). The negative control wells were also placed on the plates, being sterile medium. The biofilms were formed in microtiter plates at 30 \pm 3 $^{\circ}$ C for 24 h. After biofilm development, the content of wells was removed and the wells were washed three times with 200 μ L of NaCl (8.5 g/L) to remove reversibly adherent bacteria. The phytochemicals were added to the wells at the MIC and 5 \times MIC. The microtiter plates were incubated for 1 h. The remaining attached bacteria were analyzed using crystal violet (CV) and resazurin methods.

3.10. Biofilm Analysis

The mass quantification by CV method was based in previous studies [40,87,93]. Before (control wells) and after phytochemicals application, the inoculum in the wells was removed and the wells were washed with 200 μ L of sterile water. Later, 250 μ L of ethanol were loaded for 15 min to promote biofilm fixation. The supernatant was removed and the plates were air-dried. Subsequently, 200 μ L of CV solution (Gram color staining set for microscopy, Merck, Germany) was added for 10 min to stain the fixed bacteria. After washing in water, the plates were dried and finally, the wells were loaded with 200 μ L of acetic acid 33% (v/v) (Merck, Germany) to release and dissolve the stain. In order to analyze the biofilm, the OD of the solutions was measured at 570 nm using a microtiter plate reader (SpectraMax M2E, Molecular Devices, Norway). After obtaining the values of absorbance, the percentage of biomass removal was calculated.

$$\% \text{ Biomass removal} = \frac{OD_{\text{control}_{570}} - OD_{\text{phytochemical}_{570}}}{OD_{\text{control}_{570}}} \times 100 \quad (7)$$

where OD control₅₇₀ represents the optical density of the control at 570 nm, and OD phytochemicals₅₇₀ is the optical density of the phytochemical at 570 nm.

The resazurin microtiter plate assay was performed to evaluate the metabolic activity according to Sarker *et al.* [94]. For the resazurin method, a commercially available resazurin solution (Sigma, Portugal) was used. The plates were loaded with 190 μ L of sterile MH medium and 10 μ L of resazurin solution (0.1 mg/mL). After 20 min of incubation in darkness and at room temperature, fluorescence (λ_{ex} : 570 nm and λ_{em} : 590 nm) was measured using the microtiter plate reader. After measuring the fluorescence, it is possible to calculate the percentage of metabolic inactivation.

$$\% \text{ Metabolic inactivation} = \frac{FLUO_{control} - FLUO_{phytochemical}}{FLUO_{control}} \times 100 \quad (8)$$

where $FLUO_{control}$ represents the fluorescence intensity of biofilms not exposed to phytochemicals and $FLUO_{phytochemical}$ represents the fluorescence intensity value for biofilms exposed to phytochemicals.

3.11. Statistical Analysis

The data was analyzed using One-Way Anova and the statistical program SPSS 21.0 (Statistical Package for the Social Sciences). The results were presented as the means \pm standard deviation. Significance level for the differences was set at $p < 0.05$ and the calculations were based on confidence level equal or higher than 95%.

4. Conclusions

In order to find new antimicrobial agents, plant products were studied as substituents or complementary products of antibiotics for which bacteria already acquired resistance. Therefore, in this work, the antimicrobial effect of selected phytochemicals—7-HC, I3C, SA and SP—was evaluated as well as their ability to control biofilms of two important pathogens, *E. coli* and *S. aureus*.

This study demonstrates that 7-HC and I3C are the most promising phytochemicals against *E. coli* and *S. aureus*. The 7-HC was one of the most effective phytochemicals tested against *E. coli* and *S. aureus* with a MIC of 800 and 200 $\mu\text{g/mL}$ for *E. coli* and *S. aureus*, respectively. Regarding the biofilm control, the exposure of *S. aureus* biofilms to 7-HC at different concentrations produced significantly different percentages of inactivation (39 at MIC and 47 at 5 \times MIC). I3C was also effective against both bacteria with MIC of 800 and 400 $\mu\text{g/mL}$ for *E. coli* and *S. aureus*, respectively. Dual combinations of all the antibiotics and I3C presented a synergistic effect against *S. aureus* resistant strains. Both phytochemicals (I3C and 7-HC) affected the motility and QS activity, which means that they can play an important role in biofilm prevention and interference with cell-cell interactions. The phytochemicals also demonstrated significant potential to reverse antibiotic resistance. However, in order to apply these phytochemicals with therapeutic/clinical purposes, further studies are required to ascertain their toxicity against mammalian cells and to confirm *in vivo* their efficacy and potential side effects.

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FA1202. The authors are solely responsible for this work. It does not represent the opinion of the Community, and the Community is not responsible for any use that might be made of data appearing herein.

Author Contributions

Joana Monte conducted all the experimental section with the help of Ana Abreu and Anabela Borges. Joana Monte also wrote the manuscript which was revised by Anabela Borges, Ana Abreu, Lúcia C. Simões and Manuel Simões. Manuel Simões planned the work. All authors gave the approval to this final version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Antibiofilm Effect of Octenidine Hydrochloride on *Staphylococcus aureus*, MRSA and VRSA

Mary Anne Roshni Amalaradjou and Kumar Venkitanarayanan

Abstract: Millions of indwelling devices are implanted in patients every year, and staphylococci (*S. aureus*, MRSA and vancomycin-resistant *S. aureus* (VRSA)) are responsible for a majority of infections associated with these devices, thereby leading to treatment failures. Once established, staphylococcal biofilms become resistant to antimicrobial treatment and host response, thereby serving as the etiological agent for recurrent infections. This study investigated the efficacy of octenidine hydrochloride (OH) for inhibiting biofilm synthesis and inactivating fully-formed staphylococcal biofilm on different matrices in the presence and absence of serum protein. Polystyrene plates and stainless steel coupons inoculated with *S. aureus*, MRSA or VRSA were treated with OH (zero, 0.5, one, 2 mM) at 37 °C for the prevention of biofilm formation. Additionally, the antibiofilm effect of OH (zero, 2.5, five, 10 mM) on fully-formed staphylococcal biofilms on polystyrene plates, stainless steel coupons and urinary catheters was investigated. OH was effective in rapidly inactivating planktonic and biofilm cells of *S. aureus*, MRSA and VRSA on polystyrene plates, stainless steel coupons and urinary catheters in the presence and absence of serum proteins. The use of two and 10 mM OH completely inactivated *S. aureus* planktonic cells and biofilm (>6.0 log reduction) on all matrices tested immediately upon exposure. Further, confocal imaging revealed the presence of dead cells and loss in biofilm architecture in the OH-treated samples when compared to intact live biofilm in the control. Results suggest that OH could be applied as an effective antimicrobial to control biofilms of *S. aureus*, MRSA and VRSA on appropriate hospital surfaces and indwelling devices.

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1. Introduction

The Nosocomial Infections Surveillance System recognizes *Staphylococcus aureus* as the most frequently isolated nosocomial pathogen from patients [1]. Additionally, a high percentage of these isolates were found to be methicillin resistant (89% of identified *S. aureus* isolates). Methicillin-resistant *S. aureus* (MRSA) is the most commonly identified antibiotic resistant pathogen [2]. It is responsible for causing complicated skin and skin-structure infections and serious hospital-acquired infections [3]. Vancomycin has long been used as the antimicrobial agent for the treatment of MRSA infections in patients. However, this has led to the emergence of vancomycin-resistant *S. aureus* [4] (VRSA). It is estimated that staphylococci normally colonize 20%–25% of healthy adults permanently and 75%–80% transiently [5]. Millions of indwelling devices are implanted in patients every year, and staphylococci are responsible for a majority of infections and treatment failures linked to these devices [6]. Indwelling devices become coated with

host-derived extracellular matrix proteins that provide a rich surface for bacterial attachment [7]. This ability to bind proteins facilitates pathogen attachment to plastic surfaces and other matrices [8]. Once established, staphylococcal biofilms are resistant to antimicrobial treatment and host response, besides serving as the etiological agent for recurrent infections [9]. Biofilm-associated staphylococci can lead to several diseases, including osteomyelitis, chronic wound infection endocarditis, polymicrobial biofilm infections and indwelling medical device infections [10].

