CHAPTER SIX

The past, present and future of polymicrobial infection research: Modelling, eavesdropping, terraforming and other stories

Éva Bernadett Bényei, Rahan Rudland Nazeer, Isabel Askenasy, Leonardo Mancini, Pok-Man Ho, Gordon A.C. Sivarajan, Jemima E.V. Swain, and Martin Welch*

Department of Biochemistry, Tennis Court Road, Cambridge, United Kingdom
*Corresponding author. e-mail address: mw240@cam.ac.uk

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Abstract

Over the last two centuries, great advances have been made in microbiology as a discipline. Much of this progress has come about as a consequence of studying the growth and physiology of individual microbial species in well-defined laboratory media; so-called "axenic growth". However, in the real world, microbes rarely live in such "splendid isolation" (to paraphrase Foster) and more often-than-not, share the niche with a plethora of co-habitants. The resulting interactions between species (and even between kingdoms) are only very poorly understood, both on a theoretical and experimental level. Nevertheless, the last few years have seen significant progress, and in this review, we assess the importance of polymicrobial infections, and show how improved experimental traction is advancing our understanding of these. A particular focus is on developments that are allowing us to capture the key features of polymicrobial infection scenarios, especially as those associated with the human airways (both healthy and diseased).

1. Introduction

Recent years have seen an increasing realisation that many chronic infections are associated with not just a single species (the "pathogen") but often include a plethora of additional species, that may, or may not contribute towards the pathology of the disease. Although Pasteur noted this in the nineteenth century, it has only been with the advent of culture-independent approaches in the twenty-first century that we have really been able to mine this "microbial dark matter" (Lloyd, Steen, Ladau, Yin, & Crosby, 2018; Pasteur & Joubert, 1877). These approaches have revealed just how small the tip of the iceberg actually is (Fig. 1), revealing that a welter of species, often across different kingdoms, often co-exist in infection scenarios. In the wake of such advances, our goal here is to present an overview of how these polyspecies-associated infections are now being studied *in vitro*, with a particular focus on infections of the skin and airway epithelia.

Microorganismal ecosystems seem to display all of the ecological diversity and dynamism of their macroorganismal counterparts; qualities that are often influenced as much by the environment in the infection niche as the co-habiting species themselves (Dixon & Hall, 2015). Indeed, although deterministic, interspecies interactions are seldom one-dimensional and it is increasingly clear that co-habiting species sometimes simultaneously exhibit a range of behaviours (Ho, Nazeer, & Welch, 2023). On the epithelia of healthy humans, these interactions are typically balanced ("eubiotic"), and do not require therapeutic intervention. However, in certain disease pathologies,

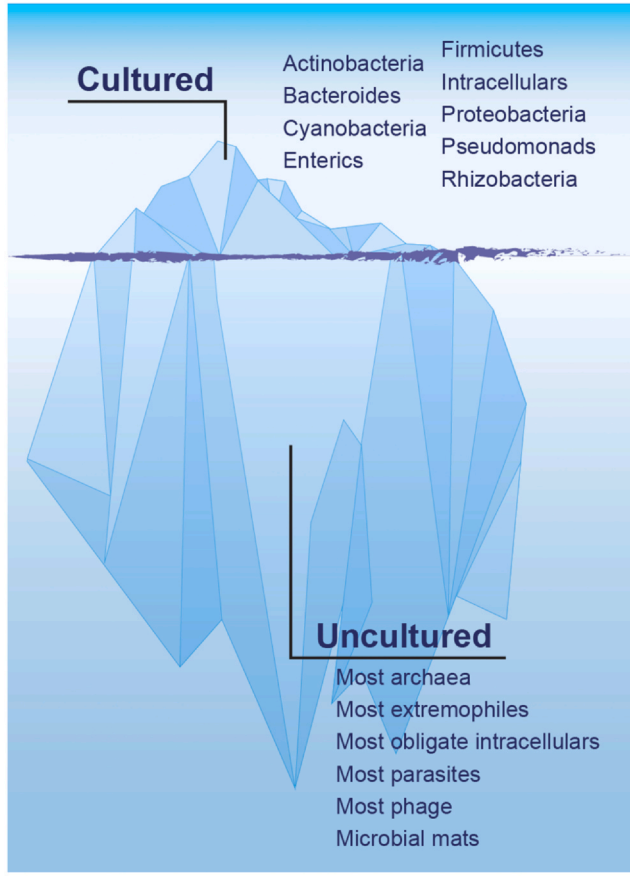

Fig. 1 The iceberg of microbiology. Recent advances have revealed how little we know of the microbial world. The tip of the iceberg represents those culturable organisms which have been studied most thoroughly, whereas the submerged part of the iceberg represents the majority of microbial life – uncultured and unstudied.

this balance becomes dysbiotic, necessitating treatment (Harriott & Noverr, 2011; McKenzie, 2006). These challenges are particularly pronounced in long-term, chronic infections, which are often described as being typically polymicrobial in nature (Young, Hussell, & Dougan, 2002).

