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1 # *Pseudomonas aeruginosa* biofilms and their partners in crime

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17 **Keywords:** *Pseudomonas aeruginosa*, biofilms, polymicrobial, chronic infections, *P.*
18 *aeruginosa* models, antimicrobials

19

20

Abstract

Pseudomonas aeruginosa biofilms and the capacity of the bacterium to coexist and interact with a broad range of microorganisms have a substantial clinical impact. This review focuses on the main traits of *P. aeruginosa* biofilms, such as the structural composition and regulatory networks involved, placing particular emphasis on the clinical challenges they represent in terms of antimicrobial susceptibility and biofilm infection clearance. Furthermore, the ability of *P. aeruginosa* to grow together with other microorganisms is a significant pathogenic attribute with clinical relevance; hence, the main microbial interactions of *Pseudomonas* are especially highlighted and detailed throughout this review. This article also explores the infections caused by single and polymicrobial biofilms of *P. aeruginosa* and the current models used to recreate them under laboratory conditions. Finally, the antimicrobial and antibiofilm strategies developed against *P. aeruginosa* mono and multispecies biofilms are detailed at the end of this review.

35 ## 1. Introduction

36 The ability of *Pseudomonas aeruginosa* to colonize medical devices and human tissues
37 while growing in resistant communities called biofilms is a worldwide public health concern.
38 Biofilms are bacterial communities that grow together embedded in an extracellular matrix
39 (ECM), which is a fundamental structural component of the bacterial community and acts as a
40 protective shield (Ma et al., 2009). Bacteria modulate their gene expression during adaptation
41 to biofilm growth, promoting phenotypically opposite behavior compared to their planktonic
42 counterparts. Bacterial communication via the quorum sensing (QS) network plays a critical
43 role during biofilm establishment, namely, in regulating the genes involved in biofilm
44 development (Schuster and Greenberg, 2006). *P. aeruginosa* biofilms have increased antibiotic
45 tolerance and are more resistant to host responses than their planktonic counterparts, which
46 makes the clearance of these biofilms difficult and infections chronic (Lebeaux et al., 2014;
47 Maurice et al., 2018).

48 A critical clinical trait of *P. aeruginosa* is its capacity to interact and coexist with other
49 microorganisms in multispecies communities. From a clinical point of view, these interactions
50 are usually detrimental to the patient, as infections caused by multiple species are often
51 associated with worse prognosis (Peters et al., 2012). On the other hand, from a biotechnological
52 perspective, there is a challenge to recreate the optimal conditions to grow multiple bacterial
53 species simultaneously. *P. aeruginosa* can interact with other bacteria, fungi and viruses and
54 together infect a wide range of human tissues (Filkins et al., 2015; Hendricks et al., 2016; Smith
55 et al., 2015). Due to the clinical challenge of *P. aeruginosa* biofilms and the recalcitrant
56 infections they cause, science has moved toward developing efficient and alternative
57 antimicrobial strategies to clear *P. aeruginosa* biofilms (Barraud et al., 2009; Guillon et al.,
58 2018; Ibaraki et al., 2020; Mwangi et al., 2019).

59 This review outlines the main aspects of *P. aeruginosa* biofilms and the clinical burden
60 they represent; describes *P. aeruginosa* infections, importantly focusing on the polymicrobial
61 interactions of this bacterium and the relative clinical outcomes; and finally, it discusses
62 the current models used to recreate *P. aeruginosa* polymicrobial biofilms under laboratory
63 conditions and the antimicrobial therapeutics used against *P. aeruginosa* biofilms.
64

2. Extracellular matrix of *Pseudomonas aeruginosa* biofilms: the structural basis of the clinical threat.

The ECM is the body of the biofilm. It maintains biofilm integrity by holding the bacterial community together and confers biofilm protection against antimicrobials and the host immune response. Hence, the ECM is a fundamental component of this multifactorial structure and is composed mainly of a mix of exopolysaccharides (EPS), extracellular DNA (eDNA) and proteins (Ma et al., 2009).

Psl and Pel are the two main polysaccharides present in the ECM of *P. aeruginosa* strains and are crucial for the integrity of the biofilm. Psl is a neutral pentasaccharide composed of D-glucose, D-mannose and L-rhamnose, while Pel is a cationic exopolysaccharide comprising 1-4 linked galactosamine and glucosamine sugars (Billings et al., 2013; Franklin et al., 2011; Jennings et al., 2015). However, during an *in vivo* infection, mutations in the anti-sigma factor encoding the *mucA* gene result in overproduction of the alginate polysaccharide and change the architecture of the biofilm's ECM (Martin et al 1993). Alginate is an anionic polymer composed of β-D-mannuronic acid and α-L-guluronic acid. Biofilms that contain bacteria overproducing alginate occupy more space than nonmucoid biofilms, which are more densely packed (Ma et al., 2012). Each polysaccharide (Psl, Pel and alginate) provides different physiological properties to the biofilm matrix, and it is associated with different stages of biofilm development. Even though *P. aeruginosa* has the capacity to produce the three types of polysaccharide, it only does one type at any given time (Franklin et al., 2011).

The initiation and maintenance of biofilms are promoted by the interaction of Psl with the matrix adhesin CdrA. Within the matrix, bound CdrA-Psl forms robust and protease-resistant bacterial aggregates that fortify the biofilm structure (Borlee et al., 2010; Ma et al., 2009; Reichhardt et al., 2018). CdrA can also attach other yet-unknown EPS, contributing to biofilm formation and stabilizing the structure (Reichhardt et al., 2018). CdrA, Psl and Pel are

90 bis-(3′-5′)-cyclic diguanosine monophosphate (c-di-GMP)-dependent molecules (Borlee et al.,
91 2010). c-di-GMP is a secondary messenger that transduces the environmental signal into
92 different cellular processes (Jenal and Malone, 2006). Under low concentrations of c-di-GMP,
93 *P. aeruginosa* biofilms disperse and become planktonic cells. High levels of cAMP have been
94 directly related to low c-di-GMP content, thus contributing to the inhibition of *P. aeruginosa*
95 biofilm formation (Almblad et al., 2019). Alginate is regulated by c-di-GMP at the post-
96 translational level (Whitney et al., 2015). Additionally, *P. aeruginosa* produces two soluble
97 lectins: LecA and LecB. The binding between LecB and mannose present in Psl favors the
98 positioning of Psl within the matrix, enhancing bacterial retention and aggregation (Passos da
99 Silva et al., 2019). LecA shows specificity for binding galactose, *N*-acetyl-d-galactosamine and
100 glucose, and it contributes to biofilm formation by cross-linking with these residues present in
101 the biofilm matrix as well as with other glycoproteins and bacterial polysaccharides (e.g., LPS)
102 (Diggle et al., 2006; Mitchell et al., 2005; Sabin et al., 2006).

103 A critical and clinically relevant event occurs *in vivo*, during the establishment of *P.*
104 *aeruginosa* infection, with the organism becoming mucoid and overproducing alginate
105 polysaccharide. From that moment on, *P. aeruginosa* induces the transition from an intermittent
106 to chronic infection (Martin et al., 1993). Mucoid strains generally produce less Psl than
107 nonmucoid *P. aeruginosa* strains. Nevertheless, the interactions between this EPS and the
108 components of the matrix are also required to keep bacteria together and form robust and mature
109 biofilms (Jones and Wozniak, 2017; Ma et al., 2012).

110 All three polysaccharides (Psl, Pel, alginate) play a role in biofilm adhesion, scaffolding,
111 and stability. However, they differ in terms of biofilm protection. While Psl confers protection
112 against the immune cells, Pel is shown to defense the biofilm against antimicrobial treatment.
113 On the other hand, alginate production protects from both antimicrobials and the host immune

114 response and confers additional protection to hostile environments as, for instance, the oxidative
115 stress created during phagocytosis (Karygianni et al., 2020).

116 eDNA is a major component of the *P. aeruginosa* biofilm matrix that changes depending
117 on the biofilm maturity and the surrounding environment (Whitchurch et al., 2002). In *P.*
118 *aeruginosa,* eDNA is produced through a process of explosive cell lysis that subsequently forms
119 membrane vesicles by engulfing DNA and other cytosolic content. eDNA is then released in a
120 mechanism that is regulated by both QS-dependent and QS-independent mechanisms (Turnbull
121 et al., 2016). Once released, eDNA interacts with extracellular Ca^{2+} and, via “cationic bridging”,
122 induces bacterial aggregation, promoting biofilm formation and subsequent maturation.
123 Therefore, eDNA is required for the initial establishment of the biofilm (Das et al., 2014;
124 Whitchurch et al., 2002). eDNA has been detected to interact with Psl (Wang et al., 2015) and
125 Pel (Jennings et al., 2015) polysaccharides. During an infection, the host also impacts the
126 presence of eDNA in the biofilm matrix through interactions with immune cells during the
127 inflammatory response. While eDNA has been shown inside biofilms in *in vitro* studies, in *in*
128 *vivo* biofilms, it has been shown to be concentrated in the external part of the biofilms (Alhede
129 et al., 2020; Ciszek-Lenda et al., 2019; Whitchurch et al., 2002). *P. aeruginosa* strains, namely,
130 the “rugose small colony variants” (RSCVs), isolated from patients with chronic infections are
131 hyperbiofilm-forming strains that, unlike common laboratory strains, have fragmented eDNA
132 within the matrix that leads to a more resistant structure (Deng et al., 2020). RSCVs are
133 associated with high levels of c-di-GMP (Malone et al., 2010), in addition, to be able to produce
134 alginate and Psl polysaccharides simultaneously (Franklin et al., 2011).