The most commonly followed approach in the management of such infections is the removal and replacement of the contaminated devices [10]. An alternative to this is the use of antimicrobials or other technologies to prevent and control bacterial biofilms on indwelling devices. A variety of antimicrobials, including plant essential oils, phages, EDTA, nitric oxide, quorum sensing inhibitors and biofilm dispersants, such as oxidizing biocides, have been evaluated for controlling staphylococcal biofilms [10,11]. Although these approaches have shown promise in the control of staphylococcal biofilms, it is essential that these compounds maintain their efficacy in the presence of host proteins.

Octenidine hydrochloride (OH) is a positively-charged bispyridinamine exhibiting antimicrobial activity against plaque-producing organisms, such as *Streptococcus mutans* and *S. sanguis* [12]. Recent studies have also demonstrated its antimicrobial effect against *E. coli* O157:H7, *Salmonella* Enteritidis, *Acinetobacter baumannii*, *Candida albicans* and *Fusobacterium nucleatum*, *S. aureus* and *Pseudomonas aeruginosa* [13–17]. Toxicity studies in a variety of species have shown that OH is not absorbed through the mucous membrane and gastrointestinal tract, with no reported carcinogenicity, genotoxicity or mutagenicity [18].

The objective of this study was to investigate the efficacy of OH for inhibiting biofilm formation by *S. aureus*, MRSA and VRSA and inactivating pre-formed *S. aureus*, MRSA and VRSA biofilms at 37 °C in the presence and absence of serum proteins on polystyrene matrix, stainless steel coupons and urinary catheters.

2. Results and Discussion

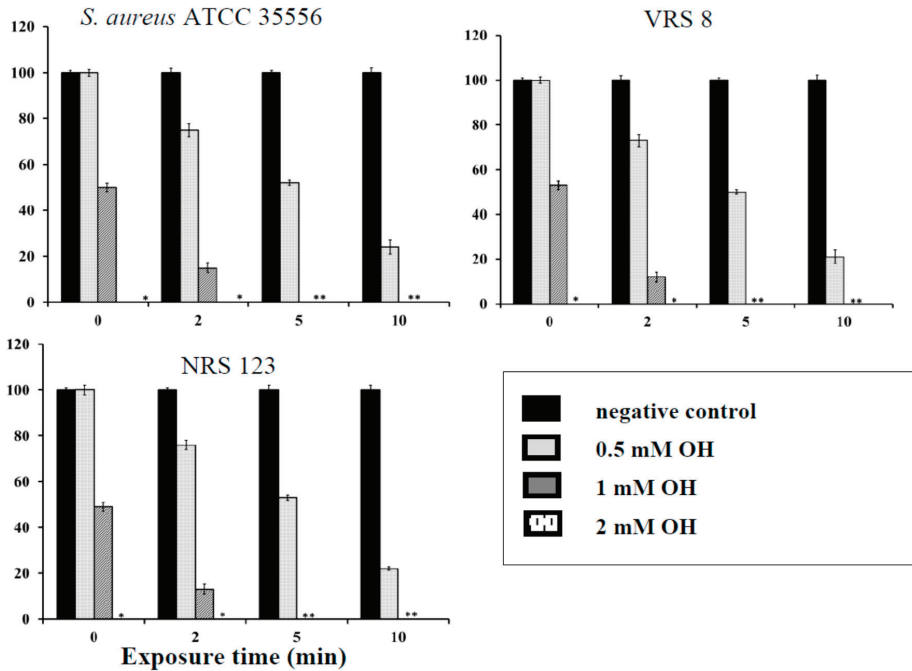
OH was found to be equally effective against *S. aureus*, MRSA and VRSA biofilms. No significant differences were observed between the different isolates ($p < 0.05$). Therefore, the results obtained with one representative isolate of *S. aureus* (ATCC 35556), VRSA (VRS 8) and MRSA (NRS 123) are provided here. OH was found not only to be effective at killing planktonic cells and preventing biofilm formation, but also at inactivated fully established staphylococcal biofilms.

2.1. Prevention of Biofilm Formation

The efficacy of OH in preventing biofilm formation on polystyrene and stainless steel coupons is depicted in Figures 1 and 2. OH was effective in rapidly inactivating planktonic staphylococci cells, thereby preventing the establishment of biofilms on polystyrene and stainless steel surfaces. With planktonic cells, 2 mM of OH completely inactivated staphylococcal populations immediately upon exposure, whereas 1 mM of OH reduced bacterial counts by greater than 3.0 log CFU/mL on

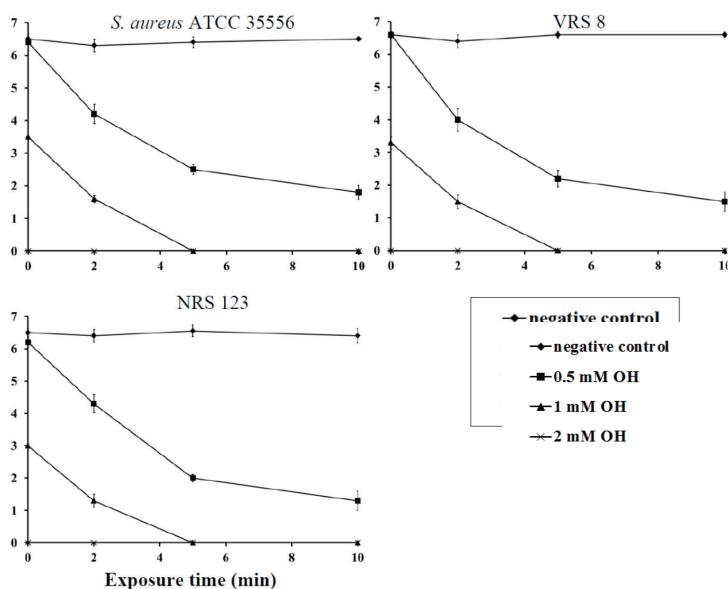
contact (Figures 1 and 2). As expected, staphylococcal populations in negative control samples remained the same throughout the sampling period. A set of samples were also assayed after 24 h to investigate biofilm formation. It was observed that the negative control samples had a fully formed biofilm, while the treated samples did not have any surviving population at 24 h (data not shown). When the efficacy of OH was tested for its ability to prevent biofilm formation in the presence of serum protein, OH retained its antimicrobial efficacy and resulted in a similar antibiofilm effect, as observed in the absence of protein (data not shown).

Figure 1. Inhibition of *S. aureus* (ATCC 35556), vancomycin-resistant *S. aureus* (VRSA) (VRS 8) and MRSA (NRS 123) biofilm formation on polystyrene by octenidine hydrochloride (OH). Duplicate samples were used for each treatment, and the experiment was replicated three times. Data are represented as the mean ± SEM.



Note: Y-axis, Biofilm (Percentage of control)

Figure 2. Inhibition of *S. aureus* (ATCC 35556), VRSA (VRS 8) and MRSA (NRS 123) biofilm on stainless steel by octenidine hydrochloride. Duplicate samples were used for each treatment, and the experiment was replicated three times. Data are represented as the mean \pm SEM (Standard Error of Mean).



Note: Y-axis, Log CFU/coupon.

2.2. Inactivation of Established Biofilm

OH was also effective at killing fully formed biofilms of *S. aureus*, MRSA and VRSA on polystyrene and stainless steel ($p < 0.05$). Staphylococcus has been demonstrated to form biofilms on stainless steel implants, such as screws and fragment implants [19]. Therefore, the antibiofilm effect of OH was also investigated on a stainless steel matrix. At 10- and 5-mM levels, OH completely inactivated the biofilm immediately after addition (0 min) and 5 min of exposure, respectively (Figures 3 and 4). As observed with planktonic cells, the biofilm inactivation by OH was not affected by the presence of serum albumin (data not shown). A similar reduction in biofilm populations was observed by Junka and others [16], who tested the antimicrobial efficacy of octenisept, a commercially available antiseptic that contains octenidine dihydrochloride. They observed a complete inactivation of the *S. aureus* biofilm on polystyrene discs within 1 min of contact time. Another study by Sennhenn-Kirchner [20] evaluated the antimicrobial efficacy of OH on biofilm formed by aerobic oral bacteria on rough titanium surfaces. Their study revealed that rinsing with OH for 8 min reduced the biofilm by 99.8%. However, our study demonstrates that exposure of the biofilm to 10 mM OH completely inactivated it immediately after addition. Besides biofilm inactivation, it is also interesting to note that the antibiofilm effect of OH was irrespective of the strains employed. It was equally effective on the antibiotic-resistant strains (MRSA and VRSA), especially in light of their association with nosocomial and community-acquired infections.