The problem is that our mechanistic understanding of inter-species interactions remains remarkably limited. The main reason for this is that stable polymicrobial communities have proven very difficult to recapitulate *in vitro* (or using *in vivo* animal models); simply mixing different species together and hoping for the best is a recipe for failure. Nevertheless, progress is now being

made, and not-too-soon either, since it is now clear that polymicrobial cultures display markedly altered virulence and antimicrobial resistance properties (Ibberson, Barraza, Holmes, Cao, & Whiteley, 2022). Moreover, it is clear that we still do not understand how antibiotics act in complex microbial communities. For example, people with cystic fibrosis (pwCF) often acquire chronic airway infections, such that by their early 'teens, their airways are colonised by a veritable "zoo" of microbes (Crousilles et al., 2015; Foweraker, 2008). Somewhat counter-intuitively, maintenance of a diverse airway microbiota in this constituency of patients is associated with a better long-term prognosis (Cuthbertson et al., 2020). However, the application of antibiotics (targeted towards the principal pathogen present, which is usually *Pseudomonas aeruginosa*) for routine infection management, or in response to the occasional flare-ups (pulmonary exacerbation episodes) that characterise CF airway disease progression, almost certainly leads to remodelling of the airway microbiome. The patient usually gets better in the short term, but in the longer-term, polymicrobial diversity (and hence, lung function) often declines. Whether this progressive decline in diversity is a direct consequence of the "collateral impact" of antibiotics on non-pathogenic co-habitants is not clear. Moreover, antibiotics are stressors that may also effect population remodelling indirectly by stimulating prophage to exit latency and enter the lytic cycle. The resulting phage blooms may themselves contribute to population remodelling (Federici, Nobs, & Elinav, 2021). Finally, if one or more species are eliminated through antibiotic/phage action, the resulting vacated niches will likely become filled, either by extant other co-habiting species, genetic variants of these, or newly-arrived interlopers from the exterior (Ashworth et al., 2024; Langdon, Crook, & Dantas, 2016; Lloyd-Smith, 2013; Naureen et al., 2020). In the absence of well-defined polymicrobial experimental systems to test these hypotheses, we still do not understand how populations remodel following antibiotic challenge.

1.1 Tapping into 'microbial dark matter'

Polymicrobial systems are often described in terms of two key parameters; richness and abundance (Rogers et al., 2004). Culture-based methods, which were traditionally the standard for identifying constituent microbiota, often fail to detect a significant portion of the species present (Delhaes et al., 2012; Lloyd et al., 2018; Surette, 2014). However, recent improvements in the throughput, cost, resolution and scalability of sequencing technologies (Ten Hoopen et al., 2017) are now circumventing this problem. In particular, two technologies have begun to dominate the

field: targeted rRNA gene sequencing, and shotgun sequencing ("metagenomics"). The former uses amplification of hypervariable regions (flanked by conserved regions, enabling the use of "universal" primers for PCR) of the gene(s) encoding ribosomal RNA (Chistoserdovai, 2010). The resulting so-called 16S amplicons contain enough sequence information to allow identification and quantification of the bacterial and archaeal species present. In the case of eukaryotes, so-called 18S rRNA and internal transcribed spacer (ITS) sequencing is used for similar purposes (Willger et al., 2014). Meanwhile, metagenomics provides a tally of the entire gene content of a sample, enabling not only phylogenetic inferences to be made, but also functional inferences (Weinstock, 2012). This notwithstanding, both approaches have their pros and cons, and are not perfect (Durazzi et al., 2021). For example, metagenomic depth of sequencing remains an issue, since much of the sequencing effort will necessarily be directed towards more abundant species, or species with larger genomes. Moreover, and rarely explicitly addressed, different species (or even different strains of a single species) often contain multiple "16S genes". For example, *Pseudomonas aeruginosa* encodes, on the average, four 16S rRNA-encoding genes, whereas other common airway cohabitants, *Rothia mucilaginosa* and *Staphylococcus aureus*, contain an average of 3 and 6 copies of the same gene, respectively. The extent to which these differences in copy number might frustrate proper analysis and quantitation is not always made clear (or sometimes, apparently even considered) (Větrovský & Baldrian, 2013). Similarly, within-organism differences in 16S gene sequence are also rarely discussed (Pei et al., 2010). Finally, many studies ignore the issue of dead cells – an issue that can be significant in antibiotic-treated samples – in spite of a simple experimental solution for the problem (involving propidium azide treatment (Nocker, Sossa-Fernandez, Burr, & Camper, 2007)). Nonetheless, both rRNA amplicon and metagenomics are serving to unlock a great deal of previously hidden information about the polymicrobial world.