135 However, eDNA is more than just a structural biofilm component; it also influences the
136 transcriptome of *P. aeruginosa*. This anionic polymer modulates the expression of antibiotic
137 resistance genes such as ß-lactamases and aminoglycoside resistance genes as well as the
138 expression of multidrug efflux pumps (e.g., EmrAB). Additionally, it alters metal homeostasis

139 by chelating cationic ions and controlling the expression of different metal uptake and efflux
140 systems. The acidification of the biofilm confers tolerance to acidic environments, making
141 biofilms able to resist the infection site or phagocytose acidified vacuoles. Importantly, eDNA
142 traps nutrients in addition to inducing genes to be able to use DNA as a nutrient source for
143 phosphate, nitrogen or carbon (Lewenza et al., 2020; Mulcahy et al., 2008; Wilton et al., 2016).
144 Altogether, eDNA promotes all the hallmark features of biofilms. A recently published mini-
145 review addresses the different aspects of *P. aeruginosa* eDNA release and interactions very
146 accurately (Sarkar, 2020).
147

148 3. *P. aeruginosa* polymicrobial coexistence

149 In nature, biofilms are often polymicrobial structures, meaning that different microbial
150 species can interact and coexist within the same biofilm community. In disease, microbial
151 interactions can affect a patient's prognosis. In this sense, the synergic interactions of organisms,
152 whereby the combined effect is more significant than that produced by individual bacteria, can
153 worsen the outcome of the patient (Murray et al., 2014). *P. aeruginosa* can grow and coexist
154 with a wide range of microorganisms, including bacteria, fungi and viruses (Figure 1).
155

156 3.1. *P. aeruginosa* with *Staphylococcus aureus*

157 One of the major partners in crime of *P. aeruginosa* is *Staphylococcus aureus.* This
158 partnership can cause severe chronic infections promoted by biofilm aggregates in infected
159 wounds and lungs, especially in diabetic foot ulcers and cystic fibrosis (CF). The presence of *S.*
160 *aureus* and the Pel production of *P. aeruginosa* have been shown to increase the surface
161 coverage and microcolony size of biofilms formed by both *P. aeruginosa* and *S. aureus*, and
162 diguanylate cyclase SiaD is required for competitiveness between them (Chew et al., 2018). In
163 CF-affected lungs, *P. aeruginosa* takes advantage of the physiology generated by the disease

164 and the lack of iron present in the environment to produce 4-hydroxy-2-heptylquinoline-*N*-oxide
165 (HQNO). HQNO inhibits *S. aureus* growth by shifting its metabolism to fermentation, which
166 eventually promotes the killing of *S. aureus*, and *P. aeruginosa* can use the iron that
167 *Staphylococcus* stored (Filkins et al., 2015; Mashburn et al., 2005; Nguyen et al., 2015). *P.*
168 *aeruginosa* produces the endopeptidase LasA, which has also been suggested to help for
169 acquiring the iron from *S. aureus* (Mashburn et al., 2005). Transcriptional profiles performed in
170 early cocultures of both bacteria reflect the metabolic adaptation and competition for glutamine,
171 as nitrogen and energy sources, that the organisms face when they grow together rather than the
172 expression of host-directed virulence factors (Tognon et al., 2019). It has also been seen that
173 during *P. aeruginosa* coinfection with Gram-positive bacteria such as *S. aureus*, *P. aeruginosa*
174 senses the N-acetyl glucosamine (GlcNAc) of its peptidoglycan as a cue to produce multiple
175 virulent factors with lytic activity against prokaryotic and eukaryotic cells, therefore enhancing
176 host killing (Korgaonkar et al., 2013). *P. aeruginosa* production of the *cis*-2-decenoic acid
177 promotes biofilm dispersal of different bacteria, including *S. aureus* (Davies and Marques,
178 2009).

179 On the other hand, *S. aureus* can use the HQNO molecule produced by *P. aeruginosa* to
180 increase its tolerance to certain antibiotics, such as vancomycin or tobramycin. Prolonged
181 coculture growth of *P. aeruginosa* and *S. aureus* or increased *S. aureus* exposure to HQNO,
182 which is abundant in CF sputum, selects for resistant *S. aureus* small-colony variants (SCV), in
183 a mechanism dependent on the transcriptional factor sigma B (SigB) (Mitchell et al., 2010).
184 Furthermore, *P. aeruginosa* influences *S. aureus* susceptibility to antibiotics such as
185 vancomycin, gentamycin or ciprofloxacin (Biswas et al., 2009; Cendra et al., 2019; Hoffman et
186 al., 2006; Orazi and O'Toole, 2017). Recent findings indicate that *S. aureus* extracellular
187 metabolites (e.g., adhesins, enzymes, polysaccharides and peptides) can suppress *P. aeruginosa*
188 growth, which leads to an increase in *P. aeruginosa* susceptibility to antibiotics such as

189 ciprofloxacin and aminoglycosides (Trizna et al., 2020). The secreted staphylococcal protein A
190 (SpA) inhibits *P. aeruginosa* biofilms through a mechanism that affects type IV pili and PsI
191 production. This protein has also been suggested to protect *P. aeruginosa* from phagocytosis
192 (Armbruster et al., 2016). In a clinical context, alginate overproduction of *P. aeruginosa* in the
193 CF environment benefits *S. aureus* as it decreases the production of anti-staphylococcal
194 molecules, enabling coexistence between these two bacteria (Limoli et al., 2017). Figure 2
195 summarizes the reciprocal effect of *P. aeruginosa* and *S. aureus* interaction on their
196 pathogenicity and the mechanism or molecule through which each beneficial or antagonistic
197 effect is done. It is worth mentioning that a review by Hotterbeekx *et al.* thoroughly compiles
198 the *in vivo* and *in vitro* interactions between these two organisms and the resulting phenotypic
199 effects (Hotterbeekx et al., 2017).
200 On the other hand, the alpha toxin of *S. aureus* has been shown to be important in *P.*
201 *aeruginosa* infection and dissemination because it mediates the disruption of host immunity and
202 the barriers promoted by epithelial cells (Cohen et al., 2016). Coinfection with *P. aeruginosa*
203 and *S. aureus* leads to rapid pulmonary decline and diminished lung function, which is why
204 these two bacteria are targets of numerous treatments to clear infections in CF lungs (Limoli et
205 al., 2016). Due to its clinical relevance, numerous studies have been performed to culture these
206 two bacteria *in vitro* to investigate their partnership better, but *Pseudomonas* dominance limits
207 its *in vitro* coexistence (see section 6 for detailed information on *in vitro* models to grow *P.*
208 *aeruginosa* polymicrobial biofilms).
209
210 **3.2. *P. aeruginosa* with *Streptococcus* spp.**
211 *P. aeruginosa* also interacts with *Streptococcus* in a CF environment. A study revealed
212 how a clinical CF isolate of *P. aeruginosa* (FRD1 strain) enhanced the capacity of *Streptococcus*
213 *parasanguinis* to colonize and form biofilms in a mechanism wherein the latter exploited the

214 exopolysaccharide production of *Pseudomonas* (Scoffield et al., 2017). Furthermore, in
215 coculture growth with *Streptococcus* spp. commonly present in the flora of the host's respiratory
216 tract, *P. aeruginosa* differentially expresses several virulence factors and drug efflux pumps,
217 which are thought to be modulated by the QS molecule AI-2. AI-1 accumulates in CF sputum
218 and could have a role in facilitating the intercommunication of *P. aeruginosa* with the patient
219 flora, exacerbating CF disease. However, this relationship is complex; for instance,
220 *Streptococcus* upregulates the production of rhamnolipids via the expression of the QS molecule
221 AI-2, but rhamnolipids kill *Streptococcus.* The effect of these gene modulations through the AI-
222 2 molecule has also been observed with other bacteria present in the flora, such as
223 *Staphylococcus* spp. (Duan et al., 2003; Peters et al., 2012). It has recently been detected that
224 both bacterial species compete for zinc, as increasing transcription of genes involved in zinc
225 uptake was detected in *Streptococcus sanguinis* during coculture growth with *P. aeruginosa.*
226 This study also showed that the zinc levels present in CF sputum correlate with the abundance
227 of these two species, indicating that zinc availability may impact *S. sanguinis* and *P. aeruginosa*
228 growth *in vivo* (Li et al., 2020). In terms of infection outcomes, coinfection with streptococci
229 and *P. aeruginosa* shows higher damage to the CF-affected lung and microbial burden than that
230 observed with any of the microbes alone (Duan et al., 2003; Filkins et al., 2012; Peters et al.,
231 2012).
232

233 **3.3. *P. aeruginosa* with other bacteria**

234 *P. aeruginosa* has been detected coexisting with a wide range of microorganisms.
235 Sputum analysis of CF adult patients showed the presence of many strict and facultative
236 anaerobic bacteria including *Prevotella*, *Veillonella*, *Propionibacterium*, and *Actinomyces*
237 together with *P. aeruginosa, S. aureus* or *Burkholderia cepacia*. Interestingly, this study
238 revealed that *P. aeruginosa* increased the presence of anaerobic microorganisms in the sputum

239 (Tunney et al., 2011). Specifically, coinfection with *B. cepacia* and *P. aeruginosa* has been
240 detected to promote a higher decline in pulmonary function and worse clinical outcomes
241 (Jacques et al., 1998). *Stenotrophomonas maltophilia* has also seen involved in polymicrobial
242 infections with *P. aeruginosa* in CF-affected lungs. This interaction benefits *S. maltophilia*
243 persistence in the lung (McDaniel et al., 2020).

244 The oral cavity is very susceptible to bacterial biofilm growth. Colonization of teeth
245 causes dental plaque formation and consequently the formation of polymicrobial biofilms that
246 can lead to infection like periodontal disease. Biofilm formation in the oral cavity can serve as
247 a reservoir of respiratory pathogens (Berger et al., 2018; Vieira Colombo et al., 2016). *P.*
248 *aeruginosa* with *S. aureus* and *Enterococcus faecalis* have been detected coexisting together in
249 oral epithelial cells of people with periodontitis, and *P. aeruginosa* with *Acinetobacter* spp. have
250 a high correlation to be found together in the oral cavity (Colombo et al., 2013; Souto et al.,
251 2014). Additionally, polymicrobial infections in the urinary tract have linked the presence of *E.*
252 *faecalis* with more severe forms of pyelonephritis caused by *P. aeruginosa* (Tsuchimori et al.,
253 1994).

