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Tracing the path of Quorum sensing molecules in cystic fibrosis mucus in a biomimetic in vitro permeability platform

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P. aeruginosa **employs specific quorum sensing (QS) mechanisms to orchestrate biofilm formation, enhancing resistance to host defences. In physiological conditions, QS molecules permeate the lung environment and cellular membrane to reach the cytoplasmic Aryl Hydrocarbon Receptor (AhR) that is pivotal for activating the immune response against infection. In pathological conditions like cystic fibrosis (CF) this interkingdom communication is altered, favouring *P. aeruginosa* persistence and chronic infection. Here, we aim to investigate the molecular journey of QS molecules from CF-*like* environments to the cytoplasm by quantifying via HPLC-MS the permeability of selected QS molecules (quinolones, lactones, and phenazines) through in vitro models of the two main biological lung barriers: CF-mucus and cellular membrane. While QS molecules not activating AhR exhibit intermediate permeability through the cellular membrane model (PAMPA) ($1.0–4.0 \times 10^{-6}$ cm/s), the AhR-activating molecule (pyocyanin) shows significantly higher permeability ($8.6 \pm 1.4 \times 10^{-6}$ cm/s). Importantly, combining the CF mucus model with PAMPA induces a 50% decrease in pyocyanin permeability, indicating a strong mucus-shielding effect with pathological implications in infection eradication. This study underscores the importance of quantitatively describing the AhR-active bacterial molecules, even in vitro, to offer new perspectives for understanding *P. aeruginosa* virulence mechanisms and for proposing new antibacterial therapeutic approaches.**

Early signs of cystic fibrosis (CF) are evident within the first 5 years of life, with CF patients often exhibiting air trapping, mucus obstruction, bronchiectasis and, in 98% of cases, infection of gram-negative pathogens, including *P. aeruginosa*[1,2]. The chronic infection of *P. aeruginosa* exacerbates different pulmonary dysfunctions, such as the presence of inflammatory biomarkers, that are directly associated with the premature death of 90% of CF patients[3–5].

The infection dynamics of *P. aeruginosa* in CF are complex, coordinated on a molecular level by a plethora of quorum sensing (QS) molecules, including quinolones, lactones, and phenazines like pyocyanin. Although pyocyanin is primarily recognized as a virulence factor, it is also considered a QS molecule due to its regulatory effects in facilitating virulence, electron transfer and modulation of specific set of genes, known as the PYO stimulon, during the stationary growth phase[6,7].

The intricate QS network collectively contributes in transitioning the *P. aeruginosa* infections from a planktonic (non-adherent) state to a sessile, chronic infection[8–10]. This transition is multifaceted, involving metabolic shifts to adapt to hypoxic and nutrient-limited conditions, the emergence of small colony variants (SCVs), hypermutation leading to antibiotic resistance, and population diversification (i.e., evolution into multiple distinct subpopulations, each with different genetic, phenotypic, or behavioural traits)[11–13]. These adaptations collectively contribute to the shift from an acute, antibiotic-sensitive phenotype to a chronic, antibiotic-resistant state[14]. In these favourable conditions, *P. aeruginosa* colonies are characterised by significant biofilm formation, in what is generally described as mucoid conversion. The enhanced exopolysaccharide production, characteristic of mucoid conversion, plays a crucial role in stabilizing biofilms and protecting bacterial communities from both the host immune system and antibiotic treatments[15–17].

The mucoid conversion is associated with inefficient antibiotic therapies for long-term eradication of infection, leading to deteriorating clinical outcomes in CF, including loss of lung function due to severe

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bronchiectasis, prolonged hospitalisation, and increased mortality[18,19]. It is therefore crucial to find alternative methods to antibiotic treatment that take into account the dynamicity of *P. aeruginosa* infections conferred by QS communication and that can counteract and/or prevent phenotypic conversions (including mucoid conversion) that empower the virulence of *P. aeruginosa*[17].

An emerging approach to address CF-associated infection is to reconsiders bacteria-derived products, including QS, as potential therapeutic agents thus exploiting strategies that the human body has developed to naturally counter the bacteria-bacteria crosstalk during infections through immune cell activation[20–22]. Recent research suggests that QS molecules could be harnessed in novel ways, either as targets for therapeutic intervention or as direct therapeutic agents, for example triggering a broad spectrum of responses such as cytokine and chemokine expression and immune cell recruitment[23]. Among different receptors, the cytoplasmic Aryl Hydrocarbon Receptor (AhR) is increasingly recognized as a pivotal sentinel-receptor for infection[24]. For this reason, QS molecules have been proposed as modulators of AhR, showing promising results in pioneering studies as a new potential therapeutic target in the context of CF[25]. Specifically, bacterial-derived metabolites of tryptophan were investigated as AhR modulators in both in vitro and in vivo models, yielding promising results in terms of restoration of drug metabolism and eradication of the bacterial infection[26,27]. However, the AhR-targeting approach can be extended to other bacteria-derived molecules and particularly those involved in QS communication. Indeed, the AhR-QS binding mechanism is naturally exploited by healthy human cells to monitor the bacterial population density in the lungs, finely tuning the activation of immune mucosal cells only at the opportune moment of the bacterial infection, thus avoiding unnecessary inflammation while hindering the transition from planktonic to sessile states (Fig. 1A, B)[28]. The potential targeting of the AhR by QS molecules as an alternative therapeutic strategy for CF-related *P. aeruginosa* infections aligns with the emerging approach of modulating the immune response to fight infections[25]. This strategy opens the possibility of shifting from traditional methods of directly attacking the pathogen to one where the host's immune system is leveraged or adjusted to better manage and control the infection. Yet, the passage of QS molecules from the CF mucus to the cytoplasm of lung cells results in being particularly challenging. In pathological conditions like CF, overproduction of mucins forms a thicker and denser mucus layer where the interkingdom signalling loses its balance and the diffusion of molecules of both natural and synthetic origin is impaired by altered mucus properties[29–31]. From a therapeutic perspective, it is hence pivotal to assess in a simple, rapid, and quantitative way, the capability of QS molecules of *P. aeruginosa* to overcome the pathological CF-mucus barrier, penetrate cellular membranes and reach the cytoplasmic target. To achieve this goal, in vitro systems may represent a fundamental asset, as they can provide information about the permeability of molecules in controlled conditions, outside of the confounding complexity of the human body. Currently, in vitro permeability assay relies on cell-free membranes like the parallel artificial membrane permeability assay (PAMPA), culture of monolayers of epithelial cells (Caco-2 cell line) or complex systems based on an ex vivo approach[32]. All these systems proved to be useful in different fields, such as the investigation of standard pharmacokinetic drugs. Nonetheless, the rich molecular diversity of QS molecules makes a critical need for the development of high throughput screening (HTS) systems to assess the permeability of a wide range of QS molecules as rapidly and informatively as possible, thus properly indicating the trajectory of new drug design and development. Recently, an HTS system was developed to quantify the permeability of drugs in a more in vivo-*like* environment that includes a bioinspired in vitro model of mucus[33,34]. The inclusion of an in vitro mucus barrier greatly influenced the permeability of selected drugs, highlighting the importance of mucus when evaluating permeability to replicate the in vivo barrier function of CF-infected mucus, which hinders molecules permeability and, consequently, drug bioavailability including AhR modulators.