Figure 3. Inactivation of *S. aureus* (ATCC 35556), VRSA (VRS 8) and MRSA (NRS 123) biofilm on polystyrene by octenidine hydrochloride. Duplicate samples were used for each treatment, and the experiment was replicated three times. Data are represented as the mean \pm SEM (Standard Error of Mean).

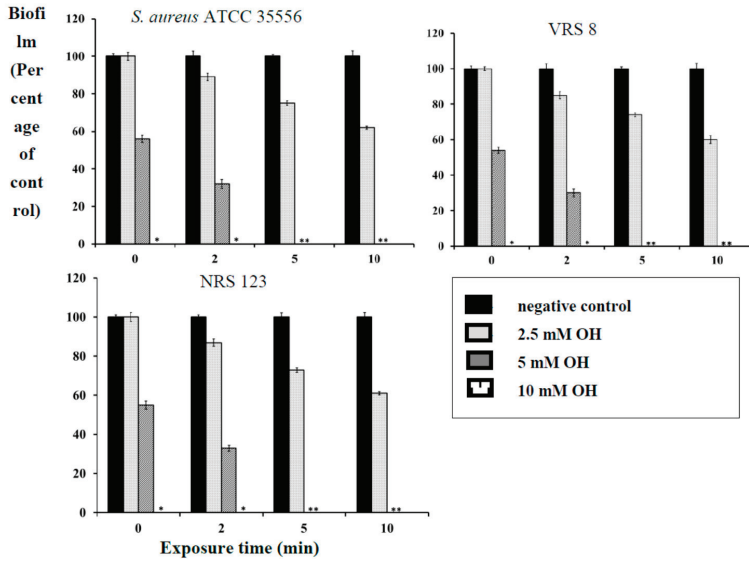
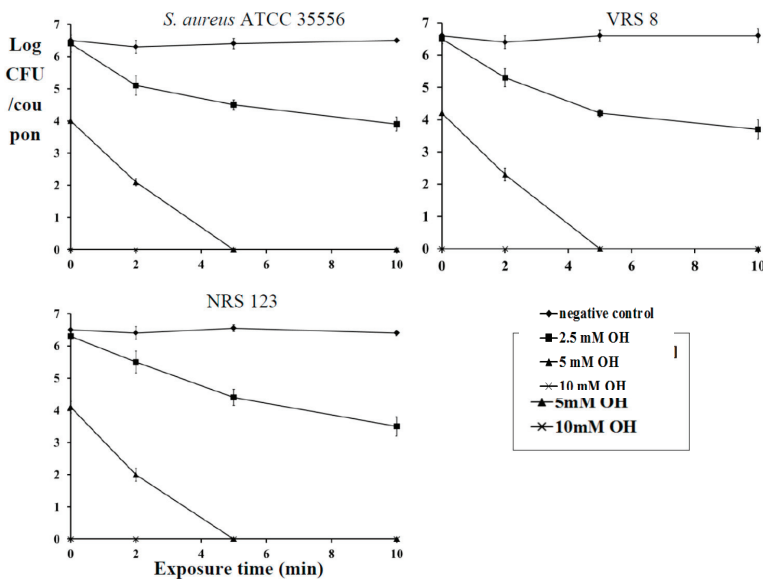


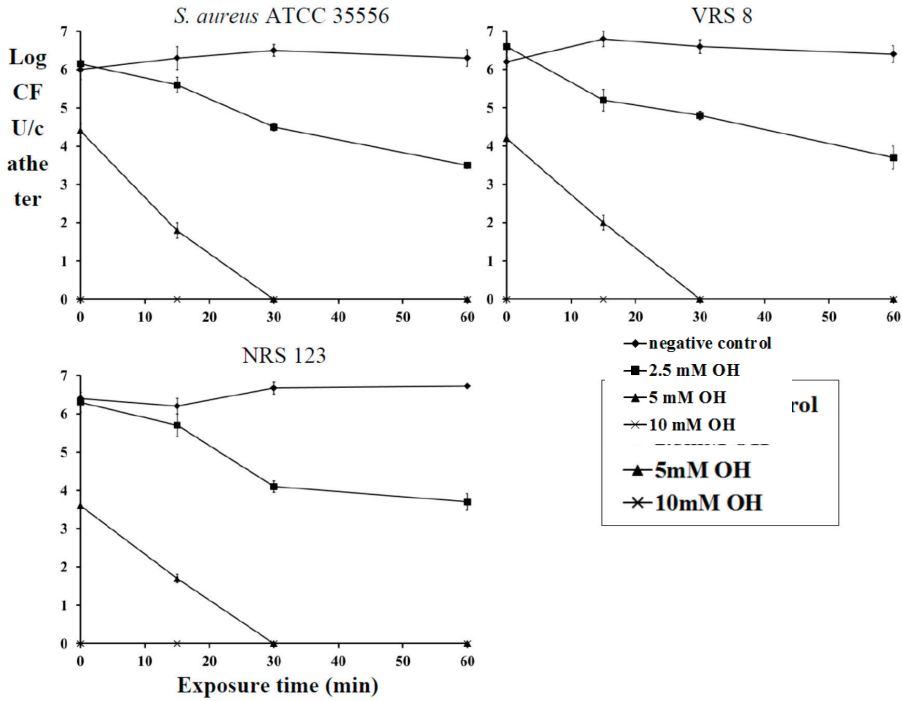
Figure 4. Inactivation of *S. aureus* (ATCC 35556), VRSA (VRS 8) and MRSA (NRS 123) biofilm on stainless steel by octenidine hydrochloride. Duplicate samples were used for each treatment, and the experiment was replicated three times. Data are represented as the mean \pm SEM (Standard Error of Mean).



Staphylococci have been shown to infect and form biofilms on orthopedic implants, stents, intravenous catheters, infusion pumps, mechanical heart valves, pacemakers and cosmetic surgical implants [19]. Treatment of these foreign-body-associated infections caused by MRSA and VRSA are difficult because of the limited availability of antibiotic options that are effective against bacterial biofilms [21]. The current treatment protocol against MRSA involves the use of vancomycin (plus rifampin when the bacteria are susceptible) [22]. However, the increase in the MICs of vancomycin and rifampicin needed for the treatment against MRSA and VRSA is of significant concern [23]. Chaudhury and others [24] investigated the ability of ethanol for the eradication of MRSA biofilms. They observed that the use of ethanol at a 40% concentration could inactivate the biofilms in 1 h. Although efficacious, there are several concerns regarding ethanol use. These include concerns about systemic exposure to ethanol, an increase in catheter dysfunction [25] and the effect of prolonged exposure to ethanol on catheter integrity, which have limited the widespread use of ethanol locks. Besides ethanol, recent study by Rosenblatt and others [26] demonstrated that the use of lock solution containing 7% citrate, 20% ethanol and 0.01% glyceryl trinitrate was able to inactivate MRSA biofilm in 2 h of exposure. Although these approaches have shown promise in the control of MRSA biofilms, it is essential that these compounds maintain their efficacy in the presence of host proteins. A study by Zumbotel and others [17] evaluated the ability of OH to prevent or delay *S. aureus* biofilm formation in OH-coated tracheostomy tubes. This study demonstrated that OH-coated tubes reduced the biofilm associated *S. aureus* population by 2 log compared to the negative control. However, reprocessing of the OH-coated tubes did not result in any significant reduction in biofilm formation. Therefore, in this present study, we investigated the antibiofilm effect of OH as a lock solution using urinary catheters as a model for indwelling devices. Inoculation of catheters with *S. aureus*, MRSA or VRSA resulted in a mature biofilm by the fifth day of incubation at 37 °C. A fully-formed staphylococcal biofilm was recovered from negative control catheters even after 24 h of incubation, whereas no biofilm was detected on catheters within 15 min of exposure to 10 mM of OH (Figure 5). After 60 min of exposure, 5 mM of OH also completely eliminated staphylococcal biofilms (Figure 5). However, staphylococcal biofilm counts on negative control catheters remained at 6.0 log CFU/mL throughout the experiment (Figure 5). A similar antibiofilm effect of OH was also observed in the presence of serum albumin. The ability of OH to retain its antibiofilm efficacy in the presence of serum albumin is of significance, since the presence of host proteins on the indwelling devices enhances the ability of pathogens to attach and form biofilms [1]. The antibiofilm effect of OH was compared with that of tetrasodium EDTA at a concentration of 40 mg/mL [11]. No significant decrease in staphylococcus populations in the biofilm was observed, even after an exposure time of 60 min to the EDTA (data not shown).