254

255 **3.4. *P. aeruginosa* with fungi**

256 *P. aeruginosa* has also shown the ability to interact with organisms of other phyla such
257 as fungi. For instance, *P. aeruginosa* has been found together with *Aspergillus fumigatus*
258 infecting lungs. The presence of *P. aeruginosa* in lung infections caused by *A. fumigatus* has
259 been shown to lead to higher loss of pulmonary function than in fungal infections alone. Patients
260 with CF suffering from *P. aeruginosa* and *A. fumigatus* infections may exhibit a poorer
261 prognosis than those patients with individual infections, as *A. fumigatus* enhances the elastase
262 production of *P. aeruginosa* (McGuigan and Callaghan, 2015; Smith et al., 2015). Furthermore,
263 *P. aeruginosa* secretes antifungal molecules that inhibit *A. fumigatus* biofilms; however, this

264 inhibitory effect differs depending on the *P. aeruginosa* strain. This inhibition is higher if it is
265 promoted by CF isolates than by non-CF isolates of *P. aeruginosa*, indicating that a selection
266 pressure of the environment may have a role in *P. aeruginosa* and *A. fumigatus* interactions
267 (Bisht et al., 2020).

268 An interaction has also been described between *P. aeruginosa* and *Candida albicans. P.*
269 *aeruginosa* can kill *C. albicans* by attaching to the hyphal areas of the fungus, but it is unable
270 to attach or to kill to the round form of the yeast (Hogan et al., 2004).

271

272 **3.5. *P. aeruginosa* with viruses**

273 *P. aeruginosa* interactions with respiratory viruses have also been detected in different
274 chronic infections. In this case, there is no mutual interaction between organisms but simple
275 coexistence. Respiratory syncytial virus (RSV) infection induces secondary infections and *P.*
276 *aeruginosa* biofilm formation by dysregulating iron homeostasis in the airway epithelium
277 (Hendricks et al., 2016). A viral infection reinforces *P. aeruginosa* adhesion to epithelial cells.
278 In this sense, RSV has been seen to be responsible for the transition of *P. aeruginosa* to chronic
279 colonization in CF patients (Brownlee and Turner, 2008). On the other hand, a recent study has
280 indicated that CF-affected people suffering from intermittent infections with *P. aeruginosa* have
281 an increased risk of being infected with human rhinovirus, in which the bacterium may be able
282 to modulate the antiviral response *in vivo,* as was detected in a clinical setting of CF (Sorensen
283 et al., 2020). Influenza virus (IAV) also correlates with secondary infections of *P. aeruginosa*
284 in CF patients as well as in people with pneumonia (Hiatt et al., 1999; Scheiblauer et al., 1992).
285 Recent findings have shown that IAV tends to exacerbate *P. aeruginosa* infection by inducing
286 deleterious inflammatory responses (Villeret et al., 2020).

287 Additionally, during the COVID-19 pandemic, severe acute respiratory syndrome
288 coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19, has shown capacity to be

289 coinfecting with other microbes. A systematic review and meta-analysis that considered thirty
290 studies, including 3834 patients, showed that 7% of hospitalized patients infected with SARS-
291 CoV-2 suffered from bacterial coinfection, of which *P. aeruginosa* appeared in 12% of the cases
292 (Lansbury et al., 2020). Even though it is early to have data about the nature of SARS-CoV-2
293 and *P. aeruginosa* coinfection, a retrospective study performed in 61 patients with COVID-19
294 in an Italian hospital detected a higher prevalence of *P. aeruginosa* colonization in severe
295 COVID-19 patients than in non-COVID-19 patients (Intra et al., 2020). Further studies will be
296 needed to elucidate the real impact of *P. aeruginosa* coinfection on COVID-19, as well as
297 possible effects of SARS-CoV-2 on *P. aeruginosa* metabolism and biofilm growth.
298

299 4. Quorum sensing in *P. aeruginosa* biofilms

300 QS is an intercellular mechanism of communication that many bacterial species use to
301 interact with each other. QS plays a critical role during the establishment of chronic infections
302 since it modulates the expression of many different virulence factors as well as biofilm
303 formation. In QS, autoinducers (e.g., acyl-homoserine lactones (AHLs)) serve as signaling
304 molecules whose production is dependent on cell density (Waters and Bassler, 2005). To date,
305 three different QS mechanisms have been described in *P. aeruginosa*: the LasI/LasR system,
306 which produces *N*-3-oxo-dodecanoyl-homoserine lactone (3O-C12-HSL) as a signaling
307 molecule; the RhlI/RhlR system, which produces *N*-butanoyl-homoserine lactone (C4-HSL);
308 and the third system, which is based on 2-alkyl-4-quinolone (AQ) signals and PqsR as a
309 transcriptional regulator. Both the LasR and RhlR systems are related to cell density, while PqsR
310 is related to the stress response. In addition to these three primary QS circuits, the factor QscR
311 has been detected binding to 3OC12-HSL and other long-chain AHLs without producing any
312 AHL molecules. Furthermore, a role of 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde in the

313 environmental stress-related *iqs* system has also been described (Gökalsın, 2019; Papenfort and
314 Bassler, 2016; Schuster and Greenberg, 2006).
315
316 **4.1. Role of QS on ECM's development**
317 QS has a considerable impact on *P. aeruginosa* biofilm formation, specifically on the
318 development of the biofilm matrix. As mentioned above, the eDNA of the matrix is generated
319 via QS-dependent and QS-independent pathways. The one mediated by QS occurs via the lysis
320 of a small population of cells, while the other liberates only basal levels of eDNA (Allesen-
321 Holm et al., 2006). There are other ways to promote *P. aeruginosa* death and the production of
322 eDNA, such as the intracellular increase in H_2O_2 due to pyocyanin exposure (Das and
323 Manefield, 2012). Furthermore, the expression of the *pel* polysaccharide operon is entirely
324 dependent on QS regulation (Sakuragi and Kolter, 2007). Biofilms formed by *P. aeruginosa*
325 expressing deficient *las, rhl* and *pqs* systems exhibit reduced levels of eDNA in the matrix,
326 resulting in an unstable biofilm (Allesen-Holm et al., 2006). *P. aeruginosa* with mutations
327 affecting the production of the 3O-C12-HSL signaling molecule has been seen to form thin
328 biofilms that lack a three-dimensional architecture (Davies et al., 1998). Recent findings have
329 shown that treatment with tobramycin increases the presence of eDNA in the biofilm matrix
330 through a mechanism dependent on QS. Subinhibitory concentrations of this antibiotic increase
331 the levels of the LasR and RhlR signaling molecules 3O-C12-HSL and C4-HSL (Tahrioui et al.,
332 2019). Additionally, rhamnolipids are amphipathic glycolipids with a critical role in the
333 establishment and maintenance of *P. aeruginosa* biofilms. They have been shown to be required
334 for maintaining the channel structures within the biofilm ECM that facilitate the access of
335 nutrients and oxygen within the surrounding microcolonies (Davey et al., 2003). QS also
336 controls rhamnolipid expression through the regulator RhlR (Medina et al., 2003). Figure 3
337 summarizes the role of the QS in *P. aeruginosa* ECM, including the factors involved in the

338 composition of the ECM and the mechanisms by which the QS modulates the ECM
339 development.
340
341 **4.2. Effect of QS on infection and *P. aeruginosa* polymicrobial interactions**
342 The QS of *P. aeruginosa* significantly impacts on the polymicrobial interactions
343 promoted by the bacterium. Using a wound-like medium, it was demonstrated that the QS of *P.*
344 *aeruginosa* is inhibited by the albumin present in the serum *in vitro*, thus making the bacteria
345 unable to produce the virulence factors that kill *S. aureus* and allowing the survival of the cocci
346 in the presence of *P. aeruginosa* (Smith et al., 2017). It has also been seen that alginate protects
347 *S. aureus* from killing by *P. aeruginosa* when both organisms are growing in coculture due to a
348 downregulation of the *pvdA* gene, which is required to produce the siderophore pyoverdine and
349 the QS system *Pseudomonas* Quinolone System (PQS) in *P. aeruginosa* (Price et al., 2020). The
350 PQS is responsible for producing the phenazine pyocyanin, which is induced by the presence of
351 *S. aureus* (among other Gram-positive species and conditions), inhibiting the oxidative
352 respiration of the cocci while promoting the selection of SCV (Biswas et al., 2009).
353 Additionally, a recent study revealed that *P. aeruginosa* suppresses its antimicrobial activity
354 against *S. aureus* through a mechanism that involves *Pseudomonas* AAA+ ClpXP protease
355 activity on critical proteins needed to produce PQS and C4-HSL QS signal molecules (Yang et
356 al., 2020). Otherwise, when *P. aeruginosa* interacts with *C. albicans*, pyocyanin secretion by *P.*
357 *aeruginosa* is toxic in *C. albicans*, which counterattacks by producing the QS molecule farnesol.
358 Farnesol downregulates the transcription of the pyocyanin mediator gene *pqsA* (Cugini et al.,
359 2007). During the colonization of the CF lung, farnesol may also have a protective role by
360 reducing the levels of pyocyanin (Peters et al., 2012). In the interaction between *P. aeruginosa*
361 and *S. maltophilia*, the latter produces a diffusible signal factor that influences the structure of
362 *P. aeruginosa* biofilms (Ryan et al., 2008). Furthermore, *S. maltophilia* produces the fatty acid

363 *cis*-9-octadecenoic that quenches the AHL signal of *P. aeruginosa,* inhibiting its biofilm
364 formation (Singh et al., 2013).
365 Additionally, mutants of the QS regulator LasR have been associated with lung disease
366 progression in CF-affected people (Hoffman et al., 2009). In these *lasR* mutant strains, QS
367 remains active by regulating RhlR in a mechanism independent of LasR (Chen, R. et al., 2019).
368 In a study that used a CF isolate, RhlR was recognized as critical for establishing chronic
369 infection and the generation of cell toxicity in a 3D lung epithelium aggregated model (Cruz et
370 al., 2020).
371

372 5. Clinical implications of *P. aeruginosa* biofilms – a public health issue

373 Microbial biofilms are involved in 65% of infectious diseases and more than 80% of
374 chronic infections. *P. aeruginosa* is a leading nosocomial pathogen associated with this type of
375 healthcare infection, and it is almost never found infecting alone (Bisht et al., 2020). The nature
376 of *P. aeruginosa* biofilms *per se* is a challenge to the current known antimicrobial treatments
377 and a nightmare for physicians.
378

