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Friends or enemies? The complicated relationship between *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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Abstract

Pseudomonas aeruginosa (*Pa*) and *Staphylococcus aureus* (*Sa*) are opportunistic pathogens that are most commonly co-isolated from chronic wounds and the sputum of cystic fibrosis patients. Over the last few years, there have been plenty of contrasting results from studies involving *P. aeruginosa* and *S. aureus* co-cultures. The general concept that *P. aeruginosa* outcompetes *S. aureus* has been challenged and there is more evidence now that they can co-exist. Nevertheless, it still remains difficult to mimic polymicrobial infections *in vitro* and *in vivo*. In this review, we discuss recent advances in regard to *Pa-Sa* molecular interactions, their physical responses, and *in vitro* and *in vivo* models. We believe it is important to optimize growth conditions in the laboratory, determine appropriate bacterial starting ratios, and consider environmental factors to study the co-existence of these two pathogens. Ideally, optimized growth media should reflect host-mimicking conditions with or without host cells that allow both bacteria to co-exist. To

further identify mechanisms that could help to treat these complex infections, we propose to use relevant polymicrobial animal models. Ultimately, we briefly discuss how polymicrobial infections can increase antibiotic tolerance.

Introduction

Pseudomonas aeruginosa and *Staphylococcus aureus* are highly prevalent pathogens found in the cystic fibrosis (CF) lung (Filkins *et al.*, 2015) and in chronic wound infections (DeLeon *et al.*, 2014), including leg ulcers (El-Naggar *et al.*, 2016), surgical site infections, burns (Pastar *et al.*, 2013), and necrotising fasciitis (NF) (Levine & Manders, 2005, Akita *et al.*, 2006). The general treatment of these infections currently involves broad-spectrum antibiotics; however, co-infections are complex, hard to treat, and often associated with poorer patient health outcomes (Pammi *et al.*, 2014, Filkins & O'Toole, 2015, Tay *et al.*, 2016, Wimmer *et al.*, 2016). It remains unclear whether this is due to increased virulence of each organism or because multiple organisms benefit from each other and therefore render antibiotic therapy ineffective. Over recent years, there has been increased evidence that the presence and interactions of *P. aeruginosa* and *S. aureus* in chronically infected lungs and wounds lead to facilitated host colonization, enhanced virulence, and differential immune response (i.e., influence wound healing) (Dalton *et al.*, 2011b, Alves *et al.*, 2018). In addition, chronic infections allow bacteria to adapt a biofilm-like growth that contributes to adaptive resistance, where bacteria can be up to 1000 times more resistant to antibiotics and host immune responses than their planktonic counterparts (Wu *et al.*, 2015, Pletzer & Hancock, 2016). Biofilms are bacterial communities encased within an extracellular polysaccharide matrix, allowing adherence to each other and a variety of different biotic and abiotic surfaces (Donlan, 2002). Bacteria within polymicrobial (i.e., multiple species) biofilms can benefit from each other through the production and secretion of molecules that other bacteria may utilize (e.g., nutrients), or compete for resources and space (e.g., production of toxins) (Lopez *et al.*, 2010, DeLeon *et al.*, 2014). This raises the question whether multispecies interaction and the release of toxins or other extracellular metabolites could confer a growth advantage for one bacterium over the other? The exact mechanisms how these bacteria interact *in vivo* still remains largely unknown. Mechanistic details are influenced and limited by host tissue, the presence of immune cells (e.g., neutrophils or macrophages), microcolony formation in biofilms, and chemical and physical interactions (such as

metabolic molecules, antimicrobials, or quorum sensing (QS) signals; reviewed in detail by Short *et al*. (Short *et al.*, 2014)).

S. aureus is one of the initial colonisers of the lung in children with CF (Hubert *et al.*, 2013, Razvi *et al.*, 2009). The general consensus is that *P. aeruginosa* overtakes *S. aureus* at a later age and becomes the major pathogen in the CF lung (Davies, 2002). However, recent studies have challenged this concept and provided evidence that *S. aureus* is often co-isolated with *P. aeruginosa* in at least a third of adult CF patients (Fischer *et al.*, 2020, Briaud *et al.*, 2020b, Briaud *et al.*, 2019, Pallett *et al.*, 2019). This is an important fact as it impacts how to effectively treat CF in children compared to adults, the antibiotics used to treat the infection, and how scientists use *in vitro* and *in vivo* models to replicate CF. Both organisms are also frequently co-isolated from chronic wound infections, where *S. aureus* tends to colonise the top layer of the wound and *P. aeruginosa* the deeper region of the wound bed (Kirketerp-Moller *et al.*, 2008, Fazli *et al.*, 2009, Serra *et al.*, 2015). The two infectious environments can be difficult to reproduce in the laboratory as during biofilm formation under static conditions, many *P. aeruginosa* strains secrete the exopolysaccharide Pel to produce a pellicle at the air-liquid interface (Friedman & Kolter, 2004), while *S. aureus* will settle at the bottom. In order to overcome these issues, shaking or flow conditions, which mimic physiological relevant conditions such as the production of exudates in chronic wounds (Duckworth *et al.*, 2018), can be used. However, in our experience shaking conditions without adequate adherence time will not allow *P. aeruginosa* to adequately attach to a surface and flow conditions often lead to massive mushroom-like biofilms by motile *P. aeruginosa* (Bjarnsholt, 2013) that are not necessarily relevant or accurately reflect chronic infections. The ability for *P. aeruginosa* and *S. aureus* to colonise different areas, in the lung or wound, is therefore challenging as the two pathogens would most likely require different treatment options.

The CDC has listed multidrug resistant *P. aeruginosa* and methicillin-resistant *S. aureus* (MRSA) as serious threats to human health in 2019 (Centers for Disease Control and Prevention, 2019). The economic burden (2018 OECD Health Policy Study) of antimicrobial resistance is predicted to cost up to $3.5 billion USD per year (OECD, 2018). Antimicrobial resistance is of emerging concern worldwide, with many bacteria developing resistance to multiple or all classes of antibiotics (World Health Organisation, 2020). Hence, there is a high demand for new strategies to tackle antimicrobial resistance.

In this review, we will be discussing known molecular interactions involved in *Pa-Sa* co-culture, their physical responses during their interaction, and the current *in vitro* and *in vivo* models used to study them. Ultimately, we will look at the complex interactions between *Pa-Sa* that hinder treatment strategies.

Molecular interactions of *P. aeruginosa-S. aureus* in co-cultures

There have been many recent *in vitro,* and some *in vivo* studies investigating the co-culture/-infection of *P. aeruginosa* and *S. aureus* (Table 1). It has been widely accepted that when *P. aeruginosa* and *S. aureus* are grown together in co-culture, *P. aeruginosa* becomes dominant, outcompeting and outgrowing *S. aureus* (Filkins *et al.*, 2015). Despite many controversial studies, there is growing evidence that would suggest that both bacteria can co-exist (Baldan *et al.*, 2014, Woods *et al.*, 2018, Limoli *et al.*, 2017, Price *et al.*, 2020).