OH exerts its antimicrobial effect by binding to the negatively charged bacterial cell envelope, thereby disrupting the vital functions of the cell membrane and killing the cell [27]. It has a high affinity towards cardiolipin, a prominent lipid in bacterial cell membranes, making it selectively lethal to bacterial cells without adversely affecting eukaryotic cells [21]. In addition, Al-Doori and coworkers [28] reported that repeated exposure of *S. aureus* to OH for up to three months did not induce resistance to the compound.

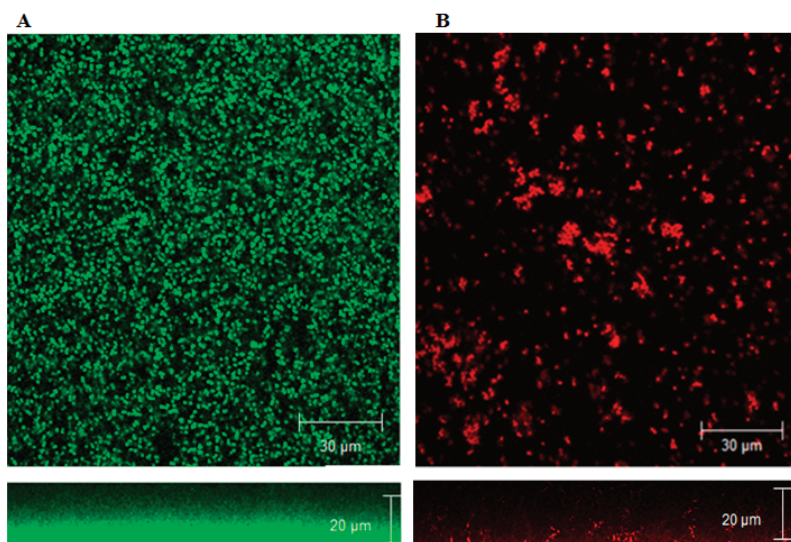
Figure 5. Inactivation of *S. aureus* (ATCC 35556), VRSA (VRS 8) and MRSA (NRS 123) biofilm on urinary catheters by octenidine hydrochloride. Duplicate samples were used for each treatment, and the experiment was replicated three times. Data are represented as the mean \pm SEM (Standard Error of Mean).



2.3. Confocal Microscopy

To investigate the effect of OH on biofilm structure, staphylococcus biofilms formed on glass coverslips were analyzed by confocal microscopy. Positive staining using SYTO[®] (Green fluorescent nucleic acid stain) and propidium iodide (PI) was used for the imaging. The confocal images of the negative control biofilm (Figure 6A) with no added OH revealed the formation of dense biofilm (average thickness 15 μ m) viewed as green cells (live) stained by the SYTO dye, while the image of OH-treated samples (Figure 6B) revealed patchy breaks in biofilm due to the loss of cells and the disruption of organization, viewed as red cells (dead) stained by PI. The average thickness of OH-treated biofilms was 1 μ m. These data collectively indicate that OH was effective at preventing biofilm formation by *S. aureus*, MRSA and VRSA, as well as rapidly inactivating pre-formed biofilms on polystyrene, stainless steel and urinary catheters.

Figure 6. Confocal microscopy of MRSA (NRS 385) biofilm without treatment (A) and after treatment with octenidine hydrochloride (B).



3. Experimental Section

3.1. Culture Preparation

All bacteriological media were purchased from Difco (Becton Dickinson, Sparks, MD, USA). The antibiofilm effect of OH was investigated on *S. aureus* (ATCC 35556, and ATCC 12600), methicillin-resistant *S. aureus* (MRSA; NRS 123, NRS 385, NRS 194) and vancomycin-resistant *S. aureus* (VRSA; VRS 8, VRS 9, VRS 10). MRSA and VRSA strains were obtained from the Network on Antibiotic Resistant *Staphylococcus aureus* (NARSA, Chantilly, VI, USA). Stock cultures were stored at -80°C in brain heart infusion broth (BHI) with 50% glycerol. Prior to each experiment, a loopful of culture was grown in 10 mL of BHI with incubation at 37°C for 24 h. The culture was sedimented by centrifugation ($3600\times g$, 12 min, at 4°C), washed and resuspended in phosphate buffered saline ($1\times$ PBS pH 7.2 consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4) and used as the inoculum. The bacterial count of the inoculum was determined by plating on BHI agar plates and incubation at 37°C for 24 h.

3.2. Octenidine Hydrochloride

OH (>99%) was obtained from Dishman USA, Middlesex, NJ, USA.

3.3. Prevention of Biofilm Formation by *S. aureus*, MRSA and VRSA on Polystyrene by OH

The efficacy of OH in inhibiting *S. aureus*, MRSA and VRSA biofilm production was investigated according to the method of Amalaradjou *et al.* [29]. Briefly, *S. aureus*, MRSA and VRSA strains were separately grown overnight in BHI at 37°C . Following incubation, the cultures

were sedimented by centrifugation (3,600× g for 15 min), washed twice with PBS and resuspended in 10 mL of BHI. Two hundred microliters of the washed culture were used as the inoculum (~6.0 log CFU). Sterile 96-well polystyrene tissue culture plates (Falcon, Franklin lakes, NJ) were inoculated with 200 µL of bacterial suspension, followed by the addition of 0 (negative control), 0.5 (0.25 µL), 1 (0.5 µL) or 2 (1 µL) mM OH (dissolved in 95% ethanol). The plates were incubated at 37 °C. Following 0, 2, 5 and 10 min of OH exposure, the surviving bacterial populations were enumerated by serial dilution (1:10 in PBS) and plating on duplicate BHI plates. When *S. aureus* was not detected by direct plating, samples were tested for surviving cells by enrichment at 37 °C for 24 h in 100 mL of BHI, followed by streak plating on mannitol salt agar (MSA). Representative colonies on BHI were confirmed as staphylococci based on colony morphology on MSA. Triplicate samples were included for each treatment, and the experiment was replicated three times.

3.4. Inactivation of *S. aureus*, MRSA and VRSA Biofilms Formed on Polystyrene by OH

The antibiofilm effect of OH was determined by microtiter plate assay [29]. Sterile 96-well polystyrene tissue culture plates (Costar, Corning Incorporated, Corning, NY, USA) were inoculated with 200 µL of the each bacterial cell suspension (~6.0 logCFU) and incubated at 37 °C for 24 h without agitation for biofilm production. Following biofilm formation, the effect of OH was tested at 0 (negative control), 2.5 (1.5 µL), 5 (2.5 µL) and 10 (5 µL) mM concentrations with an exposure time of 0, 2, 5 and 10 min. After exposure to OH for the specified time, the wells were washed three times with 200 µL of sterile PBS, dried at room temperature and finally stained with 1% crystal violet for 15 min. After rinsing three times with sterile distilled water and subsequent destaining with 95% ethanol, the absorbance of the adherent biofilm was measured at 570 nm in a microplate reader (Model 550, Bio-Rad, Hercules, CA, USA). Uninoculated wells containing BHI were used as blanks. Blank-corrected absorbance values were used for reporting biofilm production. Five replicate wells were included for each treatment, and the assay was repeated three times.