Perhaps the best studied microbiome in humans is that associated with the gut, but since this has been extensively reviewed elsewhere, here, we focus on infections affecting the airways and skin (Singh, Natalini, & Segal, 2022).

1.2 The airway microbiota

Historically, the lungs in healthy individuals were believed to be essentially sterile (Flanagan et al., 2007; Laurenzi, Berman, First, & Kass, 1964). That this belief persisted for so long, is a good example of how certain philosophies can

become entrenched, in spite of accruing evidence to the contrary. Indeed, a wealth of data now challenges this long-standing view of pulmonary sterility (Hilty et al., 2010; Rogers et al., 2004). Several authors have suggested – and we agree with them – that a key issue in the field has been an over-reliance on traditional clinical microbiology (Losada, Ghedin, Morris, Chu, & Nierman, 2011). This involves culturing clinically-derived samples on selective media. The problem with this approach is that you only grow what you're looking for. Even media commonly – although somewhat naively – perceived as allowing growth of a wide spectrum of genera (e.g., rich media such as Luria (Bertani) Broth) actually only allow the growth of a small percentage of species. [We note here that Luria Broth (more properly known as lysogeny broth) was originally developed to enable the growth of enterics such as *Shigella* sp.] However, the application of culture-independent technologies over the last two decades has drastically altered this perception (Charlson et al., 2011; Dickson et al., 2015). We now appreciate that healthy lungs are, in fact, populated by diverse communities, including bacteria, fungi, viruses, and archaea, constituting what is known as the lung microbiota (Moffatt & Cookson, 2017). This shift in understanding acknowledges the lung's contiguity with the upper respiratory tract; structures that are rich in microbes. Although, the microbial biomass in healthy lungs is very low compared with other sites such as the gut, skin or mouth, it is non-negligible (Whiteside, McGinniss, & Collman, 2021).

Another under-appreciated factor in many analyses relates to sampling methods. For example, when monitoring the lung microbiota, expectorated sputum samples have been widely used, although with the advent of highly-effective modulators, sputum from one well-studied constituency – people with cystic fibrosis – is becoming increasingly difficult to obtain. However, it is impossible to know which areas of the respiratory tract have contributed to the sample, so 'omics approaches can only report on the aggregate of material, which may also include oral bacteria (Charlson et al., 2011). Ideally, what is needed is very specific sampling e.g., of single lung lobes. If not carried out post-mortem or on explanted material, such approaches are currently highly invasive and not surprisingly, patients are not voluntarily queuing up for the procedure (Melnik et al., 2019; Sze et al., 2012). The current gold standard for spatially-resolved sampling is the bronchoalveolar lavage (BAL), which involves flushing sterile saline into the lung, followed by suction to collect the sample.

1.2.1 Understanding the healthy respiratory microbiome

Recent evidence has shown that microbial colonisation on mammalian epithelia influences the host physiology, regulating immune processes and

limiting disease in healthy individuals (Lloyd-Price, Abu-Ali, & Huttenhower, 2016). Consequently, and far from the historical understanding of the healthy lung as an essentially sterile organ, it is now clear that the pathological state may be better described as a microbial dysbiosis rather than the presence/absence of microbes *per se*. However, in the absence of a better understanding of what constitutes a healthy lung microbiome, dysbiosis is somewhat difficult to define. Recent progress in this regard has been made through metagenomic analyses, which appear to show substantial variance in composition of the healthy lung microbiota, even among groups of similar individuals (Bassis et al., 2015; Bittinger et al., 2014; Charlson et al., 2011; Martinsen et al., 2021; Van Woerden et al., 2013). Some common features are apparent though. First, and with the somewhat contentious exception of phage, bacteria are typically the most populous inhabitants of the respiratory tract. Second, the lower respiratory tract (LRT) maintains a lower microbial biomass than the upper respiratory tract (Dickson et al., 2015). This status quo is likely maintained as a consequence of efficient microbial clearance mechanisms (Man, De Steenhuijsen Piters, & Bogaert, 2017).

While interest in the bacterial microbiota of the airways has blossomed, other members of the lung ecosystem – including fungi, archaea, and viruses – have received somewhat less attention. This is largely due to the fact that such denizens are often not picked up using a single experimental platform (re: the necessity for 16S *vs* ITS analyses when comparing bacteria with fungi, for example). Moreover, DNA extraction methodology is different for different kingdoms, or even within a kingdom re: Gram-positive *vs* Gram-negative bacterial genera. There have, however, been developments in building the overall picture, including definition of the "mycobiome", "virome" and "archaeome".