The objective of this study was to investigate the permeability of selected QS molecules as a proxy of their AhR modulation potential. Firstly, we measured the permeability of *P. aeruginosa* QS molecules using a system that mimics cellular membranes (PAMPA), assessing their passive diffusion from the environment to the cytoplasm, where AhR is located. Secondly, we exploited a bioinspired in vitro model of CF mucus, including both mucins and alginate, to simulate the role of *P. aeruginosa*-infected mucus as a barrier in CF. Finally, we integrated the PAMPA and mucus models, in a simulated in vivo scenario, considering both environmental and cellular membrane barriers to evaluate the passage of QS molecules, opening the possibility of unveiling the role of both the pathological environment and the cellular membrane in the inefficient fight of the immune system against *P. aeruginosa* infections in cystic fibrosis.

Results and discussion

Modelling the cellular barrier

The cellular membrane prevents exogenous substances from reaching the intracellular compartment. Since, the modulation of AhR by QS molecules can only occur after crossing the cellular membrane, we used the parallel artificial membrane permeability assay (PAMPA) as a model of the phospholipid membrane to investigate permeability by passive diffusion of QS molecules (Fig. 2B).

We selected three classes of QS molecules (phenazines, lactones and quinolones) as molecular representatives of the QS network involved in the different stages of *P. aeruginosa* infection (Fig. 2A). The precise temporal characterization of QS molecule production and their interactions is a complex area of study that has not yet reached a universally accepted interpretation. Recently, Muora-Alves et al. reported that homoserine lactones (C4, C7, and C12) are produced by *P. aeruginosa* during the initial stages of infection, coinciding with the phase of maximum growth rate and exponential expansion of the infection[28]. Quinolones (PQS), instead, represent the intermediate stage of the infection, when the infection starts to assume a steady-state condition but a biofilm is not yet produced. Finally, phenazines such as pyocyanin represent the terminal signaling molecules within the quorum sensing network of *P. aeruginosa*, which are massively produced when the late-growth phase is reached, the bacterial density is at its maximum, and biofilm production initialises chronic infection (Fig. 1)[28].

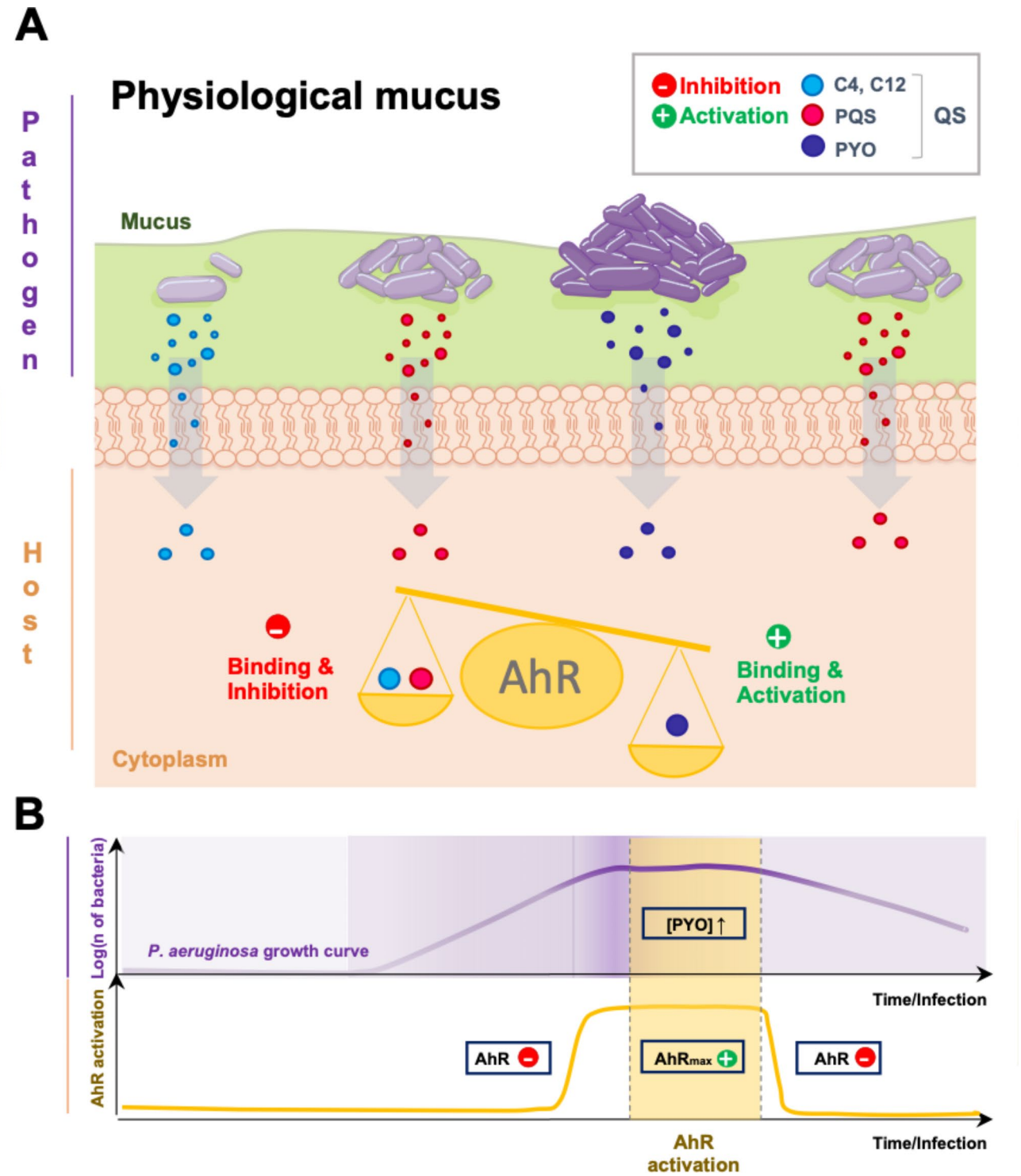

Fig. 1. Relevance of AhR in the host–pathogen interkingdom signalling. In physiological condition, QS molecules permeate through the mucus environment and the cellular membrane to bind AhR[28] (**A**). To avoid unnecessary triggering on the immune reaction, the AhR is activated by QS associated with the late-phase growth of *P. aeruginosa* (e.g., phenazine pyocyanin) while it is inhibited by other QS molecules, like quinolones and lactones, that precede terminal production of virulence factors[22,35] (**B**).