P. aeruginosa produces molecules that can affect *S. aureus* growth but do not necessarily kill the bacteria. These include 2-n-heptyl-4-hydroxyquinoline N-oxide (HQNO), an exoproduct of the PQS (Pseudomonas Quinolone Signal) quorum sensing system that targets the electron transport chain (cytochrome system) and induces small colony variant formation of *S. aureus* (Hoffman *et al.*, 2006, Filkins *et al.*, 2015) (Figure 1). *P. aeruginosa* also produces pyocyanin that inhibits *S. aureus* oxidative respiration, the LasA protease that lyses *S. aureus* cells, and Cis-2-decenoic acid and rhamnolipids which promote biofilm dispersal of *S. aureus* (Hotterbeekx *et al.*, 2017). *S. aureus* is at an even further disadvantage as it increases *P. aeruginosa* virulence through the release of the peptidoglycan component N-acetyl glucosamine (GlcNAc) upon cell lysis or cell wall turnover during growth (Korgaonkar *et al.*, 2013, Yang *et al.*, 2020). Yang *et al.* (Yang *et al.*, 2020) recently showed that GlcNAc was important for proteolytic activity in *P. aeruginosa,* and co-culture with *S. aureus* or addition of GlcNAc decreased the activity of the *P. aeruginosa* caseinolytic protease ClpXP. Interestingly, the deletion of *clpX* and *clpP* in *P. aeruginosa* PAO1, respectively, increased its *S. aureus* Newman killing capacity by over 1,000-fold (Hotterbeekx *et al.*, 2017). Mechanistically, the ClpXP system plays a critical role during QS homeostasis and interference can therefore increase QS signal production. This further results in elevated levels of virulence factors and exotoxins that can cause host tissue damage and facilitate colonisation of *P. aeruginosa* (Hotterbeekx *et al.*, 2017, Korgaonkar *et al.*, 2013). Although the ClpXP protease has been suggested as a pharmaceutical target (Moreno-Cinos *et al.*, 2019) and anti-virulence drug (McGillivray *et al.*, 2012) to specifically target *S. aureus* during *Pa-Sa* co-culture (Yang *et al.*, 2020), it remains questionable whether this is a feasible approach. The recent discovery that

ClpXP inactivation increased virulence in *P. aeruginosa* (Yang *et al.*, 2020) is of specific concern, especially in complex infections that are often polymicrobial.

Filkins *et al*. (Filkins *et al.*, 2015) developed an *in vitro* dual species co-culture system of *P. aeruginosa* and *S. aureus* on monolayers of human bronchial epithelial cells homozygous for the ΔF508 cystic fibrosis transmembrane conductance regulator. Intriguingly, *P. aeruginosa* also shifted *S. aureus* from aerobic respiration to fermentation and used the produced lactate as a carbon source (Filkins *et al.*, 2015, Tognon *et al.*, 2019). Further interaction between *P. aeruginosa* and *S. aureus* showed that both bacteria adapted metabolically (e.g., nitrogen utilization) as they started to compete for nutrients. Co-culture induced the expression of *ntrC*, a response regulator to nitrogen limitation in *P. aeruginosa*, and upregulated several genes involved in nitrogen assimilation including a glutamate dehydrogenase and synthase (*gdhA* and *glnA*). In *S. aureus*, nitrogen starvation was observed via a downregulation of the glutamate synthase genes *gltB* and *gltD*, both convert glutamine to glutamate. Interestingly, during early co-culture, *S. aureus* induced an upregulation of nucleotide synthesis genes, cell signalling, energy production, and virulence factors indicating that *S. aureus* is at a disadvantage when cultured with *P. aeruginosa* initially. However, *P. aeruginosa* clearly benefits from the presence of *S. aureus* and the additional availability of carbon and nitrogen. Hence, it may be beneficial for *P. aeruginosa* to maintain a constant source of nutrients to have an advantage during colonisation as well as long-term infection. The majority of co-infection studies on the molecular mechanisms of *P. aeruginosa* and *S. aureus* interactions are performed *in vitro*. However, Ibberson *et al*. (Ibberson *et al.*, 2017) explored their interactions *in vivo* in a murine chronic surgical wound infection model. They used a transposon mutant pool to investigate the essential genome of *S. aureus* HG003 in the presence of *P. aeruginosa* during infection, which revealed 182 uniquely essential *S. aureus* genes. The majority of these genes were involved in *S. aureus* metabolism, demonstrating that *P. aeruginosa* induces metabolic stress on *S. aureus in vivo*.

While there are several experiments that showed that *P. aeruginosa* outcompetes *S. aureus* during early co-culture, chronic, long-term infections may on the other hand promote co-existence. This would suggest that *P. aeruginosa* does not completely eradicate *S. aureus* during infection and they rather reach an equilibrium that balances the presence of both. Baldan *et al*. (Baldan *et al.*, 2014) performed co-culture experiments (1:1 ratio) of *S. aureus* Newman with *P. aeruginosa* PA14, AA2 (an early clonal isolate from a CF patient), and AA43 (a late-adapted strain from the same CF patient with several mutations in virulence factor production) (Table 1). *P. aeruginosa*

PA14 and the early CF isolate AA2 interfered with and inhibited *S. aureus* planktonic growth (>3 log reduction), biofilm formation (>5 log reduction), and *in vivo* infection (>3-fold) in co-culture. The CF isolate *P. aeruginosa* AA43 inhibited *S. aureus* at a much lower extent during growth (~1 log), biofilm (~2 log), and *in vivo* infection (no competition). This indicates that chronicity impacts the virulence of *P. aeruginosa,* and therefore promotes the co-existence with *S. aureus*. However, the study lacked experiments with late-adapted *S. aureus* strains and therefore revealed only limited insights into what drives coexistence with *S. aureus* within a host.

A hallmark of chronic pulmonary *P. aeruginosa* infections are strains that undergo mucoid conversion, which are characterised by the overproduction of the exopolysaccharide alginate. This mucoid phenotype conversion most commonly occurs via acquisition of a mutation within the *mucA* anti-sigma factor. Mucoid phenotypes of *P. aeruginosa* are associated with a decline of lung function and enhanced resistance to antibiotics (Malhotra *et al.*, 2018). Limoli *et al*. (Limoli *et al.*, 2017) discovered that the overproduction of alginate by mucoid strains inhibited *P. aeruginosa* anti-staphylococcal activity, therefore promoting *P. aeruginosa* coexistence with *S. aureus* within the CF lung. Building on those findings, Price *et al*. (Price *et al.*, 2020) showed exogenous alginate protected both planktonic cells and biofilms of *S. aureus* from antibiotics (Orazi & O'Toole, 2017, Orazi *et al.*, 2019) and *P. aeruginosa* killing in co-culture (Price *et al.*, 2020). Exogenous alginate decreased the expression of siderophores, rhamnolipids, and *Pseudomonas* quinolone signals (PQS) (Limoli *et al.*, 2017, Price *et al.*, 2020). This is interesting as it indicates that *P. aeruginosa* shifts from a competitive to a cooperative approach.

Other investigations into coexisting mechanisms were performed by Zhao *et al*. (Zhao *et al.*, 2018), using an *in vitro* co-evolution assay (1:1 ratio) of *P. aeruginosa* (PAO1) and *S. aureus* (ATCC25923) over 21 days. Intriguingly, *P. aeruginosa* dominated initially, however, reached a dynamic equilibrium with *S. aureus* after its 14th passage. The authors further investigated the *P. aeruginosa* transcriptome during co-culture, which revealed a downregulation of the QS system as the passage number increased. A quorum sensing regulator mutant in *lasR* was unable to reduce the population sizes of *S. aureus* in co-culture, confirming that the downregulation of *P. aeruginosa* QS systems contributed to the coexistence of *S. aureus* over time. Interestingly, *S. aureus* did not have any effect on the *P. aeruginosa* transcriptome, which further suggests that *P. aeruginosa* is the main pathogen that controls the infection progress. In another evolution study, Tognon *et al.* (Tognon *et al.*, 2017) investigated how *P. aeruginosa* adapts to *S. aureus* by evolving *P. aeruginosa* in the presence and absence of *S. aureus* across 150 generations.

Intriguingly, only within co-cultures the authors observed a selection for *P. aeruginosa* lipopolysaccharide (LPS) mutants, which conferred a fitness advantage and an increased resistance to β-lactam antibiotics.