379 5.1 The antimicrobial challenge of *P. aeruginosa* biofilms

380 The primary shield of *P. aeruginosa* biofilms is its ECM. The chemistry of the ECM
381 hinders the penetration of positively charged antibiotics, e.g., aminoglycosides, which are
382 sequestered by their components and impede their diffusion (Wilton et al., 2016). Biofilm
383 growth confers intrinsic antimicrobial tolerances, sometimes requiring more than 1000 times
384 the dose of antibiotic to be cleared than is needed to treat planktonic bacteria. Furthermore, the
385 clinically derived mucoid phenotype, with alginate overproduction in the ECM, is even more
386 antibiotic tolerant than the parental *P. aeruginosa.* Altogether, the high antibiotic concentrations
387 needed to treat these biofilms are hard to achieve inside the host without causing toxicity

388 (Goltermann and Tolker-Nielsen, 2017; Hengzhuang et al., 2013; Macia et al., 2014).
389 Additionally, the *P. aeruginosa* biofilm community is constituted by different subpopulations
390 of microorganisms, which have been adapted to the different microenvironments present in the
391 multicellular system. Therefore, the bacteria residing in the inner parts of the biofilms display
392 low metabolic activity due to the lack of oxygen and nutrients that are essentially consumed by
393 the cells growing at the biofilm periphery, which consequently inactivates major antibiotic
394 targets. For example, low DNA and protein synthesis affect quinolone and aminoglycoside
395 bactericidal effects, respectively (Ciofu and Tolker-Nielsen, 2019; Stewart et al., 2016). This
396 low-metabolic *Pseudomonas* can lead to the formation of persister cells, which are bacteria less
397 susceptible to antibiotics and responsible for causing reinfections (Lewis, 2010). Furthermore,
398 the hypoxic environment present in the inner parts of the biofilm is another tolerance mechanism
399 attributed to *P. aeruginosa* biofilms since the lack of oxygen impedes the ROS necessary for
400 the bactericidal effect of some antibiotics, such as tobramycin or ciprofloxacin (Borriello et al.,
401 2004; Van Acker and Coenye, 2017). Antibiotic tolerance is also attributed to genetic changes
402 occurring in these *P. aeruginosa* subpopulations, where the activation of multidrug efflux
403 pumps due to mechanisms led by the high levels of cyclic di-GMP present in the biofilm pump
404 antibiotics such as ciprofloxacin, gentamycin and tobramycin out of the bacterial cell at rates
405 ten times higher than those in planktonic cells (Gupta et al., 2014; Poudyal and Sauer, 2018).
406 Additionally, these transcriptomic changes in biofilm-forming *Pseudomonas* also occur in many
407 other genes that are absent in free-living *Pseudomonas* that contribute to specific antimicrobial
408 tolerance (Ciofu and Tolker-Nielsen, 2019).

409 Spontaneous mutations accompany the increased capacity of antimicrobial tolerance and
410 confer resistance to certain antibiotics in *Pseudomonas* due to antimicrobial pressure. In
411 addition to antibiotic resistance, the heterogeneity of the biofilm community and the different
412 stresses and pressures on the subpopulations of the bacteria induce differential spontaneous

413 mutations that benefit biofilm adaptability and persistence (Bjedov et al., 2003; Ciofu and
414 Tolker-Nielsen, 2019; Perron et al., 2007). In this sense, RSCV in CF has increased resistance
415 to antibiotics, and their persistence in the CF lung is thought to be due to the emergence of
416 multidrug-resistant (MDR) variants of the mucoid phenotype (Ciofu et al., 2015; Drenkard and
417 Ausubel, 2002).

418

419 **5.2 The battle of the host immune response to clear *P. aeruginosa* biofilms**

420 In response to a chronic infection generated by *P. aeruginosa* biofilm, the host responds
421 by attacking the bacterial community with different types of immune cells from both the innate
422 and adaptive systems (Maurice et al., 2018). There is an exacerbation of inflammation led by
423 the presence of polymorphonuclear leukocytes (PMNs). Although PMNs have been detected
424 surrounding *P. aeruginosa* aggregates, they are unable to penetrate biofilm structures and
425 therefore eradicate them (Bjarnsholt et al., 2009). PMNs are responsible for neutrophil
426 extracellular trap (NET) formation in a process called NETosis. NETs are DNA lattices
427 enmeshed with PMN granule proteins secreted by PMNs. The creation of NETs has been
428 proposed as a function of neutrophils as well as other immune cells that are still capable of
429 phagocytosis and chemotaxis (Goldmann and Medina, 2012; Yipp et al., 2012). NETs aim to
430 trap bacteria and kill them with antimicrobial proteins released from neutrophil azurophilic
431 granules (Brinkmann et al., 2004).

432 Even though NET formation is an efficient antimicrobial mechanism, depending on the
433 case, *P. aeruginosa* has the potential to overcome it. Recently, *P. aeruginosa* strains that lack
434 the LasR regulator (commonly found in CF patients) have been shown to fail to promote the
435 generation of NETosis (Skopelja-Gardner et al., 2019). Furthermore, *P. aeruginosa* can cause
436 tolerance to NETs by changing the negative charge of the outer surface either through the

437 addition of aminoarabinose to lipid A in LPS or by producing surface spermidine (Halverson et
438 al., 2015; Johnson et al., 2012).

439 Importantly, under healthy conditions, humans have effective immune mechanisms to
440 clear *P. aeruginosa* infection. For instance, mucociliary clearance in the upper respiratory
441 system and the expression of lactoferrin have been shown to effectively block the attachment
442 and microcolony formation of *P. aeruginosa*, thus preventing the establishment of *P.*
443 *aeruginosa* infection (Crabbe et al., 2014; Singh et al., 2002). However, if *P. aeruginosa* can
444 penetrate and colonize patients affected by CF or another condition that compromises the
445 immune system, then the situation can become dire. In CF-affected people, oxygen consumption
446 caused by PMN activity inhibits proper production of the metabolic burst to kill phagocytosed
447 bacteria (Bjarnsholt et al., 2009). Furthermore, the overproduction of alginate present in the
448 aggregates of CF isolates confers additional phagocytosis protection to the biofilm (Bayer et al.,
449 1991). Continuous exposure to the antigen indicates that IgG avidity against alginate does not
450 significantly increase along with the progression of chronic infection, making it difficult for the
451 immune reaction to clear the infection in CF patients (Mauch et al., 2018). Increasing the
452 problem is the capacity of clinical isolates of *P. aeruginosa* to induce the formation of biofilms
453 in the airways by upregulating EPS production through the assimilation of the host-derived
454 immunometabolite itaconate (Riquelme et al., 2020).

455

456 **5.3 *P. aeruginosa* chronic infections due to biofilm formation**

457 The ability of microbial biofilms to resist exposure to a high concentration of
458 antimicrobials and components of the host immune system makes *Pseudomonas* biofilms
459 incredibly challenging to eradicate and a public health concern (Hoiby et al., 2015). The
460 following section will focus on the most common chronic infections caused by *P. aeruginosa*
461 biofilms and the organism's interactions with other microbes. Figure 4 summarizes the

462 characteristics, effects, and consequences of biofilm growth among the different infections. In
463 the figure are present similarities and differences between infections.
464
465 **<u>5.3.1 Cystic fibrosis</u>**

466 People often associate *P. aeruginosa* infections with CF disease. The pathophysiology
467 of this disease, with increased viscosity and mucus secretions which, together with the impaired
468 mucociliary function that these patients suffer, creates a perfect environment in the airways for
469 chronic microbial colonization, is the primary cause of morbidity and mortality of this disease
470 (Rajan and Saiman, 2002). *P. aeruginosa* plays a leading role in CF, accounting for 40-60% of
471 infections of this disease (CysticFibrosisFoundation, 2019). The microaerophilic environment
472 in the CF lungs, with clear oxygen gradients promoted by abundant mucus deposition in this
473 organ, promotes *P. aeruginosa* survival and subsequent growth. In this sense, we have recently
474 shown a change in the metabolism of *P. aeruginosa* during the transition from aerobic to
475 microaerophilic and, subsequent, anaerobic growth conditions (Pedraz et al., 2019). In CF *in*
476 *vivo, P. aeruginosa* aggregates in clusters rather than in compact biofilms and grows immersed
477 within a self-produced alginate (Lam et al., 1980; Moreau-Marquis et al., 2008; Worlitzsch et
478 al., 2002). *P. aeruginosa* can undergo mutations that increase its persistence in CF-affected
479 lungs. As mentioned above, the mucoid phenotype of *Pseudomonas* is frequently isolated from
480 CF exacerbations. Additionally, during *P. aeruginosa* CF infections, microorganisms can also
481 lose the flagellum and, consequently, swimming motility. Flagellar mutants are linked to the
482 RSCV phenotype, which has been recognized to overproduce Psl and Pel exopolysaccharides,
483 thus enhancing the bacterial capacity to form biofilms and persist in CF lungs (Harrison et al.,
484 2020).

485 However, after many investigations involving culture-dependent and culture-
486 independent microbiological techniques as well as RNA-based studies, CF infections have been

487 determined to be polymicrobial and different between CF patients (Acosta et al., 2020; Filkins
488 and O'Toole, 2015). The polymicrobial community that colonizes the CF lung harbors
489 facultative as well as obligate anaerobic bacteria (Filkins and O'Toole, 2015). A recent study
490 using bioorthogonal noncanonical amino acid tagging revealed extensive heterogeneity of
491 translational activity among the CF microbiota, which is unique in every CF individual.
492 Therefore, in addition to canonical lung pathogens such as *P. aeruginosa*, there is a low
493 abundance of other members whose activity dynamics are determinants of the acute
494 inflammation occurring in CF, by either impacting the host or through the modulation of the
495 other pathogen's growth and virulence (Valentini et al., 2020). Furthermore, the existence of
496 clonal strains of *P. aeruginosa*, a consequence of patient-patient infections, which are prevalent
497 in clusters of people who live in a defined geographical area, also impacts the microbiota of CF
498 (Parkins et al., 2018). In this direction, we have recently demonstrated that reference strains and
499 clinical isolates of *P. aeruginosa* behave differently during their adaptability and intracellular
500 survival into the lung epithelium. Our work shows the importance of choosing appropriate
501 strains when studying infectious processes with relevant translational outcomes (Cendra and
502 Torrents, 2020).