The apparent permeability (P_{app}) was measured at a single time point (5 h) using a previously established protocol[34,36]. Seeking standardisation, the permeability of propranolol was used as a control reference molecule (propranolol, PROP). The recorded permeability of the QS molecules was in the range of 10^{-6} cm/s, with pyocyanin (PYO) being the most permeable with P_{app} values equal to $8.6 \pm 1.4 \cdot 10^{-6}$ cm/s and butyryl-homoserine-lactone (C4) the least permeable, with P_{app} values equal to $1.2 \pm 0.1 \cdot 10^{-6}$ cm/s (Fig. 2C). In a

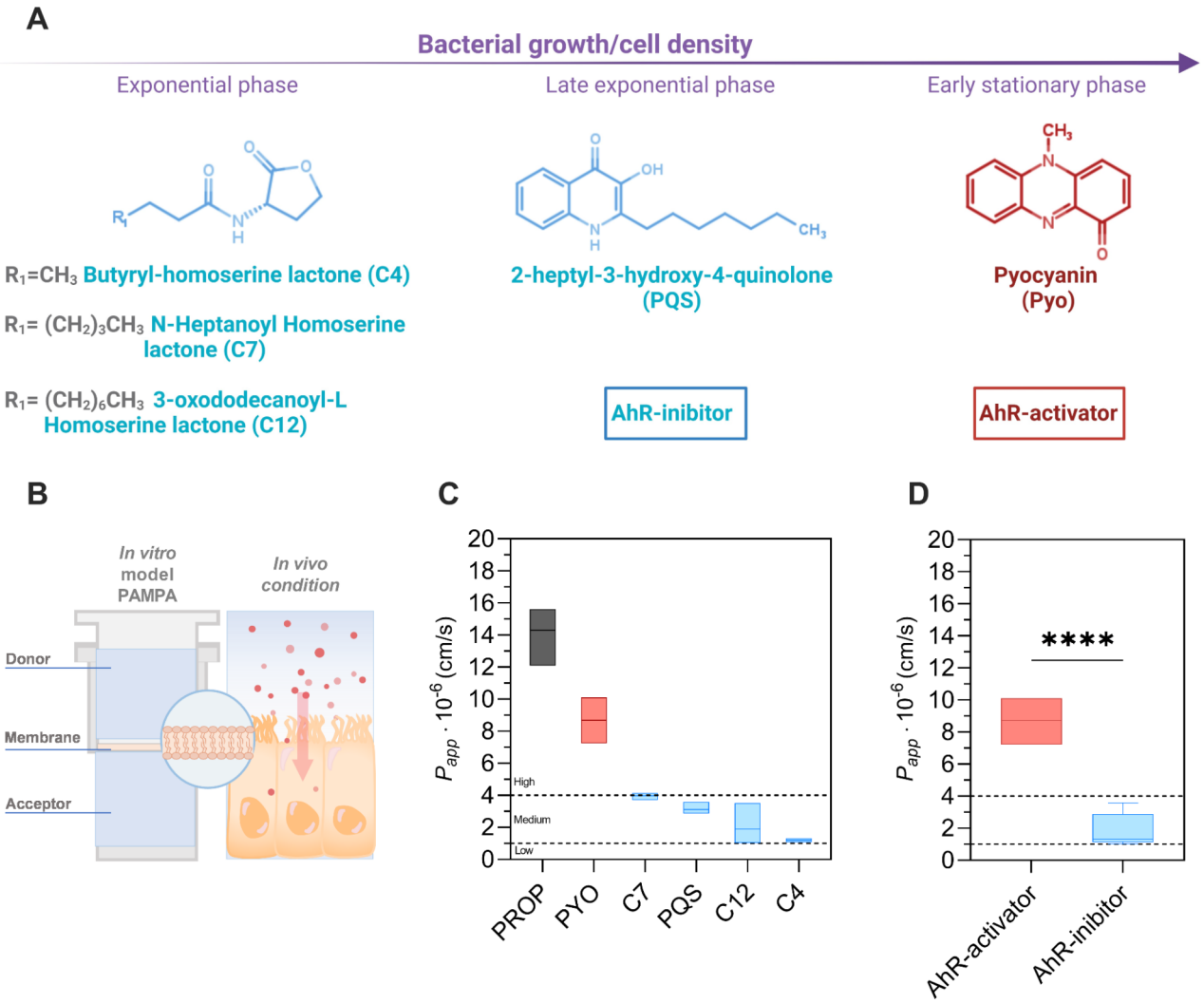

Fig. 2. Permeability evaluation of AhR-activating and AhR-inhibiting QS molecules (**A**) AhR-inhibitors (blue) and AhR-activators (red) QS molecules produced by *P. aeruginosa* at different stages of infection. (**B**) The PAMPA 96-well plate enables in vitro simulation of the cell membrane of the host that QS molecules must cross to reach the cytoplasmic receptor. The graphical insert represents the PAMPA working process, where the donor and acceptor compartments are separated by a semipermeable phospholipid membrane as a model of the phospholipid cellular barrier. (**C**) Apparent permeabilities (P_{app}) of the QS molecules: pyocyanin (PYO), N-Heptanoyl-Homoserine lactone (C7), 2-heptyl-3-hydroxy-4-quinolone (PQS), 3-oxododecanoyl-L-Homoserine-lactone (C12), butyryl-homoserine lactone (C4). Propranolol (PROP) was used as a control for testing membrane integrity. The thresholds of low-to-medium and medium-to-high permeability (1.0×10^{-6} cm/s and at 4.0×10^{-6} cm/s, respectively) are represented by the dashed lines (****$p<0.0001$). (**D**) Apparent permeability of the QS molecules able to bind and activate (red) or inhibit (blue) the AhR according to the literature[28,44].

previous study[34], we found that a permeability threshold of $4\cdot10^{-6}$ cm/s is useful to distinguish high from moderate/low permeable compounds, while $1\cdot10^{-6}$ cm/s is commonly considered indicative of permeability in numerous prior studies[37–39]. Four out of five of the QS molecules considered (i.e., C7, C4, C12 and PQS) were in the medium-permeability value range, while only PYO showed high-permeability values, with a P_{app} that was ~2.2 times higher than the medium-to-high permeability threshold. Taken together, these results indicate that all the QS molecules considered were able to permeate the PAMPA membrane and that they can be divided into two main populations. The first group, including C7, C4, C12 and PQS, exhibited a spectrum of intermediate permeabilities ranging from 1.2 to $4.0\cdot10^{-6}$ cm/s. The second group, consisting solely of pyocyanin, was more similar to the high-permeable control-molecule (PROP) in PAMPA ($P_{app}=14.3\pm1.92\cdot10^{-6}$ cm/s, comparable to literature data[34]) and with a P_{app} that was at least twofold the P_{app} of the other QS molecules.