3.5. Enumeration of Bacterial Counts in Biofilm

In addition to the microtiter plate assay, the antibiofilm effect of OH was also assayed by enumerating surviving bacterial populations in the biofilm using the viable plate count method [29]. Following exposure to OH, the wells were washed three times with PBS, and the adherent biofilm was scraped and plated directly or after serial dilution in PBS on BHI plates. The plates were incubated at 37 °C for 24 h before enumerating the bacterial colonies.

3.6. Biofilm Assay on Stainless Steel Matrix

Stainless steel (type 304 with a 4b finish) was used for making coupons (diameter: 1 cm) [30]. Stainless steel coupons were washed and cleaned prior to use, as described by Amalaradjou *et al.* [27].

3.6.1. Biofilm Assay

S. aureus, MRSA and VRSA cells were grown and diluted 1:40, as described before. Two hundred microliters of the inoculum were then dispensed onto the stainless steel coupons submerged in a 24-well plate (Falcon, Becton Dickson Labware, Franklin Lakes, NJ, USA). Biofilm was formed at 37 °C, as before, and treated with 0 (negative control), 2.5, 5 and 10 mM of OH for an exposure time of 0, 2, 5 or 10 min. A procedure described by Ayebah and coworkers [30] was used to remove, disperse and enumerate the cells in biofilm. Duplicate coupons were included for each treatment, and the experiment was replicated three times.

3.7. Biofilm Assay on Catheters

The efficacy of OH for inactivating fully-formed *S. aureus*, MRSA and VRSA biofilms on catheters was determined according to a previously described protocol [29]. A latex 12 F Foley urinary tract catheter (AtHomeMedical) was cut into 3-cm pieces. Each catheter piece was sealed at one end, filled with 1 mL of bacterial culture (~6.0 log CFU) and sealed at the other end. The catheter pieces were then incubated at 37 °C for 5 days to facilitate biofilm formation onto the catheter lumen surface. After 5 days, each catheter piece was washed with sterile saline to remove unattached cells, sealed at one end, filled with 1 mL of sterile normal saline (negative control) or saline containing 2.5, 5 and 10 mM of OH, sealed at the other end and incubated at 37 °C. The biofilm-associated bacterial population was determined following OH exposure (0, 15, 30 and 60 min) by enumerating bacteria after dislodging the biofilm from the catheter surface. This was achieved by vortexing the catheter pieces in separate tubes containing 10 mL of PBS for 1 minute, followed by sonication at 40 KHz for 5 min in a bath sonicator (Branson, North Olmstead, OH, USA). After sonication, viable bacterial counts in PBS from each tube were enumerated after serial dilution (1:10 in PBS) and plating on duplicate BHI plates. Three catheter pieces were included, and the experiment was repeated three times.

3.8. Antibiofilm Effect of OH in the Presence of Serum Protein

The efficacy of OH for inhibiting and inactivating the biofilm of *S. aureus*, MRSA and VRSA in the presence of serum protein was determined according to the method of Edmiston and others [31]. Rehydrated bovine serum albumin (20%) was used to simulate the presence of proteins on indwelling devices. To determine the efficacy of OH in preventing *S. aureus*, MRSA and VRSA biofilm from planktonic cells in the presence of serum proteins, bovine serum albumin was added to each well/stainless steel coupon prior to microbial challenge followed by OH addition (0 (negative control), 0.5, 1 or 2 mM) for 0, 1, 2 and 5 min at 37 °C. Following exposure to OH, the surviving population of bacteria was enumerated by the viable plate count, as described previously. Three samples were included for each treatment, and the assay was replicated three times. For determining the efficacy of OH for killing established bacterial biofilms in the presence of proteins, biofilms were grown in the presence of bovine serum albumin on the different matrices tested and exposed to OH (0 (negative control), 2.5, 5 and 10 mM) for 0, 2, 5 and 10 min on polystyrene and stainless steel and 0, 15, 30 and 60 min on catheters. The biofilms were assayed, as described

before. This study was done at 37 °C. Five replicate wells were included for each treatment, and the assay was repeated three times.

3.9. Confocal Microscopy

To obtain depth-selective information on the three-dimensional structure of the biofilm, *in situ* confocal laser scanning microscopy was performed. For microscopic assessment, biofilms were grown at 37 °C in BHI on a Lab-Tech 8-chambered #1 borosilicate cover glass system (Lab-Tek, Nalge Nunc International, Rochester, NY, USA). The microscopy was performed according to the method reported by Amalaradjou *et al.* [29]. The biofilms formed on cover slips were treated with OH (10 mM), and the live and dead cells were imaged after staining with 2.5 µM SYTO (Molecular probes, OR) and 5 µM propidium iodide (PI, Molecular probes, OR). Biofilms not exposed to OH (negative control) were also imaged to view the normal architecture of *S. aureus*, MRSA and VRSA biofilm. Samples were examined under a Leica true confocal scanner SP2 microscope using the water immersion lens. A krypton-argon mixed gas laser with a PMT2 (Photomultiplier tube 2) filter served as the excitation source.

3.10. Statistical Analysis

Duplicate samples were used for each treatment, and each experiment was replicated three times. For each treatment and the control, the data from independent replicate trials were pooled and analyzed using the proc mixed sub-routine of the statistical analysis software. The model included the treatment concentrations and time as the major effects. A least significant difference test was used to determine significant differences ($p < 0.05$) due to treatment concentrations and time on bacterial counts.

4. Conclusions

In conclusion, our study demonstrates that OH was effective in preventing biofilm formation by *S. aureus*, MRSA and VRSA and rapidly inactivating pre-formed biofilms on polystyrene, stainless steel and urinary catheters. In addition, OH was equally effective against biofilms in the presence and absence of serum proteins. These results suggest that OH can be potentially used as a sanitizer for hospital surfaces. Since *S. aureus*, MRSA and VRSA have the ability to persist in the hospital environment and form biofilms on a wide variety of fomite surfaces, OH can be used as a potential antimicrobial lock solution in both treatment and prophylactic modalities. However, further experiments are needed to further evaluate the efficacy of OH in comparison with other anti-MRSA therapies *in vitro* and *in vivo*. Along with improvements in catheter design and coating, the universal adoption of strict aseptic techniques and the appropriate use of novel catheter lock solutions, such as OH, that minimize catheter-related infections may help to decrease the morbidity and mortality associated with foreign-body-associated infections.

Author Contributions

Kumar Venkitanarayanan and Mary Anne Roshni Amalaradjou conceived of the idea of, designed the experiments for and wrote the manuscript. Mary Anne Roshni Amalaradjou performed the experiments and analyzed the data.

Conflicts of Interest

The authors declare no conflict of interest.

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Antimicrobial and Antibiofilm Activity of Chitosan on the Oral Pathogen *Candida albicans*

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Abstract: Oral candidiasis is particularly evident, not only in cancer patients receiving chemotherapy, but also in elderly people with xerostomy. In general, *Candida* is an opportunistic pathogen, causing infections in immunocompromised people and, in some cases, when the natural microbiota is altered. Chitosan, a natural derivative of chitin, is a polysaccharide that has been proven to possess a broad spectrum of antimicrobial activity that encompasses action against fungi, yeast and bacteria. While recent studies have revealed a significant antibiofilm activity upon several microorganisms, including *C. albicans*, little is known regarding the impact of chitosan upon the adhesive process or mature biofilms. With that in mind, the purpose of this work was to evaluate, *in vitro*, the capability of chitosan to inhibit *C. albicans* growth and biofilm formation. The results obtained showed that chitosan is capable of inhibiting *C. albicans* planktonic growth (HMW, 1 mg/mL; LMW, 3 mg/mL). Regarding biofilm growth, chitosan inhibited *C. albicans* adhesion (ca. 95%), biofilm formation (percentages above 90%) and reduced mature biofilms by ca. 65% and dual species biofilms (*C. albicans* and *S. mutans*) by ca. 70%. These results display the potential of this molecule to be used as an effective anti-*Candida* agent capable of acting upon *C. albicans* infections.