503 Within the polymicrobial nature of CF infection, *P. aeruginosa* and *Staphylococcus*
504 *aureus* have long been recognized as primary CF pathogens, with an increasing rate of MDR
505 appearance (Rutter et al., 2017). Although it is commonly assumed that *S. aureus* is an early
506 colonizer of CF lungs that is replaced by *P. aeruginosa*, a recent longitudinal retrospective,
507 single-center cohort study that included 337 patients with CF determined that *P. aeruginosa*
508 does not replace the *S. aureus*; rather, both species accumulate over time (Fischer et al., 2020).
509

510 **<u>5.3.2. Chronic wound infection</u>**

511 The skin, despite its role as a protective barrier, is susceptible to many infections,
512 especially when it is affected by burns, wounds or ulcers (Schittek, 2011). It is estimated that
513 approximately 1-2% of the population in developed countries will suffer from chronic wounds
514 during their lifetimes (Gottrup, 2004). *P. aeruginosa,* together with *S. aureus,* are common
515 bacteria usually involved in infections of skin injuries. Biofilms of *P. aeruginosa* are typically
516 associated with deep chronic wounds, which makes treatment with topical therapies difficult
517 (Fazli et al., 2009; Rabin et al., 2015). The chronicity of the wound is in part due to the
518 production of elastase by *P. aeruginosa,* which deteriorates immunoglobulin G and elements of
519 the complement system (Wilson et al., 1998).

520 In diabetes mellitus, the development of foot ulcers is a typical complication of the
521 disease. These patients tend to form nonhealing ulcers on the lower extremities with higher
522 susceptibility than nondiabetic patients (Singh et al., 2005). Insulin treatment has been shown
523 to increase the development of *P. aeruginosa* biofilms and their antimicrobial tolerance by
524 increasing the levels of eDNA through the lysis of neutrophils and other immune cells in
525 wounds. Furthermore, a direct role of insulin in this prolonged inflammatory response in the
526 wound has been demonstrated *in vitro* through the use of incremental levels of intracellular c-
527 di-GMP (Watters et al., 2014; Wei et al., 2019).

528 Chronic wound infections are primarily affected by polymicrobial communities, in
529 which *P. aeruginosa* and *S. aureus* play a central role (Serra et al., 2015). It has been recently
530 seen that the genotype of the patient influences the microbiome composition of the wound, i.e.,
531 depending on the patient's genetics, certain species are more likely to colonize the wound
532 (Tipton et al., 2020). Genomic analyses have found that genes involved in anaerobic growth,
533 metabolic and energy pathways and membrane integrity are critical for bacterial fitness in
534 wounds (Morgan et al., 2019)

535

536 **5.3.3. Keratitis**

537 Biofilm formation over contact lenses (CLs) is an important cause of corneal infections,
538 as these lenses make direct contact with the corneal surface. The annual incidence rate of
539 microbial keratitis due to CL contamination is approximately 4 per 10000 daily contact lens
540 wearers (Morgan et al., 2005). Biofilms formed over lenses enhance bacterial resistance to
541 antimicrobials (Zegans et al., 2002). *P. aeruginosa* is a leading cause of corneal infections
542 promoted by CL wearers (Stapleton and Carnt, 2012). Under normal and healthy conditions, the
543 organism can poorly colonize the cornea. Nevertheless, if there is trauma or the cornea is
544 injured, which is often mediated by continuous CL wearing, *P. aeruginosa* can penetrate the
545 epithelial layer and cause keratitis (Zegans et al., 2002). In the presence of phagocytic cells or
546 corneal epithelial debris, *P. aeruginosa* can even form denser biofilms on the CL (Burnham et
547 al., 2012; Robertson et al., 2011). While growing in biofilms, bacteria can shift their gene
548 expression to be able to persist in the ocular environment. In this sense, *P. aeruginosa* adapts to
549 the human corneal epithelium by modulating, mainly, the expression of virulence genes. This
550 corneal-adapted *P. aeruginosa* forms large biofilm-like aggregates (Evans and Fleiszig, 2013).
551 The T3SS is highly expressed in the adaptation of *P. aeruginosa* for survival on the corneal
552 surface, and it has an essential role in preserving the biofilm against the attack of host
553 neutrophils. This event leads to NET production, which inhibits the spread of the bacteria to the
554 brain by forming a barrier against the pathogen (Thanabalasuriar et al., 2019). Developing new
555 materials for contact lenses able to inhibit bacterial attachment, viability or the adaptive changes
556 in gene expression associated with bacteria growing on surfaces could have a remarkable impact
557 on reducing the risk of infection (Evans and Fleiszig, 2013).

558

559 **5.3.4. Medical device colonization**

560 *P. aeruginosa* is a major nosocomial pathogen able to colonize and form perdurable
561 biofilms on indwelling medical devices such as endotracheal tubes (EETs), catheters, and
562 orthopedic implants as well as on the inner surfaces of metal pipes in hospital water systems. *P.*
563 *aeruginosa* is responsible for 10-15% of nosocomial infections worldwide (Shi et al., 2019).

564 Biofilm formation has been found in 95% of patients intubated with EET and mechanical
565 ventilation for more than 24 h. ETT biofilms are perdurable and able to remain despite antibiotic
566 treatment. This fact increases the risk of upper respiratory tract infections that eventually lead
567 to ventilator-associated pneumonia (VAP), for which *P. aeruginosa* is the primary causative
568 agent (Gil-Perotin et al., 2012). Furthermore, the rhamnolipids produced by *P. aeruginosa*
569 isolates are associated with VAP development (Kohler et al., 2010). *P. aeruginosa* VAP has
570 increased morbidity and involves intensive care unit stays with the additional cost that they
571 represent (Safdar et al., 2005).

572 Urinary catheters are also susceptible to colonization by *P. aeruginosa* and lead to
573 catheter-associated urinary tract infections (CAUTIs), the most common hospital-associated
574 infection. *P. aeruginosa* is responsible for 12% of the CAUTIs acquired in hospitals, which
575 generally tend to be more complicated and have a worse prognosis than those caused by other
576 bacteria (Cole et al., 2014). Iron deficiency in urinary tract infections increases the expression
577 of rhamnolipids in *Pseudomonas,* promoting biofilm development (Glick et al., 2010). In
578 addition to the increased antimicrobial tolerance that bacteria have when growing in biofilms,
579 many clinical *P. aeruginosa* isolates from CAUTIs have been shown to encode for multiple
580 antimicrobial resistances (Vipin et al., 2019), complicating the clearance of this infection.

581

582 6. The challenge to reproduce *P. aeruginosa* polymicrobial chronic infections

583 Even though we now know that biofilms are the predominant type of bacterial growth
584 in nature, planktonic experiments have historically been used as a reference to study chronic

585 infections *in vitro*. Therefore, it has been an urgent need to regenerate the current knowledge
586 about these infections, with models that properly recapitulate the pathophysiology created by
587 biofilm-related infections, as the resulting phenotype obtained from experiments done under
588 laboratory conditions, using monoculture growth and rich media, is far different from what is
589 happening in nature. Additionally, during polymicrobial chronic infections, microbes interact
590 with each other and with the host, which creates an environment that is harsh to recreate *in vitro,*
591 and that often leads to having oversimplified models. Furthermore, even though multi-species
592 interactions can be found in nature, they usually have antagonistic relations due to nutrient and
593 space competition, which also complicate their co-growth *in vitro.* On the other hand, if good
594 models can be achieved, they allow high-throughput screening with the flexibility to modify
595 conditions. For this reason, there is a biotechnological challenge to mimic the polymicrobial
596 biofilms found in several chronic infections with low-cost and easy-to-set-up technologies
597 (Gabrilska and Rumbaugh, 2015).

598 Medium optimization has been used as a strategy to recreate multi-species biofilms by
599 including or excluding specific components that can increase *P. aeruginosa* polymicrobial
600 coexistence. For instance, addition of albumin in the media formulation has been used to grow
601 *P. aeruginosa* and *S. aureus* simultaneously due to the inhibitory effect of the albumin on *P.*
602 *aeruginosa*'s QS (Smith et al., 2017). Similarly, the use of L-arginine or adenosine
603 monophosphate to compromise *P. aeruginosa* pathogenesis while, consequently, increasing *S.*
604 *aureus* survival, or the use of nicotinamide adenine dinucleotide phosphate (NADPH) to combat
605 the oxidative stress created during the coculture growth, are other supplements tested to increase
606 the coexistence of both bacteria *in vitro* (Cendra et al., 2019; Sheng et al., 2012; Zhu et al.,
607 2007). Otherwise, modification of the physicochemical parameters in the coculture system has
608 been another strategy to increase both species' survival *in vitro.* For instance, oxygen is an
609 important parameter to consider, as it influences bacterial survival during the coculture biofilm

610 growth and it has a direct impact on the spatial distribution of the microorganisms within the
611 biofilm (Cendra et al., 2019). In this line, we have recently described an optimized medium and
612 coculture conditions, which consider environmental parameters such as pH and oxygen and
613 include bovine serum albumin in Dulbecco's Modified Eagle's medium, that allow the
614 coexistence of *P. aeruginosa* and *S. aureus* in stable biofilms *in vitro* (Cendra et al., 2019).
615 Different inoculation ratios of these two bacteria have also been used to increase both strains'
616 viability during coculture biofilm growth (Woods et al., 2018). These optimized mediums have
617 been tested on multispecies biofilms grown in static conditions and limited nutrients supply,
618 using microtiter plates or Calgary biofilm devices, as well as in dynamic models such as
619 microfluidic devices, bioreactors, or flow-system models. While static models are cost-effective
620 and useful in high throughput screenings, dynamic models better mimic the natural
621 environmental conditions, thus enabling the biofilm to grow closer as it does *in vivo* (Gabrilska
622 and Rumbaugh, 2015; Stewart and Franklin, 2008).