P. aeruginosa and *S. aureus* exhibit a range of interactions; from competitive to coexistence. The type of interaction between the two species is dependent on the genetic background and the environmental adaptation they undergo during infection. Camus *et al*. (Camus *et al.*, 2020) investigated the impact of *S. aureus* on the physiology of *P. aeruginosa* within a coexistence context. Transcriptomic analyses revealed that *S. aureus* downregulated *P. aeruginosa* carbon and amino acid metabolism genes and upregulated genes involved in the acetoin catabolism (*aco*) pathway. This is interesting because clinical strains of *S. aureus* produce acetoin *in vitro* and in the CF sputum, which can further be catabolised by *P. aeruginosa* and therefore promote persistence of both pathogens. The competitive pairs of *P. aeruginosa* and *S. aureus* revealed that there was a downregulation of iron metabolism genes, which is in agreement with the study carried out by Mashburn *et al*. (Mashburn *et al.*, 2005).

These studies highlight the complexity of *Pa-Sa* interactions between the different strains. It is clear that different strains of *P. aeruginosa* and *S. aureus* interact differently to one another based off many genetic factors. This eventually leads to the current controversy where one view point is that *P. aeruginosa* outcompetes *S. aureus* and the other view point where both *P. aeruginosa* and *S. aureus* are able to coexist together. Neither of these viewpoints are wrong, it is indeed true that some *P. aeruginosa* and *S. aureus* strains fall under both categories. The genetic background of each strain is the basis for the controversy however, there are also other factors such as the environment that influences the interactions between *P. aeruginosa* and *S. aureus*.

Host environment, localisation and motility during *P. aeruginosa-S. aureus* coinfections

Molecular interactions between bacteria drive the spatial organisation within polymicrobial biofilms and these interactions result in mixed (co-localisation) or segregated patterns (Figure 1). The spatial organisation can alter the progression of infection within the host, which depends on the cooperative or competitive interaction between species. This can further remodel the biofilm leading to changes in biofilm structure, differentiation and interspecies interactions. The spatial organisation and remodelling of the biofilm changes based on environmental conditions such as oxygen gradient, pH and upon treatment with drugs such as antibiotics (Stacy *et al.*, 2016). These changes to the environment alter how bacteria may interact with one another. This is highlighted

by Trizna *et al*. (Trizna *et al.*, 2020) where polymicrobial biofilms of *P. aeruginosa* and *S. aureus* without treatment revealed that *P. aeruginosa* dominated the lower layers within the biofilm whereas *S. aureus* was found within microcolonies in the upper layers. Upon treatment with vancomycin however, the spatial organisation of the biofilm changed and *S. aureus* was found within the middle-lower layers. This is interesting and suggests that although initially the spatial organisation appeared to be segregated, the pattern may change due to co-localisation upon encountering stress such as antibiotic treatment or the immune system.

Cigana *et al*. (Cigana *et al.*, 2018) utilised histopathology and IF staining and demonstrated that *P. aeruginosa* and *S. aureus* localised in different areas of the lung. This is very similar to what is observed in wound infections when *P. aeruginosa* and *S. aureus* are localised in different areas of the wound bed (Serra *et al.*, 2015, Kirketerp-Moller *et al.*, 2008, Fazli *et al.*, 2009). Primarily, *S. aureus* localised around the surface of the wound and *P. aeruginosa* was generally found in the deeper regions of the wound (Fazli *et al.*, 2009). These findings correlate with Cendra *et al*. (Cendra *et al.*, 2019) where different culture media, additives and environmental conditions were used to support the co-existence of *P. aeruginosa* and *S. aureus in vitro* using static and continuous flow biofilm models. Interestingly, the authors observed oxygen stratification when using a continuous flow biofilm with a hypoxia probe dye, which indicated that *P. aeruginosa* was found within the deepest layers of the biofilm, in contrast to *S. aureus* which was found closer to the biofilm surface. This contradicts static growth conditions in the laboratory where *P. aeruginosa* initially (usually within the first 24 h) forms biofilms at the air-liquid interface (pellicles) and *S. aureus* settles at the bottom (Figure 1).

In another study, Pernet *et al*. (Pernet *et al.*, 2014) used CF bronchial epithelial cells to demonstrate that *P. aeruginosa* eradicates *S. aureus* through the manipulation of the host environment. *P. aeruginosa* secreted the toxin ExoS into epithelial cells, which stimulated the production of the phospholipase sPLA2-IIA, an enzyme that kills *S. aureus*. This is a very interesting as it provides a mechanism behind *P. aeruginosa* taking over from *S. aureus* to become the dominant species in CF patients as they grow older. Although this is not always the case, it begs the question if *P. aeruginosa* or *S. aureus* have other interactions with the host that may facilitate their existence or coexistence, localisation and motility.

Bacterial motility is important for rapid adaptation to different environmental conditions, surface adhesion, and to escape from mature biofilms (Khan *et al.*, 2020). Pallet *et al*. (Pallett *et al.*, 2019) reported an interesting observation, where the cell-free supernatant of *S. aureus* culture

induced *P. aeruginosa* swarming and swimming motility under anaerobic conditions in an isolate-dependent manner. This would suggest that *S. aureus* influences *P. aeruginosa* behaviour and has an impact on dissemination during infection, which could influence disease due to *P. aeruginosa* colonisation at other body sites. They also found that under anaerobic conditions, *P. aeruginosa* was not able to outcompete *S. aureus*. This finding is especially relevant as the availability of oxygen in the cystic fibrosis lung is limited due to excessive mucus production and oxygen consumption by host immune cells (Bhagirath *et al.*, 2016, Pallett *et al.*, 2019). Low oxygen conditions attenuate *P. aeruginosa* virulence, which would strongly support the co-existence of *P. aeruginosa* and *S. aureus* in complex infections (Schaible *et al.*, 2017). But how does this relate to the general conception of worse treatment outcomes for CF patients infected with multiple pathogens? This concept has been challenged in a recent study by Briaud *et al*. (Briaud *et al.*, 2020a) where the authors investigated the coexistence of *P. aeruginosa* and *S. aureus* in 52 CF patients. While they identified that 65% of *P. aeruginosa* and *S. aureus* in chronically co-colonised patients predominantly coexisted, there was no difference in clinical outcomes compared to *P. aeruginosa* only infections. Niggli *et al*. (Niggli & Kümmerli, 2020) recently described that ecological factors, the starting frequency of the population, and the genetic background of *S. aureus* influences the coexistence of *P. aeruginosa* with *S. aureus*. Their work emphasises that the infectious environment plays a major role in the coexistence of *P. aeruginosa* and *S. aureus* and therefore also the disease progress. The complexity of the host environment, genetics of the infecting strains, and patient-to-patient variability could explain why not all patients experience worse disease outcomes.

Armbruster *et al*. (Armbruster *et al.*, 2016) utilised *S. aureus* supernatant and discovered that Staphylococcal protein A (SpA), a protein known for its interaction with host targets such as glycoproteins and immunoglobulins, bound to both *P. aeruginosa* exopolysaccharide Psl and the PilA protein component of the type IV pili. *P. aeruginosa* strains that did not produce Psl, such as the commonly used strain PA14, exhibited inhibition of biofilm formation upon SpA binding to the type IV pili. *P. aeruginosa* strains that produced Psl, such as PAO1 did not exhibit inhibition of biofilm formation, instead SpA bound to Psl. This is interesting since the genetic background of each strain could influence disease progression. A further investigation by the authors revealed that the interaction between SpA and Psl protected *P. aeruginosa* from phagocytosis by neutrophils. This is beneficial to *P. aeruginosa* and allows for both *Pa-Sa* to avoid killing by neutrophils within the host when SpA is attached to the *P. aeruginosa* cell surface.