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1. Introduction

Since the 1970s, there has been an increase in candidiasis incidence, mostly due to the use of plastic permanent catheters, antibiotics and immunosuppressive drugs [1]. These *Candida*-derived infections may occur in the skin, mucous membranes (such as the mouth and vagina) and in the viscera, with the main etiological agent being *Candida albicans* [1,2]. Among the various human fungal pathogens, *C. albicans* accounts for the majority of systemic infections in immunocompromised patients, with overall mortality rates ranging from 29% to 76% [2–6]. This opportunistic fungi causes great problems, as it is resistant to most antimicrobial compounds, namely amphotericin-B, which is considered the standard for the treatment of systemic mycoses. Despite still being considered the drug of choice against *C. albicans*, these antifungal agents are being increasingly reported as inefficient with numerous cases of resistances, particularly to fluconazole, being observed [1–4]. This problem has led to the search for alternative drugs and compounds to be used in the treatment and management of *C. albicans* infections.

Chitin is the primary structural component of the shells of crustaceans, arthropods and the fungal cell wall and is obtained mainly as a byproduct of the fishing industry. Partial deacetylation of chitin leads to chitosan, a polysaccharide composed of units of glucosamine (2-amino-2-deoxy-D-glucose) and *N*-acetyl glucosamine (2-acetamido-2-deoxy-D-glucose) linked by $\beta(1\rightarrow4)$ bonds. Chitosan is

the only natural polysaccharide that presents a cationic character due to its amino groups, which, at low pH, are protonated and can interact with negatively-charged compounds, such as proteins, anionic polysaccharides (e.g., alginates, carraghenates, pectins), fatty acids, bile acids and phospholipids [7]. This behavior, along with its biocompatibility, biodegradability and lack of toxicity, has led to the usage of chitosan in diverse fields, such as technology, food, cosmetics, medicine, biotechnology, agriculture and the paper industry [8,9]. However, chitosan possesses some limitations, namely its insolubility in water, high viscosity and tendency to coagulate proteins at high pH [10–12].

Chitosan's antimicrobial activity is well established against a variety of microorganisms, including fungi [10,13–15]. When considering chitosan antifungal activity, several authors have already shown that it is active upon yeasts, molds and dermatophytes [16–19]. While the antifungal activity of chitosan upon *C. albicans* is well established, the same cannot be said regarding the effect of chitosan upon *C. albicans* biofilm formation. Early reports [20–22] suggest that chitosan may be active upon *C. albicans* biofilms; however, the real effect of chitosan upon the different steps of *C. albicans* biofilms has not yet been fully explored. As such, the aim of this work was to fully assess chitosan's potential as a means to prevent *C. albicans*-derived infections through the control of its growth, adhesion and biofilm formation.

2. Results and Discussion

2.1. MIC Determination

The MIC values, obtained by broth microdilution, for chitosan activity upon *C. albicans* were relatively low. In fact, HMW chitosan presented a MIC value of 1 mg/mL and LMW chitosan a MIC value of 3 mg/mL. The antifungal activity of chitosan upon *C. albicans* is well established, with several authors [16–19] presenting various MIC values for different chitosans against this yeast. Tayel, Moussa, El-Tras, Knittel, Opwis and Schollmeyer [2] previously reported a MIC of 1.25 mg/mL (32 kDa, deacetylation degree (DD) 86%). Qin *et al.* [23] reported an even lower MIC of 0.8 mg/mL (2.91 kDa, DD 86.4%), and Şenel *et al.* [24] reported a MIC of 10 mg/mL (1,000 kDa, DD 80%). Comparing these results with the ones obtained, it is possible to see that for LMW chitosan, the MIC value obtained was slightly superior to those previously reported [2,23], with this differences being probably due to the higher DD used in those assays. On the other hand, for HMW chitosan, the values here obtained were significantly lower than those reported by Şenel, İkinici, Kaş, Yousefi-Rad, Sargon and Hincal [24]. From here, the ½ and the ¼ of the MIC were calculated to be used in the biofilm assays, as previously described by Cerca *et al.* [25].

2.2. Adherence to Coated Surfaces

The effect of chitosan upon *C. albicans* adhesion to surfaces can be seen in Figure 1. The results obtained showed that both MW and the times tested were capable of producing adhesion inhibition percentages above 90%. In fact, the lowest inhibition percentage was obtained for LMW chitosan after only 30 s of exposure. When considering the differences between 30 s and 90 s of exposure, there were no significant statistical differences ($p > 0.05$) found, either for HMW or LMW chitosan.

On the other hand, when considering the impact of the MW and the exposure time, some differences are ascertainable; 90 s of exposure for HMW presented statistically significant ($p < 0.05$) higher inhibition values than both LMW assays; LMW, at 30 s of exposure, presented a significantly lower ($p < 0.05$) inhibition value than the one registered in both HMW assays. These results are in line with those previously reported by Carlson, Taffs, Davison and Stewart [20], who showed that chitosan reduced *C. albicans* adhesion up to 99%.

2.3. Microtiter-Plate Test

When considering the impact of chitosan upon *C. albicans* biofilm formation (Figure 2), here analyzed indirectly through biomass production, one can see that, as with the previous assay, the highest inhibition percentage (66.94%) was obtained for HMW chitosan (0.5 mg/mL) and the lowest inhibition percentage (37.97%) was obtained for LMW chitosan (0.75 mg/mL). When comparing the results obtained for the $\frac{1}{2}$ and $\frac{1}{4}$ of the MIC of both MWs, no statistically significant ($p > 0.05$) differences were found when considering the effect of the MW upon chitosan's activity. On the other hand, when considering the effect of the MW in conjunction with the concentration, one can see clear differences in behavior (Figure 2). In fact, 0.5 mg/mL of HMW chitosan presented significantly higher ($p < 0.05$) inhibition values than the remaining assays, while no significant ($p > 0.05$) differences were found between the $\frac{1}{4}$ of the MIC for HMW (0.25 mg/mL) and $\frac{1}{2}$ of the MIC for LMW (1.5 mg/mL). On the other hand, 0.75 mg/mL of LMW chitosan presented statistically significant ($p < 0.05$) lower inhibition percentages than the remaining assays. These results are in line with those registered by Martinez, Mihu, Tar, Cordero, Han, Friedman, Friedman and Nosanchuk [22], who reported that chitosan was capable of reducing *C. albicans* biofilm formation by a 2.5 factor, and by those of Cobrado *et al.* [26] and of Cobrado *et al.* [27], who showed that chitosan was capable of reducing *C. albicans* biomass production up to 90%.

Figure 1. Inhibitory effect of chitosan upon *C. albicans* adhesion. Values obtained given as the percentage of adhesion inhibition. Different letters represent the statistically significant differences found ($p < 0.05$). All assays performed in triplicate. HMW, high molecular weight; LMW, low molecular weight.