Polarity-related descriptors (e.g., hydrogen bond acceptors, r = -0.66) were found to correlate with permeability. In particular, the topological polar surface area (TPSA) is the molecular descriptor that better correlates with the P_{app} (r = -0.84, Fig. S1).

Previous studies reported that most homoserine lactones (i.e., C4, C7, C12 in this study) are diffuse QS molecules, which passively permeate through the cell membrane (diffusible QS). Differently, others QS molecules like PQS require active transport (i.e., secreted QS) via vesicles-production although retaining diffusible properties under favorable environmental conditions[40].

In *P. aeruginosa* infections, the length and the degree of substitution of the N-acyl side chain determine whether the homoserine lactone molecule is freely diffusible or subjected to active transport[41]. Yet, it is unclear if similar transporters are exploited to cross the host's cellular membrane. In our experimental setup, the homoserine lactones showed comparable permeabilities to each other and to quinolone (PQS)[42,43]. This indicates that the AhR inhibitors (i.e., C4, C7, C12, and PQS)[28] have similar permeabilities, which were significantly lower when compared to the AhR activator pyocyanin (Fig. 2D). Among the QS molecules considered in this work, PYO is also the only QS molecule capable of easily reaching the AhR. Importantly, the high permeability of PYO is in line with the in vivo data that report the activity of PYO on AhR[28], which describe the AhR as strategically highly sensitive to pathological molecules (PYO) compared to other non-activating ligands (PQS and lactones) to elegantly avoid the use of unnecessary resources during mild infection stages and concentrate a targeted and powerful immune reaction only when the bacteria's presence becomes harmful[28,44].

Assessing in vitro the CF mucus barrier

Modelling only the cell membrane is not sufficient to replicate the complex CF environment. After secretion, the QS molecules are released within the pathologically thick and dense mucus network, which affects their diffusion to the lung epithelium[33]. This pathological scenario is further exacerbated in the case of chronic *P. aeruginosa* infections, where mucoid conversion of the pathogen generates overabundant biofilm production through massive secretion of calcium-crosslinked alginate[16], whose specific physico-mechanical properties impact the mass transport of compounds, including therapeutics[45,46]. For this reason, understanding the role of mucus on the mobility of QS molecules could be highly relevant for elucidating new therapeutic strategies within the CF context[34].

We used an in vitro bioinspired CF-mucus model incorporating mucins and alginate as key components of pathologically infected CF-mucus[33]. Then, we incorporated the CF-mucus model into a Transwell system to investigate the diffusion and retention of QS molecules through mucus (Fig. 3A). All the QS molecules passed through the mucus barrier, albeit at different diffusion rates, ranging from a minimum to a maximum of 26 and 64%, respectively (Fig. 3B). This impact can be explained through two possible mechanisms: steric and chemical interactions with mucins[47]. From a steric perspective, the mucus network can impair the passage through a physical filtering effect of the mucins forming a network with a nanometric mesh size. This network is capable of reducing the mass transport of compounds and, consequently, their diffusion[48]. Although the mesh size of the CF-mucus model is comparable to the mesh of cystic fibrosis mucus (54.7 ± 5.35 nm)[33], the molecular dimension of the considered QS molecules is far smaller, indicating that the retention within mucus predominantly originates from the chemical barrier and not steric filtration. We observed that hydrophobicity (cLogP), solubility (cLogS), and molecular size (VDW Volume) are critical properties governing the diffusion of QS molecules through mucus (Fig. 3C, D, E). In particular, small and polar compounds are less retained than large and hydrophobic molecules (Fig. 3F). Such a result should not be surprising, as hydrophobicity and solubility have an implied inverse correlation. Moreover, previous studies characterising molecular binding to mucus showed that increased hydrophobicity is associated with strong interactions with mucus[49,50].

Integrating the cellular barrier and mucus

Thanks to the integration of PAMPA with the bioinspired CF-mucus, we were able to measure the permeability of the selected QS molecules in a pathological-*like* scenario, as this set-up includes models of both environmental and cellular membrane barriers (Fig. 4A). Interestingly, the effect of mucus on molecule permeability appears to vary depending on their structure. Quinolones (PQS) and lactones (C4, C7, and C12) were not significantly impacted in terms of permeability, as their P_{app} values with or without mucus were comparable, ranging from $9.02 \pm 3.96 \cdot 10^{-7}$ cm/s for C4 to $2.89 \pm 0.67 \cdot 10^{-7}$cm/s for C7, and within the same permeability range (Fig. 4B). Differently, the phenazine pyocyanin was highly influenced by mucus, showing a reduction in P_{app} of 60% (P_{app} of $3.50 \pm 1.07 \cdot 10^{-6}$ cm/s) (Fig. 4C). Unfortunately, quantitative studies in the literature that monitor AhR activity as a function of pyocyanin concentration are lacking. However, cellular-based models of CF treated with pyocyanin concentrations comparable to those found in the PAMPA system with and without mucus (6 and 1 µM) have shown approximately a 50% reduction in AhR activity[28]. These in vitro findings indicate a not-trivial role of mucus in the inefficient fight of the immune system against *P. aeruginosa* infections in cystic fibrosis. Indeed, the reduced permeability of PYO suggests a diminished AhR targeting and activation in the presence of CF mucus, especially the mucoid *P. aeruginosa*-infected mucus (Fig. 4D, E). Since AhR activation triggers recruitment of neutrophils[44], the reduced AhR activation may hinder neutrophils recruitment to infection sites, suggesting that *P. aeruginosa* may have evolved to exploit the QS crosstalk and biofilm mass production in mucoid-infections to evade human AhR-dependent immune defence and to facilitate chronic bacterial permanence. In this perspective, the permeability results here reported align with other in vitro observations indicating that *P. aeruginosa* might exploit multifaceted strategies (e.g., reduced motility and endotoxin production) to dampen inflammatory response following mucoid conversion aiding in escaping immune clearance[51].