Limoli *et al*. (Limoli *et al.*, 2019) used live time-lapse phase contrast microscopy to show that *P. aeruginosa* transitioned from group motility to single cell exploratory motility in the presence of *S. aureus*. The mechanism towards the change in motility was further associated to the *S. aureus* accessory gene regulator AgrB, which was responsible for the secretion of various factors that could increase both type IV pili and flagella mediated motility in *P. aeruginosa* (Limoli *et al.*, 2019). Although these results may shed some insights into *Pseudomonas-Staphylococcus* co-infection, the actual compounds remain to be identified and may not be of much relevance in the host environment due to molecule diffusion and low concentration inside the body. Despite these papers uncovering key mechanisms behind *Pa-Sa* coinfection and interaction, the majority of research only focuses on the effect of *P. aeruginosa* supernatant on *S. aureus* but not vice versa. It is also interesting because it would indicate an unknown mechanism that allows *P. aeruginosa* to switch from a chronic (non-motile) state to an acute, infectious mode after co-infection with *S. aureus*.

Traditionally, *S. aureus* has been known to be non-motile, although there has been some evidence of certain motility behaviour for some strains (Pollitt *et al.*, 2015, Pollitt & Diggle, 2017). Nevertheless, the non-motile phenotype may bring some disadvantages during infection and colonisation. So how does *S. aureus* cause deadly diseases or disseminate when present during co-infection? Samad *et al*. (Samad *et al.*, 2017) shed some light into staphylococcal dispersal with their SEM micrograph studies, where they visualised the co-localisation of *P. aeruginosa* and S. *aureus*. They observed that S. *aureus* had the ability to 'hitchhike' with swimming *P. aeruginosa* cells up to the air-liquid interface (Figure 1) on a vertical insert within an *in vitro* biofilm assay. Using fluorescently labelled *P. aeruginosa* and *S. aureus* in a microchamber combined with live confocal microscopy they found that *S. aureus* was associated with *P. aeruginosa* cells and that they moved together. *P. aeruginosa* can associate with various molecules presumable through chemical interactions, however, the exact mechanisms are still unknown, but one might speculate that it is due staphylococcal interaction with the flagellum of motile bacteria. This is in agreement with Dean *et al*. (Dean *et al.*, 2015), which utilised laser ablation electrospray ionisation mass spectrometry and confocal laser scanning microscopy to show that *P. aeruginosa* and *S. aureus* were found co-localised together within an *in vitro* polymicrobial biofilm.

The localisation of *P. aeruginosa* and *S. aureus* is still controversial with many studies that observe localisation together or clear segregation between the two species. Since the localisation of *P. aeruginosa* and *S. aureus* is dependent on the host environment, disease progression, and the

selective pressures that arise during an infection, we emphasize to consider the final spatial organisation when replicating infection models and potential treatment strategies in the laboratory.

Mimicking polymicrobial interactions *in vitro* and *in vivo*

The host environment plays a crucial role during infection and it remains challenging to accurately mimic the human host in the laboratory or small animals. Nevertheless, several strategies have been undertaken to replicate infectious conditions and we will highlight the most recent ones for *Pa-Sa* co-infections.

One important factor to consider when mimicking *in vivo* conditions *in vitro* is the use of tissue culture medium. However, the optimum growth conditions that are relevant for growth and biofilm formation of all involved bacteria can be challenging. Nutrient rich broth might cause overgrowth or prevent certain bacteria, such as *P. aeruginosa,* to form biofilms, while nutrient-deficient broth might limit the potential of certain other bacteria to attach and/or form biofilms at all. The influence of tissue culture medium on the competitive interaction between *S. aureus* and *P. aeruginosa* in biofilms, was assessed by Alves *et al.* (Alves *et al.*, 2018). To quantify the competitive interaction, they measured bacterial aggregates and the presence of keratinocytes. In contrast to Cigana *et al*. (Cigana *et al.*, 2018), Alves *et al.* found that *S. aureus* dominated in biofilms and non-attached bacterial aggregates. Moreover, in Dulbecco's Modified Eagle Medium (DMEM) *S. aureus* increased adhesion of *P. aeruginosa* to keratinocytes, and co-infection (1:1 ratio) impaired the proinflammatory response and delayed wound healing (Alves *et al.*, 2018). Smith *et al*. (Smith *et al.*, 2017) investigated what factors and mechanisms are involved in the co-existence of *S. aureus* with *P. aeruginosa* within the adapted Lubbock chronic wound medium, and identified that albumin prevented the killing of *S. aureus* by *P. aeruginosa*. Mechanistically, they used isothermal titration calorimetry and found that various QS molecules, including homoserine lactone's (HSL), had an affinity to bind to and sequester albumin.

Although there have been several studies that used co-cultures of *P. aeruginosa* and *S. aureus*, there are still plenty of contrasting findings. This highlights the complexity of polymicrobial infections caused by these two bacteria and the difficulty of mimicking them *in vitro* and *in vivo*. Further studies are required to find optimum conditions to accurately mimic infectious diseases in the laboratory. While there is clearly no golden path to reliably reproduce polymicrobial infections in different strains or environments, we think it is important to use optimized growth media (i.e., not nutrient rich broth) with or without host cells to create host-mimicking conditions. In this context, Cornforth *et al.* (Cornforth *et al.*, 2020) recently provided a quantitative framework,

based on gene expression data, evaluating *in vitro* and *in vivo* conditions that could aid scientists in selecting the appropriate infection model to use in their laboratory. Although they used *P. aeruginosa* and CF infection as a proof-of-principle of their framework, their approach is promising and may be extended to the functionality of polymicrobial communities. The use of transcriptome data from *in vivo* infection models and compare them to *in vitro* models to predict or test adequate host mimicking conditions will help to obtain more consistent data to help identify overlapping mechanisms to successfully treat complex infections.

Mimicking the CF lung *in vitro* and *in vivo*

To mimic a phenotype similar to the CF lung, where *S. aureus* is often the dominant species during adolescence before *P. aeruginosa* colonisation occurs (Hubert *et al.*, 2013, Davies, 2002), Woods *et al*. (Woods *et al.*, 2018) developed an *in vitro* model where *S. aureus* biofilms are established on polystyrene wells and silicone tube reactors for five days, before inoculation with *P. aeruginosa* (Table 1). Using a 250:1 starting ratio of *S. aureus* to *P. aeruginosa*, they demonstrated that *S. aureus* was not outcompeted as suggested by several other studies (Mashburn *et al.*, 2005, Filkins *et al.*, 2015). Millette *et al.* (Millette *et al.*, 2019) further developed an *in vivo* mouse lung co-infection model showing that *P. aeruginosa* promoted colonisation of *S. aureus* in the lung, even with *P. aeruginosa* strains that affected *S. aureus* viability *in vitro*. *P. aeruginosa* induced the expression of the cell surface receptor ICAM-1 (intercellular adhesion molecule 1; CD54) and fibronectin and fibrinogen receptor ITGA-5 (integrin alpha 5), which have previously been shown to be important surface receptors for *S. aureus* colonisation (Sinha *et al.*, 1999). This emphasises that *in vitro* results may not reflect *in vivo* conditions without the presence of host cells or host-mimicking conditions. This is also one of the few studies that showed that *S. aureus* benefited from the presence of *P. aeruginosa* during lung colonization.