623 Additionally, during *P. aeruginosa* polymicrobial biofilm growth, it has been detected
624 that continuous flow removes toxic products that inhibit the coexistence between species. It
625 provides fresh and oxygenated medium to the system, helping the maintenance of the pH~7 (in
626 static conditions, pH rapidly increases over pH=8) therefore allowing *P. aeruginosa*
627 multispecies' coexistence (Cendra et al., 2019; O'Brien and Welch, 2019). 3D printed models
628 of *P. aeruginosa* and *S. aureus* biofilms have also been developed using gelatin-based
629 multiphoton lithography, which demonstrated that aggregates of *S. aureus* can increase their
630 tolerance to ß-lactams when enclosed within a shell formed of *P. aeruginosa* (Connell et al.,
631 2013).

632 Even though several methods have been described *in vitro*, we are still far from
633 mimicking the bacterial infection as it is really occurring during human or animal chronic
634 infections. In a chronic infection promoted by biofilm growth *in vivo*, the host response is

635 activated by immune cells that battle against the infection. Some models of lung infections use
636 2D monolayers or lung organoids that mimic the features of a full-sized lung (Nadkarni et al.,
637 2016). However, these models lack vasculature and immune cells and, therefore, fail to
638 recapitulate the complex disease environment at the organ level completely. Similarly, the lung-
639 on-a-chip model greatly mimics the lung's mechanical properties, and it can include endothelial
640 cells in the microfluidic system (Wu et al., 2020). Nevertheless, it has a high manufacturing cost
641 and the limitation to do high-throughput screening. Overall, there is still a long way to go to
642 have proper *in vitro* models able to mimic the pathophysiology generated in chronic infections.
643 CF and wound infections are the two infectious environments that have been more
644 investigated to reproduce *P. aeruginosa* biofilm and polymicrobial infections.
645
646 **6.1 CF models.**
647 An artificial sputum media has been developed to mimic the sputum generated in CF.
648 However, transcriptomic profiles have revealed differences between *P. aeruginosa* grown in
649 this medium and those isolated directly from CF sputum samples (Palmer et al., 2007).
650 Furthermore, as it is a medium created by analyzing the metabolic pathways of *P. aeruginosa*
651 isolates, it tends to favor *P. aeruginosa* growth, limiting polymicrobial coexistence. The use of
652 agar and alginate-beads to grow *P. aeruginosa*, alone or with other microbes found in CF
653 infections, has also been a strategy to simulate the CF infectious environment (Sonderholm et
654 al., 2017). 2D models of coculture experiments growing *P. aeruginosa* and *S. aureus* on lung
655 cells affected with CF have also been used to study this microbial interaction (Hogan et al.,
656 2004; Orazi and O'Toole, 2017). However, the infection virulence limits the monolayer integrity
657 and impedes proper biofilm formation as extended incubation times cannot be achieved. To
658 overcome it, many of these experiments use bacterial supernatants instead of live-bacteria,
659 which can be an approximation but fail to recreate the *in vivo* disease completely.

660 On the other hand, mice and rat models have also been used to generate the CF-disease
661 environment to subsequently study *P. aeruginosa* infections alone and other CF pathogens such
662 as with *S. aureus* (Cigana et al., 2018; Millette et al., 2019). However, murine secretions differ
663 from those in humans, which hinders the direct translation of the results (Benahmed et al., 2014).
664 *ex vivo* pig lung models are used as a replacement for live animal infection as they are clinically
665 realistic and more ethical models (Harrington et al., 2020).

666

667 **6.2 Wound models.**

668 The Lubbock chronic wound biofilm (LCWB) model was created to mimic chronic
669 wounds infected with biofilms under laboratory conditions (Sun et al., 2008). This model uses
670 a chopped-meat-based medium supplemented with heparinized plasma and red blood cells to
671 grow 24 h old multispecies biofilm *in vitro*. Initially, wound-*like* biofilms grown using this
672 model were analyzed over the abiotic surface where the bacterial strains were growing. Later,
673 in a work that investigated *P. aeruginosa* and *S. aureus* interactions, it was detected that the
674 LCWB coagulated after 16 h of bacterial inoculation in a mechanism triggered by a *S. aureus*
675 coagulase-positive strain. This observation was used to employ the coagulated plasma of the
676 LCWB directly as a scaffold to grow the mixed biofilm, thus resembling more the *in vivo*
677 conditions (DeLeon et al., 2014). LCWB is still the basis of a wide range of wound infection
678 models. *P. aeruginosa* and *S. aureus* coculture biofilms grown in simulated wound fluid over
679 collagen matrices has also been used as a wound infection model (Werthen et al., 2010).
680 Recently, tryptic soy broth enriched with NaCl and glucose was used to grow biofilms of these
681 two bacteria on cell-derived matrices. This model allowed us to obtain the competitive
682 distribution of these two organisms as it occurs *in vivo* and identify the SCV morphology of *S.*
683 *aureus* driven by *P. aeruginosa* (Gounani et al., 2020).

684 *In vivo* models have also been used to study wound biofilm infections, however, they
685 are limited by their elevated cost and ethical dilemmas. Still, murine wound infection models
686 have been used to investigate the progression of *P. aeruginosa* and *S. aureus* coinfection and
687 wound healing, as well as to test topical treatments (Hoffmann et al., 2019). Otherwise, the
688 porcine skin wound healing closely resembles human healing, hence, porcine burn models have
689 also been used to study *P. aeruginosa* polymicrobial burn infection with *S. aureus* (Pastar et al.,
690 2013).
691

692 7. Current strategies against *P. aeruginosa* biofilms

693 Biofilms are a terrible public health threat. However, this is not the only issue, as these
694 bacterial communities can also cause tremendous economic losses in the industry. *P. aeruginosa*
695 is a common biofilm producer in food, water, and textile industries (Vishwakarma, 2020). To
696 date, numerous strategies have been developed to prevent *P. aeruginosa* biofilm formation for
697 both medical and industrial purposes.
698 Due to the emergence of antimicrobial resistance, antimicrobial peptides (AMPs),
699 biofilm-degrading agents, QS inhibitors, as well as other compounds that target specific *P.*
700 *aeruginosa* molecules have been developed as an alternative to antibiotics. All these
701 antimicrobials are used against *P. aeruginosa,* killing the bacterium directly or
702 preventing/eradicating its biofilms. The main mechanisms of action that these molecules follow
703 are: i) inhibition of bacterium's attachment to the surface, ii) biofilm disruption with consequent
704 bacterial dispersion, iii) inhibition of the production of specific molecules that promotes biofilm
705 formation, or iv) increasing the antibiotic diffusion within the biofilm. Recent reviews address
706 these types of antimicrobial therapies and tackle the emergence of antimicrobial resistance
707 (Makabenta et al., 2021; Pinto et al., 2020; Pircalabioru and Chifiriuc, 2020; Rumbaugh and

708 Sauer, 2020; Verderosa et al., 2019). In Table 1, we have summarized some of the currently

709 investigated antibiofilm treatments.

710

711 **Table 1.** Antibiofilm strategies against *P. aeruginosa* biofilm formation.

Antibiofilm strategy	Mechanism of action	Examples
Inhibition of QS	Use of compounds to inhibit QS signaling	• Furanones interfere with AHLs (Wu et al., 2004) • Terrein blocks QS receptors and inhibits the production of virulence factors such as elastase, rhamnolipid or pyocyanin (Kim et al., 2018) • A benzimide-benzimidazole compound inhibits the MvrF regulator, inhibiting biofilm formation (Maura et al., 2017) • Baicalin hydrate is an AHL-targeting inhibitor (Brackman et al., 2011)
Inhibition of adhesion	• Surface coating with antimicrobial NPs/molecules that prevent bacterial adhesion • Modification of the surface material to inhibit biofilm formation	• Furanones are used to coat medical devices thus preventing bacterial adhesion (Baveja et al., 2004) • Coating devices with antibiotic hydroxyapatite-based coatings (Veerachamy et al., 2014) • Hydrophilic polymers as hyaluronic acid, hydrogel coating, and heparin coating have been used for catheters coating (Chen et al., 2013) • Functionalization of surfaces with antiadhesive high-density polymers (Neoh et al., 2015) • Nanosilver coatings have been used in catheters, heart valves, and wound dressings (Khatoon et al., 2018) • Gold, titanium, and diamond nanoparticle coating treatment (Veerachamy et al., 2014) • Modification of the properties of the biomaterial, as the surface roughness, energy or hydrophilicity can alter bacterial attachment (Bazaka et al., 2012)
Inhibition of c-di-GMP	Molecules that inhibit, decrease or sequester c-di-GMP promoting biofilm dispersal	• Terrein (Kim et al., 2018) • c-di-GMP-sequestering peptide (Hee et al., 2020) • Nitric oxide (Barraud et al., 2009) • Diguanylate cyclase inhibitors (Rumbaugh and Sauer, 2020)
Antibiofilm and antimicrobial molecules and peptides	Molecules and peptides with direct antimicrobial or antibiofilm properties	• poly-L-lysine cationic polypeptides have a mucolytic activity that disrupts the biofilm (Guillon et al., 2018) • liposomes have high antimicrobial activity and antibiofilm effect (Ibaraki et al., 2020) • antimicrobial peptides (AMPs) have quick bacterial killing and a low tendency to induce resistance, for instance, AMP ZY4 kills planktonic, biofilm and persister *P. aeruginosa* cells by permeabilizing the membrane (Mwangi et al., 2019). • Enzymes to disturb biofilms such as DNases, glycosidases, alginate lyases, and proteases to digest the proteins involved (Baelo et al., 2015; Blanco-Cabra et al., 2020; Rumbaugh and Sauer, 2020)
Bacteriophage therapy	*Pseudomonas*-targeted bacteriophages that infect and kill the bacterium	• ϕMR299-2 and ϕNH-4 phage therapies have been shown to kill clinical *Pseudomonas* isolates colonizing a murine lung as well as in biofilms in a pulmonary cell line (Alemayehu et al., 2012)
Bioacoustic effect	Ultrasonication increases antibiotic diffusion across biofilms	• Ultrasonication combined with gentamicin increased the killing of *P. aeruginosa,* forming a biofilm (Pinto et al., 2020)

712

713 Drug delivery systems (DDS) were created to control the release of therapeutic agents
714 at the target site. As detailed in section 5, *P. aeruginosa* biofilms' structure and physiology
715 challenge their treatment and, in this sense, DDS have been of great help. DDS can be used as
716 a surface coating to prevent biofilm formation, as well as carriers to deliver the antimicrobial
717 inside or over the biofilm efficiently. DDS enhance the antimicrobials' pharmacodynamic and
718 pharmacokinetic effect, increasing the effective concentration in the inner parts of the biofilm
719 for its complete clearance (Liu et al., 2020). Even though different types of DDS have been
720 developed to improve antimicrobial treatment, this review will focus on nanoparticles (NP)-
721 DDS. NPs are an important DDS group, which in continuous development and improvement.
722 Nevertheless, Table 1 also includes other types of DDS (e.g., phage-delivery systems).