To produce effective therapeutic alternatives, it is therefore crucial to investigate the QS-AhR interactions in a more holistic approach, particularly considering how QS molecules interact within the unique CF environment, which influences the virulent potency of *P. aeruginosa*. Thus, we investigated further into the interactions between the pathologically active molecule considered in this study, PYO, and the CF-mucus model.

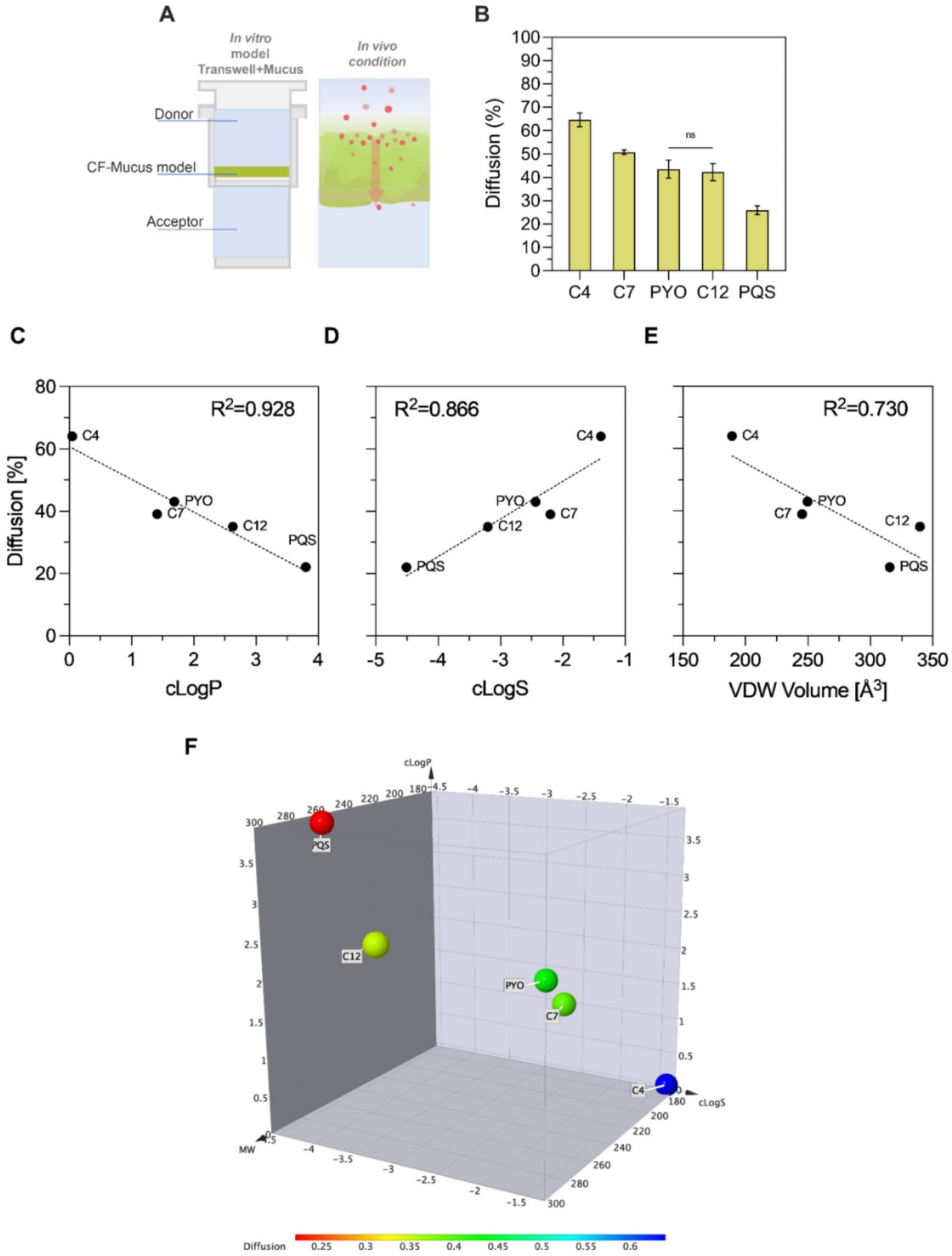

Fig. 3. In vitro evaluation of the effect of CF-mucus on QS molecules diffusion. (**A**) Plate enables to assess in vitro the impact of mucus on the diffusion of QS molecules. The concentrations in the acceptors were measured using HPLC-MS after five hours; (**B**) Diffusion (%) of the QS molecules. Correlation plots between diffusion and (**C**) partition coefficient (cLogP), (**D**) solubility (cLogS), (**E**) and Van der Waals volume (VDW Volume) of the tested QS molecules. (**F**) 3D chemical space defined by cLogP, cLogS, and VDW Volume. A colour range is used to display diffusion rates of the QS molecules.