Cigana *et al*. (Cigana *et al.*, 2018) adopted a novel approach to mimic the sequence of events of pulmonary disease in CF patients. Utilising an *in vivo* mouse model with agar-beads embedded with bacteria, the authors established a *S. aureus* culture prior to the addition of *P. aeruginosa* at a 1:1 ratio. Intriguingly, they found that the early presence of *S. aureus* increased the capacity of *P. aeruginosa* to establish a chronic infection. *P. aeruginosa* yielded a higher recruitment of immune cells at both bronchial and parenchymal levels, stimulating a much stronger host inflammatory response in comparison to *S. aureus* (Cigana *et al.*, 2018). These findings support the long-lasting pathological outcomes of *P. aeruginosa* infection. Although immune cells are vital for the clearance of pathogens from the site of infection (Chaplin, 2010), strong inflammatory responses

triggered by *P. aeruginosa* are associated with impaired bacterial clearance. Furthermore, enhanced proinflammatory cytokines have been correlated with immune-mediated destruction of parenchyma (Lin & Kazmierczak, 2017). In this regard, Bernardy *et al*. (Bernardy *et al.*, 2020) hypothesised that *S. aureus* isolated from the CF lung would have lost many of its virulence phenotypes in order to establish chronicity in the CF lung. The authors undertook sequencing of 64 clinical *S. aureus* isolates, allowing them to identify various virulence factors and antimicrobial resistance genotypes. They found that most isolates were in fact still portraying virulence abilities such as the production of polysaccharides and haemolysis but varied in their interaction with *P. aeruginosa*. The authors utilised a co-culture assay to assess the interactions between the two strains directly, investigating whether *S. aureus* isolates could survive in the presence of mucoid and non-mucoid *P. aeruginosa* strains. Intriguingly, they found three distinct *S. aureus* phenotypic groups, based on whether they were killed by non-mucoid PAO1 only, killed by non-mucoid and mucoid PAO1, and killed by mucoid PAO1 only. These findings are of interest as previous work has shown that non-mucoid PAO1 kills *S. aureus* isolate JE2, while mucoid PAO1 did not (Limoli *et al.*, 2017). Since isolates were killed by mucoid PAO1, this further provides new understanding and depth to how we believe *S. aureus* and *P. aeruginosa* interact. While this is an exciting development, naturally the study comes with limitations such as that a simple co-culture assay with the two bacterial strains cannot accurately represent the CF lung.

Cornforth *et al.* (Cornforth *et al.*, 2020) used RNA-seq and quantitatively analysed several *in vitro* CF lung models, including the synthetic CF sputum medium (SCFM2) and the CF airway epithelial model. These two models displayed the highest genome-wide accuracy, although individual models captured different aspects of infection physiology. The SCFM2 had limitations in functional categories including porins and polyamine biosynthesis whereas the airway epithelial model lacked in protein synthesis. Intriguingly, this study found that a combination of SCFM2 and the acute pneumonia mouse model outperformed all other model combinations and improved the accuracy of mimicking the CF lung. Although this study only focused on *P. aeruginosa* within the CF lung, we believe that this framework could be optimized to investigate the implications of *Pa-Sa* interaction within host mimicking conditions.

Mimicking chronic wounds *in vitro* and *in vivo*

The wound environment has a pH 7.15-8.9 (Kumar & Honnegowda, 2015), very different from the CF lung which is rather acidic in nature (Gallagher *et al.*, 2019). Thus, the environmental differences might already suggest the development of different polymicrobial interactions. The

Lubbock Chronic Wound Biofilm (LCWB) model is an *in vitro* model that mimics the structural, behavioural and functional properties of a chronic wound (Sun *et al.*, 2008). The LCWB model is made up of laked horse red blood cells, plasma, and a chopped meat-based medium (Bolton broth). The chopped meat based medium mimics the nutrients found within the wound bed, and the laked horse red blood cells mimic the degraded and damaged host tissues, while bovine plasma mimics plasma found within a chronic wound infection. The LCWB is an efficient model to assess new antimicrobial and antibiofilm compounds and can easily be adapted/modified to suit specific chronic wound conditions within the host (DeLeon *et al.*, 2014), which makes it an appealing model to implement in the laboratory.

There are a few *in vivo* models that mimic wound infections. The model described by Dalton *et al.* (Dalton *et al.*, 2011a) uses a surgical full thickness wound model where a 1.5 x 1.5 cm patch of skin from the back of a mouse is removed to create a wound. This allows for the topical application of bacteria or the transplantation of a preformed polymicrobial biofilm on top of the wound before it is covered with a semipermeable polyurethane dressing. Although this chronic wound model is commonly used, the transplantation of a preformed biofilm onto the wound bypasses the progression and development of the infection within a host. Moreover, the polymicrobial interaction strongly depends on the *in vitro* culturing methods including bacterial numbers and environmental conditions. The polymicrobial *in vivo* model by Chaney et al. (Chaney *et al.*, 2017) establishes full thickness burn wounds in a porcine model via electrical heating and topically inoculated 10^8 CFU of *P. aeruginosa* and *S. aureus* onto the wounds three days post-burn. Promisingly, five weeks post infection, the co-infected wounds recapitulated chronic wounds similar as observed in humans. The presence of both bacteria resulted in larger wound sizes with increased influx of neutrophils and wound healing impairment.

We recently developed a chronic subcutaneous abscess infection model that involves the injection of a high bacterial density ($>10^7$) without the necessity to establish a wound first (Pletzer *et al.*, 2017, Pletzer *et al.*, 2018). Our data on polymicrobial infection with similar ratios of *P. aeruginosa* LESB58 and *S. aureus* LAC USA300 showed the establishment of a chronic infection where both bacteria co-existed for at least three days (unpublished data). Although we also need to culture the bacteria and inject at a high density to establish an abscess, our model has the advantage that bacteria need to adapt to the host environment as well as to the other bacterial species *in vivo*.

It remains difficult to reproduce the infectious environment, but with defined host-mimicking conditions and the availability of reproducible animal models, we are closer to further understand complex diseases. While animal models have limitations and strengths in different areas, they are crucial to further link the gap of understanding polymicrobial infections in humans and the laboratory.

Understanding antibiotic tolerance in polymicrobial infections

The polymicrobial biofilm environment consists of bacterial compounds and exoproducts that can assist with bacterial aggregation and reduce the ability of antibiotics to reach bacteria (Beaudoin *et al.*, 2017). Antibiotic tolerance encompasses the ability of bacteria to survive the potentially lethal actions of bactericidal antibiotics at high doses (Windels *et al.*, 2019). Understanding how *P. aeruginosa* and *S. aureus* interact within a polymicrobial infection may lead to more effective methods to combat antibiotic tolerance.

DeLeon *et al.* (DeLeon *et al.*, 2014) modified the LCWB model to further mimic 'host-like' conditions by adapting a host-derived matrix as the scaffold for bacteria to adhere to. The authors showed that the antibiotic tolerance of *S. aureus* increased by more than 30% against gentamicin and tetracycline when grown planktonically in co-culture with *P. aeruginosa and* were further enhanced when grown together in the wound model. The enhanced tolerance to gentamicin was attributed to both the host-derived matrix components such as carbohydrates and proteins, and bacterial extracellular polymeric substances (EPS) such as *P. aeruginosa* alginate. This highlights once again that *P. aeruginosa* and *S. aureus* may benefit from each other during co-infection. However, the host-derived matrix components could not be linked to tetracycline tolerance. A study performed by Briaud *et al*. (Briaud *et al.*, 2019) discovered a mechanism behind the tetracycline tolerance/resistance observed by DeLeon *et al.* (DeLeon *et al.*, 2014). A transcriptomic approach was used to analyse how the co-cultivation with non-competitive *P. aeruginosa* altered *S. aureus* gene expression. They found that several genes that encoded transporters were overexpressed in *S. aureus,* the most significant upregulated transporter was *tet38* which is involved in resistance against tetracycline. In addition, this transporter also interacts with the CD36 receptor on epithelial cells, increasing *S. aureus* internalization (Truong-Bolduc *et al.*, 2017). A comparison of the internalisation rate into A549 epithelial cells of *S. aureus* alone and when in coculture with *P. aeruginosa* revealed a three-fold increase of *S. aureus* uptake in the presence of *P. aeruginosa* (Briaud *et al.*, 2019). We see that not only does *P. aeruginosa* help *S.*

aureus to become tolerant to antibiotics it also helps *S. aureus* to hide in epithelial cells and escape the attack from the immune system or *P. aeruginosa* itself.