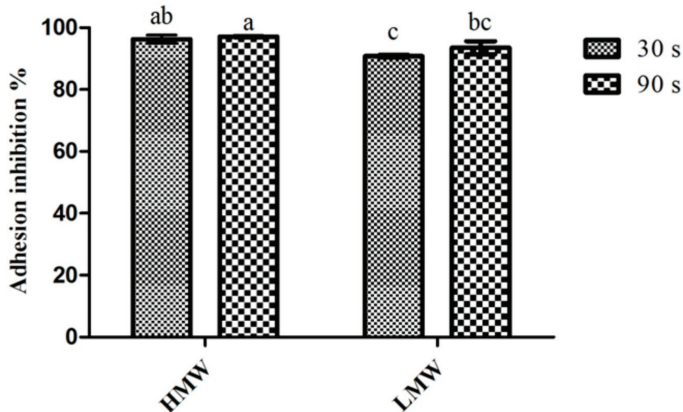
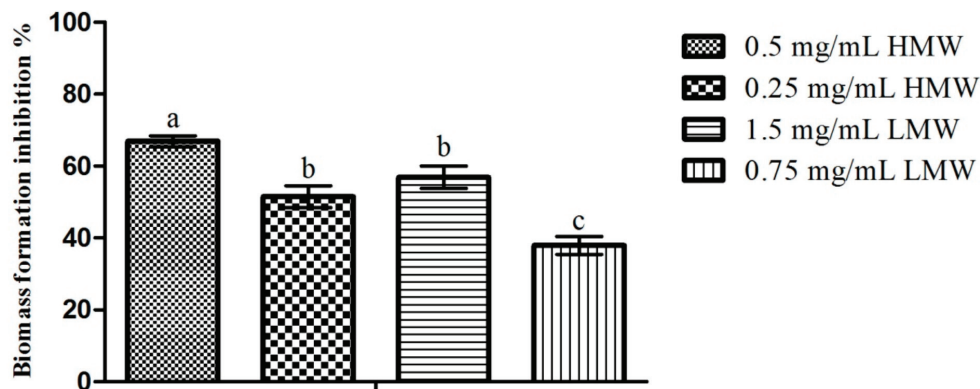


Figure 2. Effect of sub-MIC concentrations of chitosan ($\frac{1}{2}$ and $\frac{1}{4}$ of the MICs; values in mg/mL) upon *C. albicans* biofilm formation. Values obtained are given as the percentage of biofilm formation inhibition. Different letters represent the statistically significant differences found ($p < 0.05$). All assays were performed in triplicate.



2.4. Mature Biofilms Assays

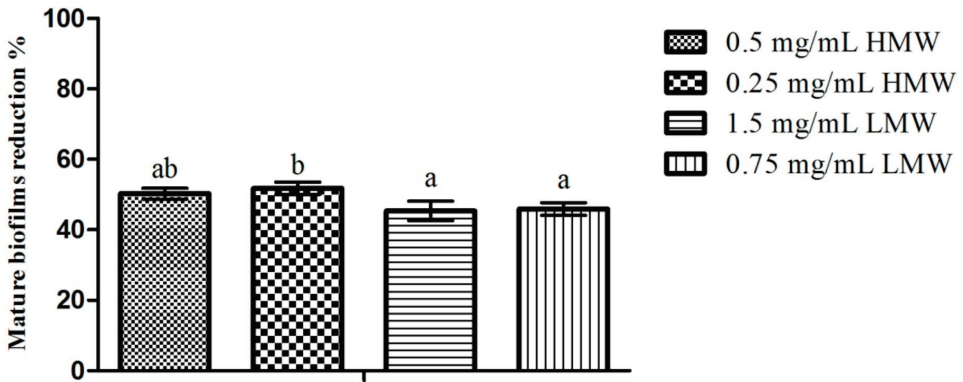
Regarding the effect of chitosan upon *C. albicans* mature biofilms, the results obtained can be seen in Figure 3. Once again, the highest inhibition percentage was obtained for HMW chitosan (51.77% for 0.25 mg/mL), and the lowest inhibition was registered for LMW chitosan (45.37% for 1.5 mg/mL). Statistical analysis of the results showed that when considering the effect of the MW, there were statistically significant differences ($p < 0.05$) between HMW chitosan at 0.25 mg/mL and both LMW concentrations tested. Simultaneously, when considering the effect of the MW in conjunction with concentration (Figure 3), differences were also observed with 0.25 mg/mL HMW chitosan, presenting significantly ($p < 0.05$) higher inhibition values than the assays that utilized LMW chitosan. Between the remaining assays, no statistically significant ($p > 0.05$) differences were found.

2.5. Dual-Species Biofilms

Results obtained regarding the activity of chitosan upon *C. albicans* mature biofilms can be observed in Figure 4. Contrary to the pattern observed in previous assays, LMW chitosan presented the highest biofilm inhibition percentage (66.77% for 0.75 mg/mL) and HMW chitosan the lowest (55.10% for 0.25 mg/mL). Statistical analysis of the results showed that the only statistically significant difference observed was for 0.25 mg/mL of HMW chitosan, which presented an inhibition value significantly lower than the inhibition values obtained in the remaining test conditions. When considering the differences in chitosan's activity between single species *C. albicans* biofilm and dual species *C. albicans* and *S. mutans* biofilms (Figure 5), the statistical analysis shows that there are statistically significant differences ($p < 0.05$) in LMW chitosan's activity between a single and a dual species population. On a closer look, LMW chitosan presents a statistically significant ($p < 0.05$) increase in activity between single species and dual species biofilms. This translates as an increase

of biomass production inhibition of *ca.* 10%, for 1.5 mg/mL, and of *ca.* 31%, for 0.75 mg/mL of chitosan, for LMW chitosan between populations.

Figure 3. Effect of sub-MIC concentrations of chitosan ($\frac{1}{2}$ and $\frac{1}{4}$ of the MICs; values in mg/mL) upon *C. albicans* mature biofilms. Results are presented as biofilm reduction percentages. Different letters represent the statistically significant differences found ($p < 0.05$). All assays performed in triplicate.



Despite the lack of previous results regarding the effect of chitosan upon *C. albicans* mature and dual species biofilms, the inhibitions here registered are quite interesting, especially when considering that *C. albicans* biofilms produce an exopolymeric matrix that serves as a diffusion barrier to antimicrobials and that, under these conditions, *Candida* cells overexpress efflux pumps to enhance antifungal resistance [28]. This mechanism may be the reason why HMW chitosan possessed higher activity than LMW upon mature biofilms, as it is known that the latter must enter the cells in order to be active [15]. In the dual species biofilms, one cannot underestimate the importance of *S. mutans*, as it is known to be crucial to *C. albicans* colonization of the oral cavity, mainly due to providing adhesion sites and producing lactate that can be used as a carbon source by yeasts. This symbiosis has been well established in several studies, which have shown that there is a strong coadherence between these microorganisms [29]. Considering that, both *S. mutans* and *C. albicans*, have been described as being more sensitive to HMW chitosan [16–19,30], it is somewhat surprising that LMW chitosan presented higher inhibition percentages than HMW chitosan for the dual species biofilms. It is possible that an unknown mechanism, possibly located at the adhesins level, as hypothesized by Azcurra *et al.* [31], or at the cell to cell communication level, where the larger HMW molecules are incapable of acting, is responsible for the higher activity registered for LMW chitosan.

Figure 4. Effect of sub-MIC concentrations of chitosan ($\frac{1}{2}$ and $\frac{1}{4}$ of the MICs; values in mg/mL) upon biofilms formed by *C. albicans* and *S. mutans*. Results presented as the percentage of biofilm formation inhibition. Different letters represent the statistically significant differences found ($p < 0.05$). All assays performed in triplicate.

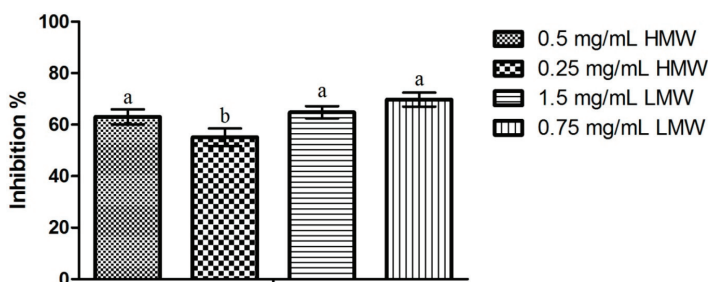
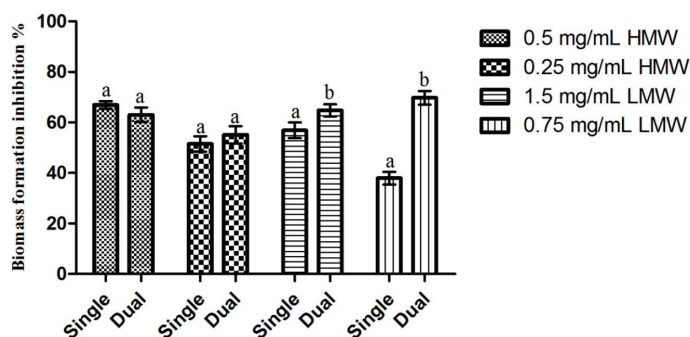


Figure 5. Comparison of the effect of sub-MIC concentrations of chitosan upon *C. albicans* single species and dual species biofilms. Results presented as the percentage of biofilm formation inhibition. Different letters represent the statistically significant differences found ($p < 0.05$). All assays performed in triplicate.