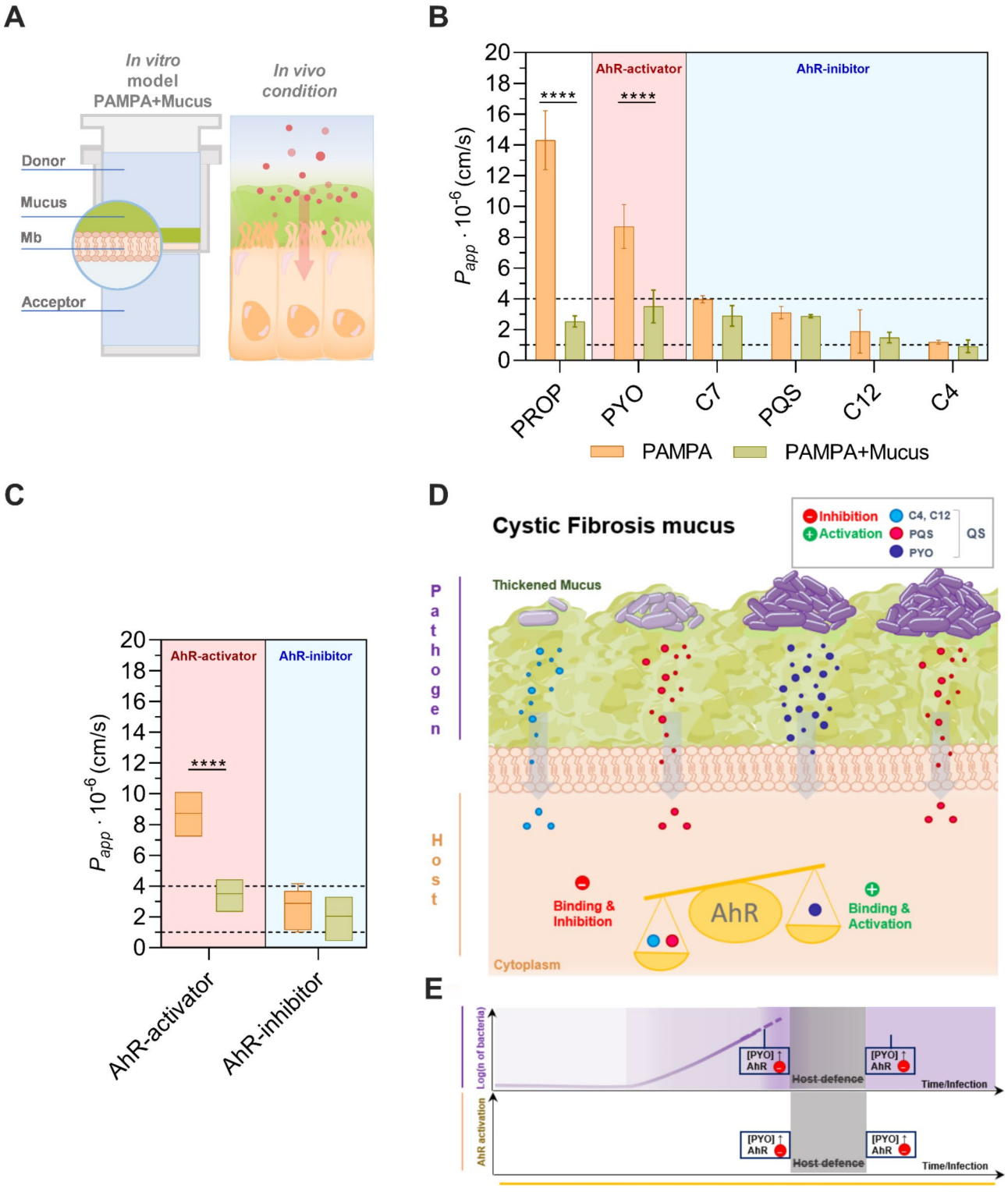

Fig. 4. Evaluation of the permeability of QS molecules in the integrated biomimicking in vitro permeability platform. (**A**) The PAMPA 96-well plate coupled with the CF mucus model enables the in vitro simulation of the two barriers that QS molecules must cross to reach the cytoplasmic receptor: the cell membrane and the mucus. (**B**) Apparent permeability (P_{app}) of Quorum sensing in absence (orange) and presence of the CF mucus model (green). Propranolol (PROP) was the internal standard. (**C**) Apparent permeability of the QS molecules able to activate (red) or inhibit (blue) the AhR in absence (orange) and presence of the CF mucus model (green). (**D**,**E**) In the pathological condition of CF, the presence of a thickened mucosal barrier can greatly influence the permeability of QS, therefore impacting their binding with AhR.

Pyocyanin is a heterocyclic compound that exhibits different colours depending on the pH values. In a neutral or alkaline environment, it appears blue in its zwitterionic form with a neutral charge, while in an acidic environment it exhibits a positive charge resulting in a red-shift in the UV-visible spectrum (Fig. 5A). In CF, the environmental changes lead to a pathological reduction of the pH[52], further exacerbated during infections[53]. Since the CF-mucus model used in this work has comparable pH than in vivo data, we investigated the possibility that the acidic environment of CF may induce alterations in the molecular interactions between pyocyanin and mucins.

From the absorption spectra of pyocyanin, it is possible to observe that at physiological pH the deprotonated form of pyocyanin is prevalent, as indicated by the peak at 684 nm. Differently, when pyocyanin is introduced into the CF-mucus both neutral and cationic species coexist in the matrix, as two absorption peaks (at 523 nm and 684 nm) are present and an isosbestic point (at 584 nm) can be identified (Fig. 5B, C).

The coexistence of both protonated and deprotonated forms of pyocyanin in the CF-mucus model provides valuable insights into the mechanisms governing the mass transport of this pathologically significant molecule through the mucus barrier. When *P. aeruginosa* infects CF-mucus, the acidic pH of the environment may shift pyocyanin towards its protonated form, resulting in stronger binding with the negatively charged mucins[6] (Fig. 5D). This unique interaction between pyocyanin and mucins significantly impacts the permeability of pyocyanin compared to other QS molecules, whose chemical properties are less affected by the environment (Fig. S2).

In the development of new therapeutic strategies for CF, particularly those targeting the AhR, the presence of mucus can no longer be neglected, as it may affect the molecular mechanisms by which both pathological and potentially therapeutic molecules reach their cytoplasmic targets. The design of mucus-including in vitro