Other investigations often use cell free supernatants to identify if secreted products of a bacterium have an effect on the growth of other species. A study by Orazi *et al*. (Orazi & O'Toole, 2017) utilised *P. aeruginosa* supernatant and identified that secreted HQNO and the siderophores pyoverdine and pyochelin protected *S. aureus* from several antibiotics across multiple strains. The authors proposed that the inhibition of the electron transport chain, mediated by HQNO and siderophores, resulted in a disruption of the electrochemical gradient. Consequently, this disruption prevented the entry of protein synthesis inhibitor antibiotics such as tetracyclines. In addition to the aforementioned upregulation of *tet38* in *S. aureus*, this contributes to a phenotype of *S. aureus* that is highly resistant to certain classes of antibiotics during coinfection. This study also revealed a potential scenario where *P. aeruginosa* shifts *S. aureus* to fermentative growth, which slowed down cell proliferation, and therefore decreased the susceptibility to cell wall targeting antibiotics (Orazi & O'Toole, 2017, Orazi *et al.*, 2019). Radlinski *et al*. (Radlinski *et al.*, 2017) provided further evidence that HQNO reduced *S. aureus* intracellular ATP, which contributed to antibiotic resistance via induction of persister cells. Orazi *et al.* (Orazi *et al.*, 2019) also showed that HQNO produced by *P. aeruginosa* was responsible for increased sensitivity of *S. aureus* biofilms against multiple antimicrobial compounds. HQNO increased *S. aureus* membrane fluidity in biofilms to membrane-permeabilizing antimicrobials either due to direct membrane interaction or due to changes in gene expression of fatty acid synthesis. While this still remains to be answered, these studies highlight the complexity of co-infections, such as that HQNO induced antibiotic tolerance, but also increased antimicrobial susceptibility in biofilms.

Recent studies also suggested that *S. aureus* is capable of enhancing antibiotic resistance in *P. aeruginosa*. Building on the findings from Armbruster *et al.* (Armbruster *et al.*, 2016) on the interaction between *S. aureus* SpA and *P. aeruginosa* Psl, Beaudoin *et al.* (Beaudoin *et al.*, 2017) have demonstrated that Staphylococcal protein A (SpA) bound and aggregated with the *P. aeruginosa* exopolysaccharide Psl (Figure 1). This consequently increased tobramycin resistance in *P. aeruginosa*. From a clinical point of view this would suggest that tobramycin treatment in CF patients, which are potentially co-colonized with *S. aureus*, might be ineffective. If that is the case, tobramycin should not be frequently used to treat pulmonary *P. aeruginosa* infections in the clinic (Hamed & Debonnett, 2017). However, on the other hand, tobramycin might be effective to eradicate *S. aureus* from co-infections. Radlinski *et al.* (Radlinski *et al.*, 2017) showed that the

production and secretion of surfactant molecules such as rhamnolipids increased the uptake of tobramycin and efficacy against *S. aureus* persister cells that are depleted of PMF. A *P. aeruginosa* PA14 rhamnolipid synthesis mutant conferred full protection of *S. aureus* against killing by tobramycin. Tobramycin uptake requires the PMF, however, rhamnolipids disrupt and damage the cell membrane of *S. aureus* (Bharali *et al.*, 2013) providing a PMF-independent method of tobramycin uptake. Although these findings would suggest a co-administration of tobramycin with rhamnolipids to eradicate persistent *S. aureus* cells, it could potentially lead to poorer patient outcomes due to increased virulence of *P. aeruginosa*. Hence, it is worth to propose that effective treatment of co-infections would require a course of multiple different antibiotics and future studies should investigate antibiotic resistance development after combinatorial drug treatment (in multiple pathogens).

All together, these studies highlight the complexity of treating polymicrobial infections. None of these studies have considered the complex host environment, which most likely will further reveal novel insights and potentially contradicting results. It will be of utmost importance to study these observations in animals to further confirm these results. One thing becomes very clear from these investigations, and this is that either organisms can benefit from the other and move into a more tolerant state. The increase of antibiotic tolerance due to interspecies interactions highlights the necessity of finding a new treatment strategy to fight complex polymicrobial infections.

Recently Belanger *et al*. (Belanger *et al.*, 2020) have shown that physiologically relevant conditions increased the susceptibility of *P. aeruginosa* to the macrolide antibiotic azithromycin (AZM) and enhanced synergy between AZM and host defense peptides DJK-5 and IDR-1018 *in vitro* and *in vivo*. They identified that transcriptional changes in gene expression in *P. aeruginosa* within their host-like conditions of RPMI/serum had strong overlap with *P. aeruginosa* isolated directly from cystic fibrosis patients. This study provides evidence that indeed the host environment impacts the assessment of novel treatment strategies. They highlight the relevance of tissue culture media supplemented with serum that mimic conditions of wound exudated or blood. In order to better mirror and mimic the host environment, we propose that such *in vitro* conditions should be optimized and used to study polymicrobial infections and antimicrobial susceptibility.

Conclusion and Future Perspective

The relationship between *P. aeruginosa* and *S. aureus* is complex, and their role in polymicrobial infections is still not fully understood. Understanding the interaction between *P. aeruginosa* and *S. aureus* in polymicrobial infections will provide further insights on how to treat them efficiently

and effectively. Recent work has begun to discern the roles and interactions between *P. aeruginosa* and *S. aureus* within polymicrobial biofilms and infections. Within only a few years, there have been many new *in vitro* and a few *in vivo* models published that explore *Pa-Sa* co-infections. More robust and reproducible models will help to investigate and generate new targets to inhibit bacteria in a polymicrobial environment. We strongly encourage researchers to optimize and implement polymicrobial infection models in their studies and treat *P. aeruginosa* and *S. aureus* simultaneously.

Conflict of Interest Statement

The authors declare no conflict of interest.

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Figure legends

Figure 1. *P. aeruginosa* and *S. aureus* interactions in a microwell plate. A) *P. aeruginosa* forms a biofilm (pellicle) at the air-liquid interface, while *S. aureus* settles at the bottom. B) *S. aureus* assisting *P. aeruginosa* attachment (Alves *et al.*, 2018). C) *P. aeruginosa* and *S. aureus* localise together (Dean *et al.*, 2015, Samad *et al.*, 2017) with some of the molecular interactions between the products *P. aeruginosa* and *S. aureus* highlighted: 1) Staphylococcal protein A (SpA) binding to *P. aeruginosa* exopolysaccharide Psl forming an aggregation (Beaudoin *et al.*, 2017, Armbruster *et al.*, 2010). 2) SpA binding to *P. aeruginosa* type IV pili. 3) *P. aeruginosa* HQNO and siderophores inhibiting *S. aureus* electron transport chain (Hoffman *et al.*, 2006). 4) Overproduction of alginate by *P. aeruginosa* (Limoli *et al.*, 2017). D) *S. aureus* hitchhiking with *P. aeruginosa* (Samad *et al.*, 2017) and formation of an air-liquid interface polymicrobial biofilm.

Tables

Table 1. Overview of *P. aeruginosa* (*Pa*) and *S. aureus* (*Sa*) co-culture experiments.