3. Experimental Section

3.1. Sources of Chitosan and Microorganisms

High and low molecular weight chitosan were obtained from Sigma-Aldrich (St. Louis, MO, USA). High molecular weight chitosan was characterized by a DD > 75% and a MW of 624 kDa. Low molecular weight chitosan was characterized by a DD between 75% and 85% and a MW of 107 kDa. Chitosan solutions were prepared in 1% (v/v) solution of glacial acetic acid 99% (Panreac, Barcelona, Spain). Chitosan was added to 1% acetic acid to the desired concentration. Afterwards, the solution was stirred overnight at 50 °C to promote complete dissolution of chitosan. The pH was adjusted with NaOH (Merck, Darmstadt, Germany) to a final value of 5.6–5.8, and solutions were stored at refrigerated temperature.

Candida albicans used in this study was obtained from the culture collection of the Göteborg University (CCUG) (Sweden) (CCUG 49242). Inocula were prepared in yeast malt broth (YMB)

(Difco, Franklin Lakes, NJ, USA) and incubated at 37 °C for 24 h. Viable counts were performed in yeast malt agar (YMA) (Difco, Franklin Lakes, NJ, USA).

3.2. Determination of Minimal Inhibitory Concentration

Determination of the MIC was performed as described by Costa, Silva, Pina, Tavaría and Pintado [10]. Briefly, an inoculum of 0.5 in the MacFarland scale (1.5×10^8 CFU/mL) of *C. albicans* was prepared from overnight cultures and inoculated in YMB with chitosan concentrations ranging from 0.1 mg/mL to 7 mg/mL. Two controls were simultaneously assessed: one with 0.1 mg/mL chitosan, but without inoculum, and another where chitosan was replaced by sterile water and with added inoculum. The MIC was determined by observing the lowest concentration of chitosan that inhibited microbial growth. All assays were performed in triplicate.

3.3. Adherence

The effect of chitosan on *C. albicans* adhesion to surfaces was performed as described by Costa, Silva, Tavaría and Pintado [30], tested using 24-well microplates. Briefly, 1 cm aluminum disks were dipped for 30 or 90 s in a well containing either 1% (v/v) HMW or LMW chitosan. Following that, the disks were rinsed with sterile water and submerged in a well containing inoculum for 60 s, after which disks were placed into wells containing the appropriate medium and incubated for 24 h at 37 °C. Two controls were simultaneously assessed. In the first one, disks were dipped in sterile water and then inoculated and incubated. In the second one, disks were dipped in the test solutions and, after rinsing in sterile water, were then incubated without inoculum. After 24 h, the disks were recovered, and after serial dilutions, viable counts were assessed by the drop method, as described by Miles *et al.* [32], in YMA. Plates were then incubated at 37 °C for 24 h under aerobic conditions. Results were given as inhibition percentages using the following formula:

$$\% I = 100 - (\log \text{CFU sample} / \log \text{CFU control}) \times 100$$

All assays were performed in duplicate.

3.4. Microtiter-Plate Test

Biofilm quantification was carried out by adapting the protocol of Stepanovic *et al.* [33]. Briefly, in a flat bottom, 96-well microplate, wells were filled with 200 μ L of test solutions with chitosan added at sub-MIC concentrations ($\frac{1}{2}$ and $\frac{1}{4}$ of the MIC) and inoculated at 1% (v/v). The plate was then incubated at 37 °C for 24 h in aerobiosis. All assays were performed in triplicate in the appropriate media supplemented with 5% sucrose.

To visualize adhesion, the contents of each well were discarded and then washed 3 times with sterile deionized water in order to remove non-adherent cells. The remaining attached microorganisms were fixed with 200 μ L of ethanol (Panreac, Barcelona, Spain) for 15 min. Ethanol was discarded, and the wells were air dried. After that, 200 μ L of crystal violet solution were added to the wells for 5 min, the excess stain removed by rinsing the plate under tap water followed by air

drying. Adherence was quantified by measuring the OD at 630 nm using a microplate reader (FLUOstar, OPTIMA, BGM Labtech).

Optical density values from wells with liquid media, chitosan and no inoculum were used as negative controls, while OD from wells with liquid media, deionized water and inoculum were used as positive controls. Additionally, a control with 1% (v/v) acetic acid for each microorganism was used.

Results for this test were given as the percentage of biofilm formation inhibition applying the following formula:

$$\% \text{ biofilm formation inhibition} = 100 - (OD_{\text{assay}}/OD_{\text{control}}) \times 100$$

3.5. Mature Biofilms Assay

The assessment of chitosan's effect on mature biofilms was performed through adaptation of the microplate protocol described by Stepanovic, Vukovic, Dakic, Savic and Svabic-Vlahovic [33]. Briefly, in a flat bottom 96-well microplate, wells were filled with 200 μL of medium, inoculated at 1% (v/v) and incubated 48 h at 37 °C. After 48 h, the medium was carefully aspirated, and the wells were rinsed with phosphate buffer. Following that, 200 μL of medium, with chitosan at sub-MIC concentrations, was added and incubated at 37 °C for 24 h.

To visualize biofilms, the contents of each well were discarded and the wells washed 3 times with sterile deionized water in order to remove non-adherent cells. The remaining attached microorganisms were fixed with 200 μL of ethanol (Panreac, Barcelona, Spain) for 15 min. Ethanol was then discarded and the wells air dried. After that, 200 μL of crystal violet solution (Merck, Darmstadt, Germany) were added to the wells for 5 min. Excess stain was removed by rinsing the plate under tap water followed by air drying of the plate.

Adherence was quantified by measuring the OD at 660 nm using a microplate reader.

All experiments were done in triplicate for each microorganism. OD values from wells only with YMB were used as negative controls. A positive control with media and sterile deionized water was used. Additionally a control with 1% (v/v) acetic acid for each microorganism was used.

Results for this test were given as the reduction of the present biofilm, applying the following formula:

$$\text{Mature biofilm inhibition percentage} = 100 - (OD_{\text{assay}}/OD_{\text{control}}) \times 100$$

3.6. Dual-Species Biofilms

Quantification of the effect of chitosan upon biofilms formed by two different microorganisms was performed as previously described by Costa, Silva, Tavaría and Pintado [30]. Briefly, a test solution, with chitosan at sub-MIC concentrations, was inoculated with *C. albicans* and *Streptococcus mutans* (CCUG 45091) (1:1) to achieve a 2% (v/v) inoculum concentration. Impact upon biofilm formation was evaluated using the biofilm microtiter plate assay as described above. Results were obtained as referred above, and all assays were done in triplicate.

3.7. Statistical Treatment

The statistical differences in the methods were evaluated using PASW Statistics v. 21.0.0.0 (New York, NY, USA). The normality of the results' distribution was evaluated through Shapiro–Wilk's test. The differences were assessed using the one-way ANOVA test associated with Scheffe's test (for normal distributions). The differences were considered significant at a 0.05 significance level.

4. Conclusions

In conclusion, chitosan showed remarkable potential as a possible anti-candidiasis agent, as it was active upon *C. albicans* in the planktonic state and, more importantly, upon its sessile growth, with significant activity upon the several phases—adhesion, formation, mature and co-aggregation—of biofilm establishment and growth.

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Author Contributions

Eduardo Costa was responsible for the experimental planning and execution, data processing and writing of the article; Sara Silva was responsible for the experimental execution, data processing, statistical analysis and writing of the article; Freni Tavaría was responsible for the experimental planning, data validation, article proofing and validation; Manuela Pintado was responsible for the experimental planning and article proofing and validation.

Conflicts of Interest

The authors declare no conflict of interest.

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