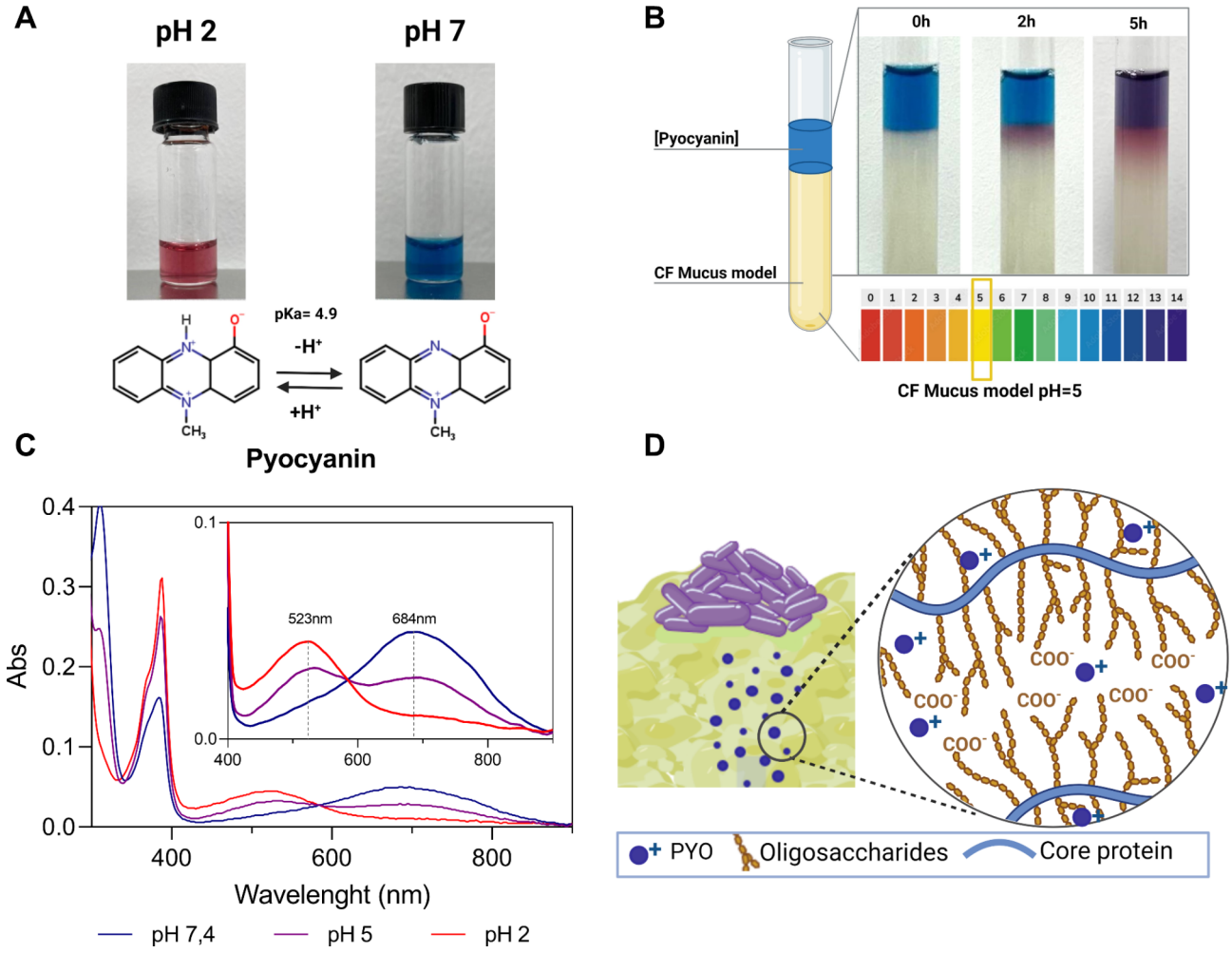

Fig. 5. Characterisation of the interaction between the AhR-activating pyocyanin and mucins in the CF-mucus model. (**A**) Pyocyanin solutions at 800 µM in acidic (pH 4) and neutral conditions (PB pH 7.4) (**B**) Color transition from blue to purple of pyocyanin solution (800 µM) deposited onto a tube containing the CF mucus model with slight acid pH (pH 5). (**C**) UV–Vis absorption spectra of pyocyanin in acidic conditions (pH 2), neutral conditions (pH 7.4) and in mucus (pH 5). (**D**) The positive charge form of protonated pyocyanin (blue) interacts with the negative charges of the mucins (COO^-). This interaction significantly impacts the permeability of pyocyanin compared to other QS molecules.

tools, like the one proposed in this work, that can give insight of pathological chemical interactions represents a strategic advance for the screening of new therapeutic molecules, enabling the fast identification of the potential therapeutic molecules that are permeable to both the cell membrane and the mucus barrier in an *in vivo-like* condition.

Conclusion

In Cystic Fibrosis (CF) *P. aeruginosa* exploit Quorum Sensing (QS) molecules to coordinate infection progression from an acute to a sessile phase, characterised by overproduction of exopolysaccharide (mucoid conversion) that is associated with ineffective antibiotic therapeutic outcomes. Novel therapeutic approaches aim at targeting the Aryl Hydrocarbon Receptor (AhR), a cytoplasmic sentinel receptor capable of recognizing QS molecules and coordinating an appropriate immune response in the host upon binding with the QS during infection stages.

In this study, we investigated for the first time the in vitro molecular journey of QS molecules produced by *P. aeruginosa* targeting the cytoplasmic AhR, focusing on the impact of two main barriers on the permeability of these molecules.

To do so, we used in vitro systems of increasing complexity. Initially, we quantified the permeability of QS molecules through a representative model of the cell membrane alone (PAMPA), finding that the AhR-activator QS molecule (i.e., pyocyanin) have much higher permeability than AhR-inhibitor QS molecules. By exploiting a bioinspired CF mucus model based on mucin and alginate, both components of native infected CF mucus, we were able to quantify the impact of the pathological barrier of mucoid *P. aeruginosa*-infected mucus on molecule diffusion. This revealed that infected mucus possesses a potent barrier effect, reducing the diffusion of all considered QS molecules of at least 25%. Finally, we integrated the two in vitro tools, PAMPA and CF mucus model, assessing how CF-mucus pathological conditions exert a potent limiting factor on the permeability of pyocyanin (AhR-activating molecules), due to the interaction between the negative charges of the mucus and the positive charge of pyocyanin at pH around 5[51,54,55]. Mucus is not only a barrier in this case, but it also acts as a dynamic filter[50,56].

The integrated system presented here allows for the study of the molecular journey of QS molecules in a simple and rapid way, opening up opportunities to investigate AhR-targeting as a novel therapeutic approach for CF infections. Although in vitro systems do not fully model the in vivo situation, they exclude many confounding factors, enabling quick determination of permeability. This supports the selection of various molecules, including QS and other bacterial-derived compounds, by assessing their efficiency in targeting this key receptor. In principle, this approach could be further expanded to consider molecules targeting other cytoplasmic receptors involved in host-pathogen interactions in CF, offering a new avenue for identifying alternative strategies to fight cystic fibrosis.

Materials and methods

Materials

Diffusion experiments were performed on Transwell supports, while permeability was measured using a pre-coated PAMPA (i.e., parallel artificial membrane permeability assay) plate system (Corning). The quorum sensing (QS) molecules N-(3-oxododecanoyl)-L-homoserine lactone (C12), N-heptanoyl-L-homoserine lactone (C7), N-butanoyl-L-homoserine lactone (C4), 2-heptyl-3-hydroxy-4(1 H)-quinolone (PQS), pyocyanin (PYO) and propranolol (Prop) were purchased from Merck. Stock solutions were prepared at a concentration of 10 mg/mL in DMSO and were stored at 4 °C until use. Further dilutions were prepared in Millipore grade water (mQ; resistivity 18.2 MΩ · cm at 25 °C) from an in-house Millipore system. Acetonitrile, ammonium acetate, and dimethyl sulfoxide (DMSO) of analytical grade were purchased from Sigma-Aldrich. Porcine gastric mucin (PGM) (type III, bound sialic acid 0.5–1.5%, partially purified powder), sodium salt of alginic acid, sodium chloride, calcium carbonate ($CaCO_3$) and D-(+)-gluconic acid δ-lactone (GDL) purchased from Merck were used for mucus model production.

Modelling the cellular barrier

The working solutions of QS molecules (C12, C7, C4, PQS and PYO) and propranolol (Prop) were prepared by diluting the stock solutions to a concentration of 40 µM in 10 mM phosphate buffer (PB) (pH 7.4, 5%DMSO).