Model type	*Pa:Sa* ratio	Quantification method	Summary	Reference
In vitro growth kinetics *In vivo* mouse model of acute pneumonia	1:1	CV[1], CFU[2]	*S. aureus* was outcompeted *in vitro* and *in vivo* during co-infection with a *P. aeruginosa* reference strain and strains isolated early at the onset of chronic infection. Pathoadaptive strains of *P. aeruginosa* isolated several years after the onset of chronic infection had a reduced capacity to outcompete *S. aureus*.	(Baldan *et al.*, 2014)
In vitro adapted Lubbock chronic wound biofilm model *In vivo* mouse wound model	1:1	CFU	Increased survival of planktonic *P. aeruginosa* and *S. aureus* when grown together in co-culture. Enhanced antibiotic tolerance when grown together within the wound model attributed to both host-derived and bacteria-derived matrix component.	(DeLeon *et al.*, 2014)
In vitro cell culture human bronchial cell line IB3-1 using primary	1:1	CFU	Secretion of *P. aeruginosa* ExoS into CF bronchial epithelial cells stimulated production of sPLA2-IIA that kills *S. aureus.*	(Pernet *et al.*, 2014)

bronchial epithelial cells collected from CF and non-CF patients *In vivo* transgenic mice overexpressing sPLA2-IIA *In vivo* guinea pigs model				
Laser ablation electrospray ionisation mass spectrometry	1:1000	Confocal laser scanning microscopy	Co-localisation observed within mixed biofilms	(Dean *et al.*, 2015)
In vitro co-culture system on monolayers of human bronchial epithelial cells homozygous for ΔF508 cystic fibrosis transmembrane conductance regulator mutation	1:1	CFU	*P. aeruginosa* and *S. aureus* coexist initially, however, extended co-culture led to a reduction of *S. aureus* viability. *S. aureus* reduction is dependent on HQNO and siderophore production in extended co-culture.	(Filkins *et al.*, 2015)

In vitro polyvinylchloride tubing	1:1	CV, CFU	*P. aeruginosa* outcompeted *S. aureus*	(Ammann *et al.*, 2016)
In vitro biofilm inhibition assay	*S. aureus* supernatant	CV	*S. aureus* SpA inhibited specific *P. aeruginosa* clinical isolate biofilm formation, and inhibited phagocytosis by neutrophils. Interactions were mediated by binding to *P. aeruginosa* Psl and type IV pili.	(Armbruster *et al.*, 2016)
In vitro biofilm slide chamber model	*S. aureus* supernatant[5]	CV	Different *P. aeruginosa* biofilm architecture is formed in the presence of *S. aureus* exoproducts. Binding of *P. aeruginosa* Psl to *S. aureus* SpA caused aggregation and increased tobramycin resistance in *P. aeruginosa* isolated from children with CF that failed eradication therapy.	(Beaudoin *et al.*, 2017)
In vitro scratch wound closure assays *In vivo* mouse wound healing	1:99 1:1000	CV, CFU	DRGN-1 healed polymicrobial *Pa-Sa* murine wounds faster than untreated and wounds treated with other peptides	(Chung *et al.*, 2017)
In vivo murine chronical surgical wound infection	1:10		*P. aeruginosa* converted ~25% of *in vivo* mono-culture essential *S. aureus* genes to non-essential. 182 *S. aureus*	(Ibberson *et al.*, 2017)

			genes essential in co-infection.	
In vitro coculture model	*P. aeruginosa* supernatant[6]	CFU	*P. aeruginosa* supernatant decreased *S. aureus* susceptibility to vancomycin.	(Orazi & O'Toole, 2017)
In vitro antibiotic survival assays *In vivo* burn wound model	*P. aeruginosa* supernatant[7] 1:100	CFU	Production of *P. aeruginosa* LasA endopeptidase, rhamnolipids and HQNO varies between clinical isolates each can alter *S. aureus* susceptibility to antibiotics. LasA producing *P. aeruginosa* populations potentiated vancomycin treatment of *S. aureus* within the mouse burn.	(Radlinski *et al.*, 2017)
In vitro biofilm assay with vertical insert	1:1, 1:100	CFU	*S. aureus* gained motility by hitchhiking onto *P. aeruginosa.*	(Samad *et al.*, 2017)
In vitro adapted Lubbock chronic wound biofilm model	1:1	CFU	Physiological levels of serum albumin inhibited *P. aeruginosa* quorum sensing, reducing production of virulence factors that kill *S. aureus*.	(Smith *et al.*, 2017)
In vitro coculture and competition assays	1:1	CFU	Overproduction of alginate by mucoid *P. aeruginosa* strains reduced anti-*S. aureus* activity.	(Limoli *et al.*, 2017)

In vitro co-evolution assays *In vitro* killing assays and fitness assays	1:100-1:1000, *P. aeruginosa* supernatant[10]	Whole genome sequencing, CFU	*P. aeruginosa* coevolution with *S. aureus* leads to mutations in *P. aeruginosa* LPS.	(Tognon *et al.*, 2017)
In vitro immortalised human keratinocyte infection model	1:1	CV, CFU	*S. aureus* predominated within biofilm, non-bacterial aggregates and promoted *P. aeruginosa* attachment to human keratinocytes.	(Alves *et al.*, 2018)
In vivo mouse with agar embedded bacterial beads[3]	1:1	CFU	*S. aureus* promoted abscess formation and subsequent chronic *P. aeruginosa* infection. During chronic infection *P. aeruginosa* influenced inflammatory responses independent of *S. aureus*.	(Cigana *et al.*, 2018)
In vivo *Drosophila melanogaster* model based on systemic infection	3:25	CFU	*S. aureus* proliferation compromised during co-infection with *P. aeruginosa.*	(Lee *et al.*, 2018)
In vitro polystyrene well and silicone tube	1:250	CFU	Timing of inoculation and bacterial concentration is important, *S. aureus* was not outcompeted.	(Woods *et al.*, 2018)

reactor biofilm model[4]				
In vitro coevolution assay *In vitro* short period competition assay	1:1 1:9, 9:1	CFU	*P. aeruginosa* quorum sensing regulation decreased during evolution and formed the basis of interspecific coexistence with *S. aureus.*	(Zhao *et al.*, 2018)
In vitro biofilm	1:1	Confocal laser scanning microscopy, Atomic force microscopy	Peptide Hss02 reduced bacterial cells within pre-formed *Pa-Sa* mixed biofilms of *Pa-Sa*.	(Bessa *et al.*, 2019)
In vitro competition assay Transcriptomic analyses	1:1	CFU mRNA fold change	*S. aureus* coexistence with *P. aeruginosa* affected *S. aureus* antibiotic resistance, internalisation into epithelial cells and altered the transcriptome.	(Briaud *et al.*, 2019)
In vitro static *In vitro* continuous flow	1:1	CFU	*S. aureus* survival is dependent on oxygen stratification within mixed biofilm.	(Cendra *et al.*, 2019)
In vitro time kill assays with a mechanism-based model	1:1	CFU	*P. aeruginosa* attenuated clindamycin activity against *S. aureus.*	(Lenhard *et al.*, 2019)
In vitro coverslip agarose pad	1:1, *S. aureus* superna	Contrast time-lapse microscopy	*P. aeruginosa* transitioned to single-cell motility (exploratory motility) from	(Limoli *et al.*, 2019)