723 NPs are DDS widely used for *Pseudomonas* biofilms' treatment. NPs are classified
724 depending on the material used in their synthesis. In this sense, they could be: i) metallic NPs,
725 ii) non-metallic NPs, iii) polymeric NPs, iv) lipid-phased NPs, v) ceramic NPs, and vi) quantum
726 dots (Buch et al., 2019). Recently, smart nanomaterials have been developed, which have the
727 particularity that the delivery of the antimicrobial cargo is done in response to a stimulus as, for
728 instance, pH or light (Chen, M. et al., 2019). NPs size is critical to penetrating within the biofilm
729 properly, and it has to range between 5 – 500 nm, being ideally 200 nm the maximum diameter.
730 NPs smaller than 5 nm prone to be filtrated by the kidney while higher than 500 nm to be
731 recognized and cleared by the human complement system (Liu et al., 2019). The shape of the
732 NP is another critical element, as it can influence contact killing as, for instance, it occurs with
733 sharp NPs, which can make a hole in the bacteria and cause cytoplasmic leakage. Otherwise,
734 including mucolytic or other degrading agents in the NPs, together with using electrostatically
735 neutral coating, increase the efficiency of NPs penetrance into the biofilm (Tan et al., 2020). In
736 this line, our group has demonstrated the degrading effect of DNAse coated NPs loaded with

737 ciprofloxacin on *in vivo*-like biofilms of *P. aeruginosa*, as well as unraveled the effect of specific
738 alginate lyases (Alg2A and A1-II') as dispersing agents of *P. aeruginosa* biofilms (Baelo et al.,
739 2015; Blanco-Cabra et al., 2020).

740 On the other hand, it has also been detected that cationic nanoparticles can have a good
741 distribution across the biofilm matrix (Baelo et al., 2015; Makabenta et al., 2021).
742 Biocompatibility and cell toxicity are major concerns when using these DDS as depending on
743 the material they are made of, NPs can cause important cytotoxicity, being useless for human
744 treatment. For example, this is one drawback of metallic NPs as high metal doses are toxic for
745 human cells, which is why these NPs are not the preferred choice to use against CF-infections
746 due to the long-term treatment that these infections require (Makabenta et al., 2021; Vandebriel
747 and De Jong, 2012). NPs used in medical applications are usually coated with liposomes, silica
748 or biopolymers to enhance their biocompatibility, as well as to improve their elimination from
749 the body. Poly-D-L-(lactic-co-glycolic acid), polylactic acid, polyethylene glycol,
750 poly(caprolactone, dextran, chitosan, poly(urethanes), poly(ethylene imine) or poly(*N*-
751 isopropylacrylamide) are some biopolymers frequently used for NPs coating (Tan et al., 2020).
752 An important disadvantage of the current NPs is the amount of cargo that they can load. The
753 vast majority only support low concentrations of the drug, making them suitable as a proof-of-
754 concept but not feasible for clinical application. The high number of NPs needed to achieve
755 efficient antimicrobial concentration limits the balance between antimicrobial activity and
756 cytotoxicity.

757 NPs can load different antibiotics/antimicrobials to be released within the biofilm.
758 Tobramycin, ciprofloxacin, colistin, levofloxacin, amikacin, and gentamicin are known
759 antibiotics delivered by NPs to treat *P. aeruginosa* infections. Some of them have been used
760 with inhaled systems, although this technology is usually inefficient to eradicate *P. aeruginosa*
761 biofilm infection (Ho et al., 2019). Otherwise, when using NPs directly against biofilms, it has

762 been detected that the small size of these nanocarriers allows the particle diffusion across the
763 thick and dense mucus of CF mucoid biofilms. In this sense, delivery of amikacin antibiotic by
764 nanoscale liposomes has been used to treat *P. aeruginosa* biofilms in lung infections (Meers et
765 al., 2008). Delivery of nitric oxide (NO) is an additional strategy used against *P. aeruginosa*
766 biofilms. NO, as endogenous free radical, acts as a broad-spectrum antimicrobial with low
767 toxicity and a great capacity to eradicate biofilms. Silica NPs conjugated with NO have been
768 tested against *P. aeruginosa* and *S. aureus* coculture biofilms (Slomberg et al., 2013). Heat
769 generation on NPs has also been seen to improve antibiotics delivery into *P. aeruginosa* mono
770 and polymicrobial biofilms (Teirlinck et al., 2018). Table 1 summarizes additional strategies
771 employed against *P. aeruginosa* biofilms using NPs as DDS.
772

773 **8. Conclusions**

774 *P. aeruginosa* biofilms are complex structures that become even more intricate when
775 they are formed together with other microorganisms. They represent a clinical and
776 biotechnological burden from different perspectives; therefore, only with the continuous
777 development and improvement of efficient antibiofilm strategies we can tackle the recurrence
778 and chronicity caused by *P. aeruginosa* biofilm infections.
779

780 **Acknowledgments**

781 The group is supported by grants from the Ministerio de Economía, Industria y
782 Competitividad, MINECO, and Agencia Estatal de Investigación (AEI), Spain, co-funded by
783 Fondo Europeo de Desarrollo Regional, FEDER, European Union (RTI2018-098573-B-100),
784 the CERCA programme and *AGAUR-Generalitat de Catalunya* (2017SGR-1079), the European
785 Regional Development Fund (FEDER), Catalan Cystic Fibrosis association and Obra Social
786 "La Caixa".

787

788 ## Author contributions

789 MC and ET have designed, written and approved the final version of the review.

790

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1427 ## Figure legends

1428 **Figure 1**. **Scheme of the clinically relevant microbial biofilm interactions of *P. aeruginosa*.**
1429 *P. aeruginosa* can interact and coexist simultaneously with a wide range of microbes from
1430 different phyla. The figure shows the main interactions described for *P. aeruginosa* with
1431 bacteria *(Burkholderia cepacia, Staphylococcus aureus, Prevotella spp., Enterococcus faecalis,*
1432 *Streptococcus spp., Acinetobacter baumannii, Stenotrophomonas maltophilia, Veillonella spp.,*
1433 *Actinomyces spp.*, and *Propionibacterium spp.*), fungi (*Aspergillus fumigatus* and *Candida*
1434 *albicans*) and viruses (respiratory syncytial virus (RSV), human rhinovirus, severe acute
1435 respiratory syndrome coronavirus 2 (SARS-CoV-2) and influenza). Many of these interactions
1436 have been found in the disease environment. The figure was created using biorender.com.

1437

1438 **Figure 2. Mutual effect of *P. aeruginosa* and *S. aureus* pathogenicity and interaction on**
1439 **each other phenotype.** The scheme summarizes the effect that the products of *P. aeruginosa*
1440 and *S. aureus* cause on each other, as well as that caused by the products generated as
1441 consequence of their interaction. Blue arrows indicate a beneficial effect, while red lines indicate
1442 an inhibitory effect. In black is specified the mutual effect that the organisms receive as a
1443 consequence of their interaction. Next to each arrow is denoted the action caused by the products
1444 generated, responsible for the inhibitory or beneficial effect promoted to the receptor.

1445

1446 **Figure 3. Role of QS on the development of the ECM.** The plot shows the effect of the main
1447 circuits of *P. aeruginosa* QS on the development of the biofilm's ECM. The principal factors
1448 described affecting QS modulation, with the respective increased autoinducers and the
1449 consequent effect on rhamnolipids (orange), Pel (red), and eDNA (blue) production, are
1450 included in the figure.

1451

1452 **Figure 4. Main features of *P. aeruginosa* biofilms and the respective consequences on the**
1453 **infection progression.** The figure shows the major infections caused by *P. aeruginosa* biofilms:
1454 cystic fibrosis, wounds, infections due to biofilm growth over medical devices, and keratitis
1455 with the representative features of each one. The scheme shows the principal causes that lead to
1456 *P. aeruginosa* growth in biofilms. Hence, a lung with the alveoli completely filled with an
1457 excess of mucus is presented for CF infections, heart valves, urinary catheters, stents, and
1458 orthopedic devices are shown as examples for medical devices-related infections, a skin wound,
1459 and a foot ulcer are shown for wound infection and, finally, an eye is shown for keratitis
1460 infection. The figure summarizes the main characteristics of each infection detailed through
1461 section 5.3. Created with biorender.com.