The permeability assay was conducted using PAMPA. Prior to use, the membrane was equilibrated by adding 300 µL of phosphate buffer (PB) to the acceptor compartment. After half an hour, 300 µL of fresh PB (pH 7.4, 5% DMSO) were added to the acceptor compartments, while 200 µL of working solutions were added to the donor compartments. The PAMPA was then stored at room temperature for 5 h. Then, the total volumes of the donor and acceptor compartments were collected and the amount of passively diffused QS was quantified by high performance liquid chromatography mass spectrometry (HPLC-ESI MS). The apparent permeability coefficient (P_{app}) was computed according to Eq. (1), derived from Fick's law for steady state conditions.

$$P_{app} = \frac{dQ/dt}{C_0 \times A} \tag{1}$$

where dQ is the amount of drug expressed in moles permeated into the acceptor compartment at time t (5 h), C_0 is the initial concentration in the donor well, and A is the area of the well membrane (0.3 cm^2).

LC-MS quantification

The concentration of the QS in each acceptor well was determined using a Varian 212-LC HPLC equipped with a Prostar 410 autosampler and a Phenomenex Luna C18 column (150×2 mm, 3 µm) and detected on a Varian 320 MS triple quadrupole (TQ) mass spectrometer hyphenated with an electrospray ionisation (ESI)

source operating in positive and negative ion modes. The detector was used in multiple reaction monitoring (MRM) mode. The experimental details of each analytical transition are reported in Table S1. Mobile phases consisting of acetonitrile and water, 0.1% formic acid (FA), or ammonium acetate (5 mM, pH 6.6), were pumped at 200 µL/min, following a gradient programme. The injection volume was 5 µL. Quantification was performed according to a seven-point calibration curve specific for each compound. The solutions from PAMPA acceptor compartments were diluted 1:2 in mQ water.

Assessing in vitro the CF mucus barrier

The CF respiratory pathology mucus model was prepared as previously described[23,24,29]. Briefly, a 21 mg/mL solution of alginate was dissolved in a NaCl solution (16.3 mg/mL), under slow magnetic stirring. Mucin solution (43.7 mg/mL) was prepared in mQ water and stirred under slow agitation for at least 4 h. The alginate and mucin solutions were mixed with $CaCO_3$ (7.0 mg/mL) and GDL (70.0 mg/mL), freshly prepared in NaCl solution (16.3 mg/mL), in a volume ratio of 4:1:1:1:1. Immediately after mixing, the mucus model (40 µL) was directly pipetted onto the membrane of the transwell. Gently orbital shaking was used to distribute the mucus volume over the surface and to remove air bubbles. Then, the mucus was stored overnight at 4 °C to complete gelation before performing the diffusion measurements.

Stock solutions of the drugs were prepared at 10 mg/mL in DMSO; working solutions were prepared by diluting the stocks to a concentration of 40 µM in a 10 mM phosphate buffer (PB) (pH 7.4, 5% DMSO). Then, 200 µL of working solutions were placed in the donor compartment of the Transwell plate, while 600 µL of PB (pH 7.4, 5% DMSO) water were placed in the acceptor compartment. The donor and acceptor compartments were then co-incubated at room temperature for 5 h. After the incubation period, the entire acceptor volume was collected. The amount of passively diffused QS was quantified by high-performance liquid chromatography mass spectrometry (HPLC ESI MS), as described above. Before quantification, the samples were diluted 1:2 in water.

Integrating the cellular barrier and mucus

The permeability evaluation with the integrated system (Mucus + PAMPA) was performed similarly to what was performed with PAMPA.

40 µL of freshly prepared CF-mucus models were directly pipetted onto the PAMPA membrane in the donor compartment. The integrated system (Mucus + PAMPA) was gently orbitally shaken to optimise distribution and bubble removal and stored at 4 °C overnight to complete gelation. Then, 300 µL of phosphate buffer (PB) were used to equilibrate to the acceptor compartment. Upon removal of the equilibrating PB, another 300 µL of fresh PB (pH 7.4, 5%DMSO) were added to the acceptor compartments, while 200 µL of working solutions of QS molecules were added with particular care to the donor compartments containing the completely crosslinked mucus layer. The diffusion experiment of QS molecules in the integrated system (Mucus + PAMPA) was maintained for 5 h at room temperature. Then, the total volumes of the acceptor compartments were collected and the apparent permeability (P_{app}) computed by quantifying the amount of passively diffused QS though high-performance liquid chromatography mass spectrometry (HPLC ESI MS).

UV–Vis spectroscopic characterization of pyocyanin

Pyocyanin stock solution was diluted in 10 mM Phosphate Buffer (PB) (pH 7.4) to a final concentration of 800 µM.

3 mL of freshly prepared CF-mucus model were pipetted onto a tube and stored at 4 °C overnight to complete gelation. Then, to evaluate the characteristic of pyocyanin in mucus, 1 mL of working solution 800 µM in 10 mM Phosphate Buffer (PB) (pH 7.4), was added onto the tube containing the CF mucus and left for 1 h. After 1 h the content of the tube was mixed using a syringe, and then filtered with a filter (Whatman® Puradisc syringe filters, 0.45 µm, PTFE). The filtered solution was then diluted to a final concentration of 20 µM. The 800 µM in Phosphate Buffer (PB) (pH 7.4) was diluted to a final concentration of 20 µM. Pyocyanin stock solution was diluted in 80 mM Ammonium formate Buffer (pH 4) to final concentration of 20 µM. The three absorption spectra of pyocyanin in mucus, PB (pH 7.4) and Ammonium formate Buffer (pH 4) were measured using a Hitachi spectrophotometer, model number UH5300, at room temperature.

Statistical analysis

At least three replicates were performed per experiment. All quantitative data are presented as the mean ± standard deviation (SD). The D'Agostino-Pearson test was used to investigate the normality of the data distribution. Depending on normality test outcomes, Student t-test/Mann-Whitney test or ANOVA/Kruskal-Wallis test were used to compare two or more groups of data, respectively. The statistical analysis was performed by using GraphPad Prism 9.3.0 (GraphPad Software, USA). Statistically significant differences are graphically represented as follow: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****).

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

S.V. designed the study with the contribution of O.V.G., L.S. and C.S.B.; O.V.G. collected the data and performed data analysis with the contribution of L.S. and C.S.B.; O.V.G. and E.F. performed the mass-spectrometry tests with the supervision of C.M. and F.D.B.; O.V.G., E.F., L.S. and C.S.B. wrote the paper under the supervision of C.M., F.D.B and S.V.; S.V. funding acquisition; all authors revised the paper and approved the submission.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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