In vitro macroscopic assays	tant[8]		collective upon sensing *S. aureus*.	
In vitro growth kinetics *In vivo* mouse lung infection model	1:1	CFU	*In vitro* not all *P. aeruginosa* strains affected viability of *S. aureus*. *P. aeruginosa* promoted *S. aureus* colonisation of lung tissues.	(Millette *et al.*, 2019)
In vitro coculture model	*P. aeruginosa* supernatant[6]	CFU	*P. aeruginosa* exoproducts enhanced various antimicrobial compounds to kill *S. aureus* within biofilms.	(Orazi *et al.*, 2019)
In vitro competition assays aerobic and anoxic *In vitro* mixed biofilm aerobic and anoxic	1:1	CFU	Anoxia reduced *P. aeruginosa* CF isolates to dominate over *S. aureus*. *S. aureus* exoproducts restored and enhanced *P. aeruginosa* motility under anoxia and normoxia (isolate dependent)	(Pallett *et al.*, 2019)
Transcriptional profiling RNA-seq *in vitro* co-culture competition	1:1	RNA-seq	*P. aeruginosa* and *S. aureus* competed for resources and adapted metabolically.	(Tognon *et al.*, 2019)
In vitro quantitative co-culture assay	1:1	CFU	Different genotypes and phenotypes of *S. aureus* CF isolates interacted differently with *P. aeruginosa.*	(Bernardy *et al.*, 2020)
In vitro	1:1, *S.*	CFU, RNA-seq,	Clinical strains of *S. aureus*	(Camus *et*

transcriptomic studies mono- and co-cultures of competitive and coexisting pairs of *P. aeruginosa* and *S. aureus* *In vitro* survival assays	*aureus* supernatant[9]	whole genome sequencing	produced acetoin. *P. aeruginosa* catabolised acetoin which improved survival of both species.	*al.*, 2020)
In vitro planktonic and biofilm susceptibility *In vitro* collagen diabetic foot ulcer 3D model	1:1	Microscopic analysis	Application of a combination of pexiganan and nisin within a biogel eradicated *S. aureus* from the model.	(Gomes *et al.*, 2020)
In vitro competition assays under different environmental conditions (shaking, static, viscous)	1:1, 9:1, 1:9	CFU, Flow cytometry	Ecological details have important effects on competitive dynamics during coinfection and determined coexistence or invasion.	(Niggli & Kümmerli, 2020)
In vitro planktonic and biofilm co-culture model	1:1	CFU	Exogenous alginate protected *S. aureus* from *P. aeruginosa* killing.	(Price *et al.*, 2020)

In vitro static 48-hour biofilm polystyrol plates and cell imaging cover slips *In vitro* antimicrobial assays and drop plate assays	1:1	CV, confocal light scanning microscopy, atomic force microscopy	*S. aureus* modulated susceptibility of *P. aeruginosa* to antibiotics in co-culture.	(Trizna *et al.*, 2020)
In vitro mutant analyses, genetic complementation	1:10	CFU	*P. aeruginosa* ClpXP protease suppressed *P. aeruginosa* anti-Staphylococcal activity. *S. aureus* induced decrease in ClpXP activity.	(Yang *et al.*, 2020)

[1] Crystal violet, [2] Colony forming units, [3] *P. aeruginosa* added seven days after *S. aureus*, [4] *P. aeruginosa* added five days after *S. aureus,* [5] 48-hour old culture, [6] overnight culture, [7] 20-hour old culture, [8] overnight culture, [9] 4-hour old culture.

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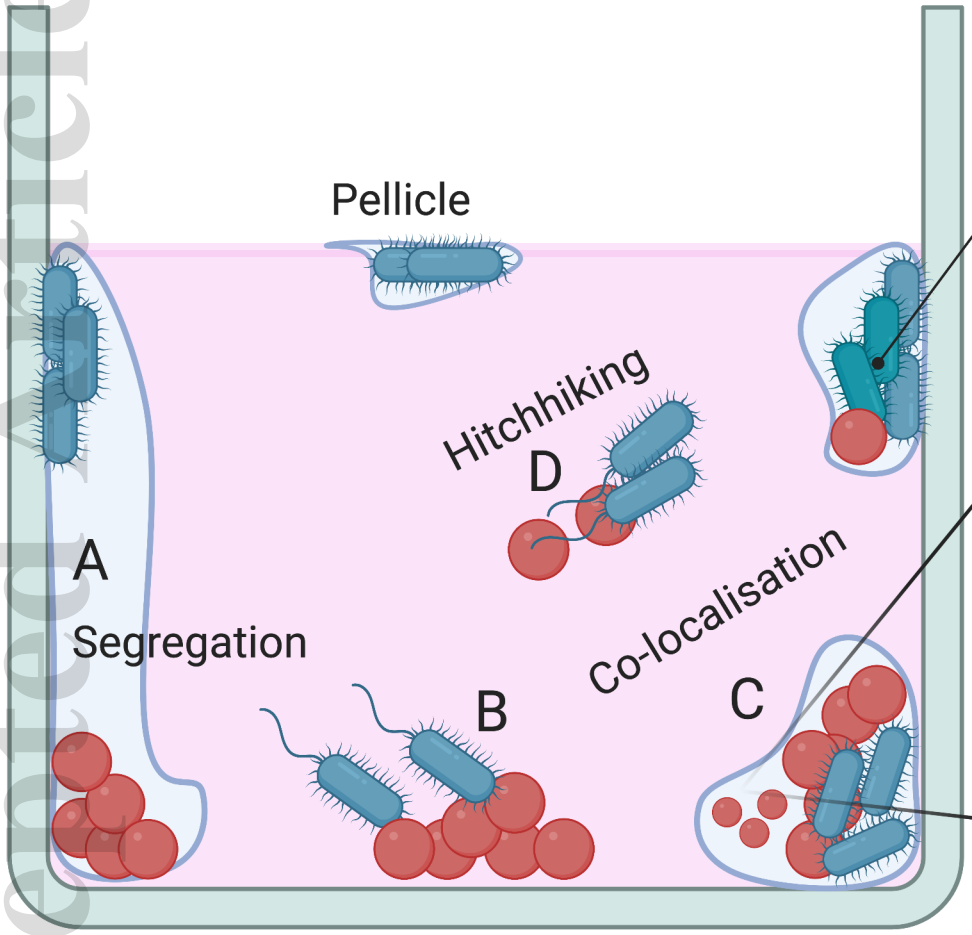
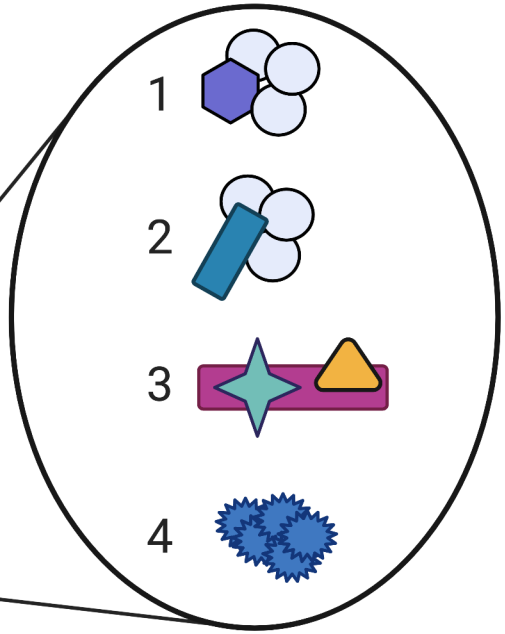


LPS mutants
Confer fitness advantage and resistance to β-lactam antibiotics



SpA & Psl
Bind together causing aggregation
Inhibit phagocytosis by neutrophils

SpA & type IV pili
Bind together inhibit biofilm formation

HQNO & siderophores
Inhibit *S. aureus* ETC inducing SCV formation

Alginate
Overproduction protects *S. aureus* from *P. aeruginosa* anti-staphylococcal activity

P. aeruginosa
P. aeruginosa LPS mutant
S. aureus
S. aureus small colony variants (SCV)
Matrix
P. aeruginosa exopolysaccharide Psl
S. aureus Staphylococcal protein A
P. aeruginosa type IV pili
P. aeruginosa HQNO
P. aeruginosa siderophores
S. aureus electron transport chain (ETC)
P. aeruginosa alginate overproduction