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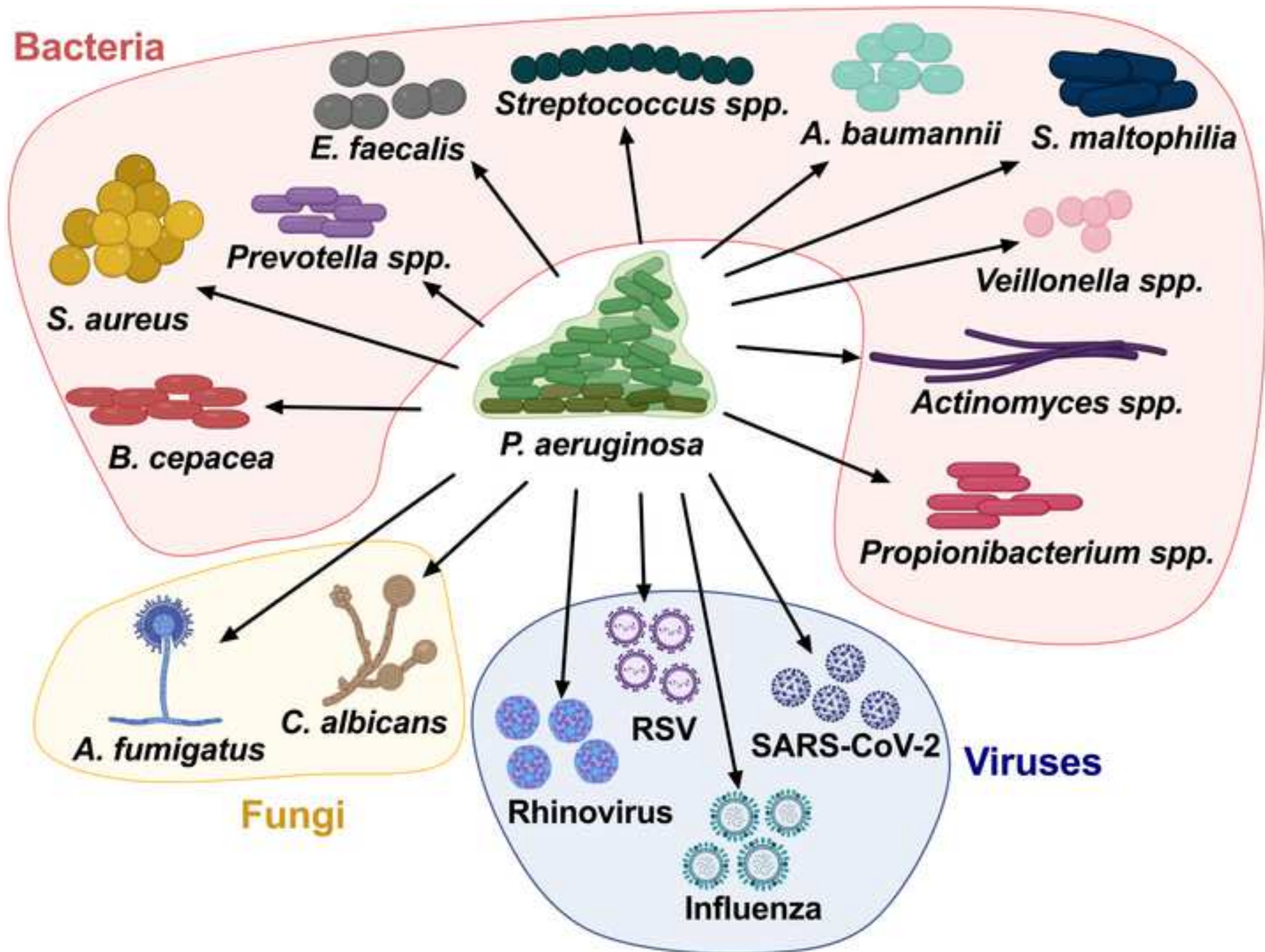

Figure 2

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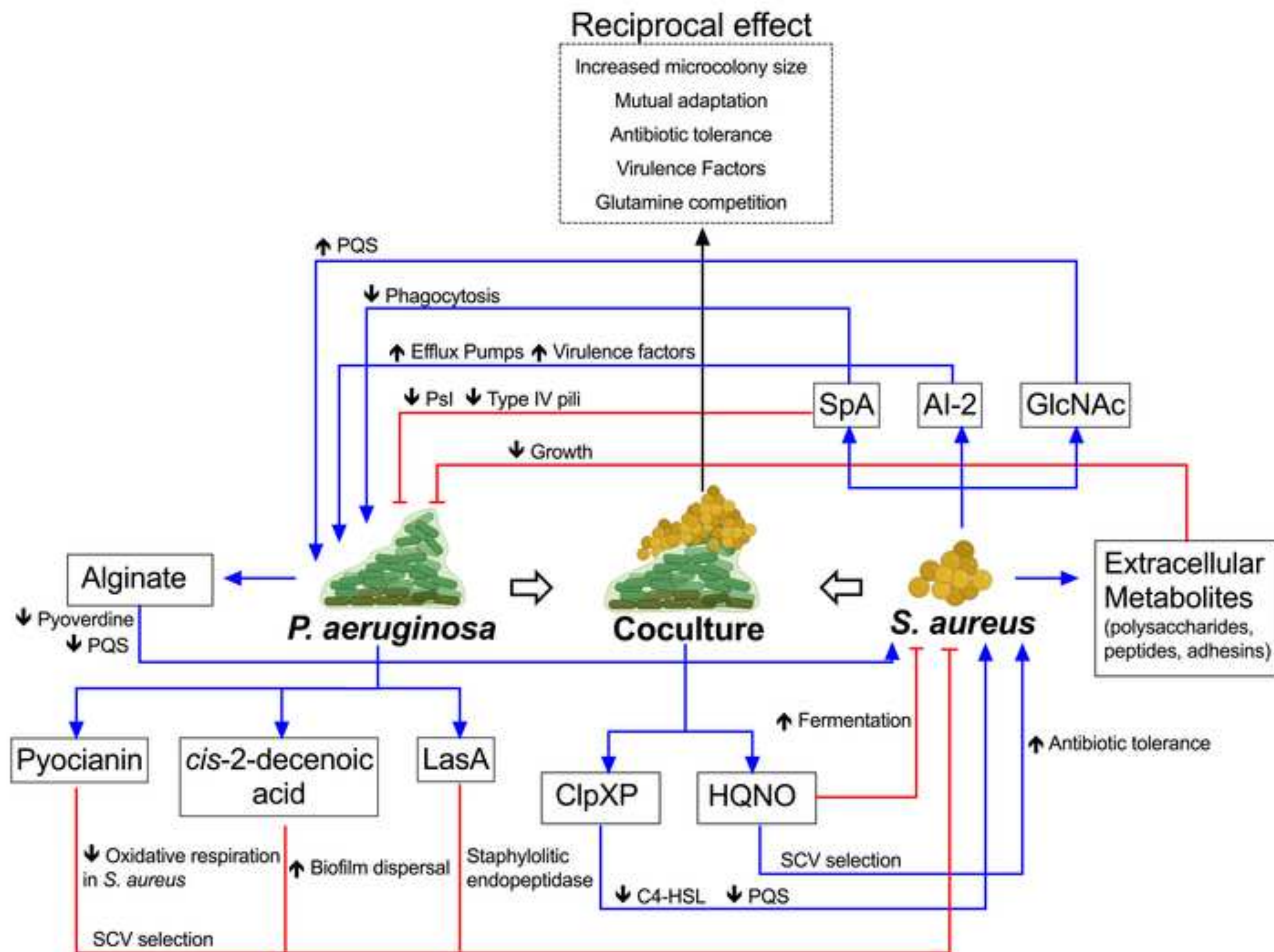

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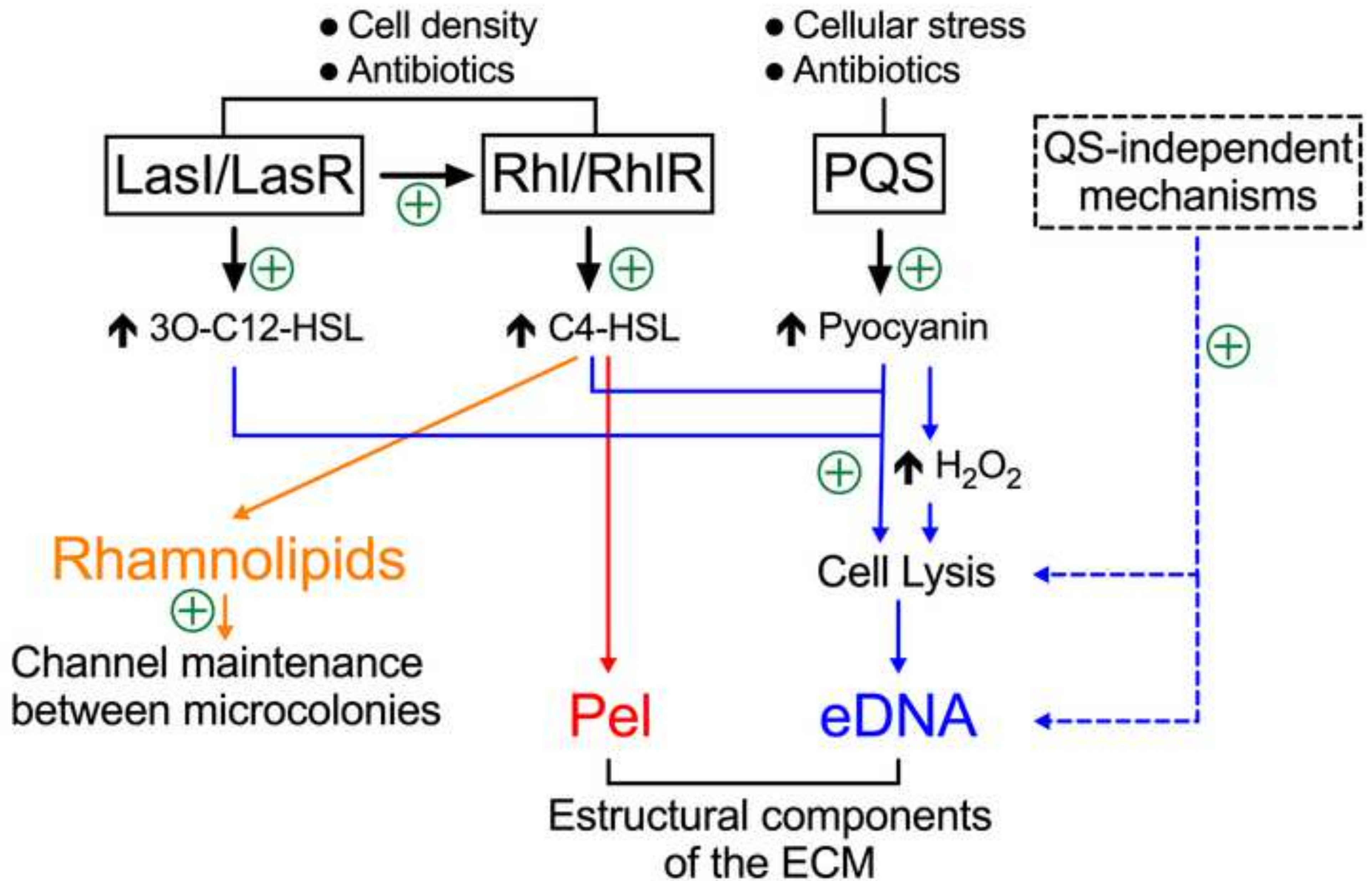

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