Exposure to prokaryotic molecular signatures, crucial for immune system maturation, start early in life (Pattaroni et al., 2018). The delivery mode at birth significantly influences initial microbial colonisation of the airways; vaginal delivery usually leads to a higher abundance of *Ureaplasma*, whereas caesarean delivery is more commonly associated with *Staphylococcus*. As neonates mature, their lung microbiome undergoes a shift towards a richer mixture of oral commensals such as *Streptococcus*, *Porphyromonas*, *Prevotella*, and *Veillonella*. Early enrichment of the latter two genera has been linked to the development of reactive airway diseases such as asthma later in life (Thorsen et al., 2019).

There are also interesting hints that lifestyle choices in otherwise healthy individuals can influence the airway microbiota. For example, recent studies

on the effect of smoking have demonstrated perturbations in the phage population (Gregory, Sullivan, Segal, & Keller, 2018; Tong et al., 2019). The health implications of such findings are not yet clear, although given that phages are known to drive population remodelling, our suggestion is to "watch this space" (Federici et al., 2021; Rolain, Fancello, Desnues, & Raoult, 2011). In a similar vein, lung microbiota eubiosis can also be affected by interventions aimed at promoting health, such as antibiotic treatments. In healthy individuals, lung eubiosis is normally maintained by a complex web of inter-species interactions, as well as by host-dependent processes such as mucociliary clearance and basal immune activity (Nelson et al., 2020). Antibiotics perturb this balance, since they often have as much of an effect on the commensal microflora as they do on the perceived pathogen(s) present.

In summary, and in spite of the fact that there is currently no consensus on what constitutes a healthy, eubiotic "core microbiome", it is increasingly obvious that microbial 'dysbiosis' can be a signature (and even a cause) of pathology. Dysbiosis is not only characterised by altered abundances of individual species, but also by changes in the total microbial carrying capacity of the lung. Qualifying this, it is also clear that certain diseases such as CF, chronic obstructive pulmonary disorder (COPD), and idiopathic pulmonary fibrosis may also be associated with their own unique microbiotal signatures (Amati et al., 2022; Cuthbertson et al., 2020; Jankauskaitė, Misevičienė, Vaidelienė, & Kėvalas, 2018; Leitao Filho et al., 2019; Van Der Gast et al., 2011).

1.2.2 The respiratory microbiota in disease

Lung dysbiosis, whether a symptom or cause of disease, has become an important parameter in understanding pulmonary dysfunction (Chen et al., 2023). CF is a genetic condition characterised by malfunctioning/mistargeting of an epithelial chloride/bicarbonate pump, the cystic fibrosis transductance regulator (CFTR) (Basics of the CFTR Protein, Cystic Fibrosis Foundation). PwCF manifest a broad range of health conditions, most obvious of which is usually the production of thicker airway mucus and impaired mucociliary clearance. This phenotype is thought to be a major underlying cause of lung dysbiosis in CF (Mika et al., 2016; Moran Losada et al., 2016). The most frequently encountered CFTR genotype in Europe and the USA is the ΔF508 variant. People homozygous for ΔF508 (or heterozygous for this variant, but carrying another CFTR inactivating mutation on the other allele) exhibit impaired airway function and a progressive decline in lung capacity (Françoise & Héry-Arnaud, 2020;

Kosorok et al., 2001; Orkin Lewin, Byard, & Davis, 1990). Moreover, and despite medical advancements, lung infections remain a leading cause of mortality in pwCF (Françoise & Héry-Arnaud, 2020).

As in other conditions, the CF lung microbiota display significant inter-individual variability in terms of composition and diversity (Magurran & Henderson, 2003; Surette, 2014). This makes it difficult to define a "core CF-associated microbiota", although commonly found inhabitants include species from the genera *Streptococcus*, *Staphylococcus*, *Pseudomonas*, *Burkholderia*, *Stenotrophomonas*, *Achromobacter* and *Haemophilus*, with the latter found mostly in paediatric samples. Unexpectedly for an aerobic organ, the CF lung microbiota also often include an abundance of anaerobes (Cuthbertson et al., 2016; Lamoureux, Guilloux, Beauruelle, Jolivet-Gougeon, & Héry-Arnaud, 2019). The airways of pwCF also typically exhibit a higher prevalence of common respiratory viruses than the general population, potentially contributing to increased morbidity (Jankauskaitė et al., 2018). The presence of these viruses often correlates with bacterial co-infections, including those associated with *Pseudomonas aeruginosa*. It has been speculated that these co-infections may be linked with acute pulmonary exacerbations and impaired lung function (Billard et al., 2017; Jankauskaitė et al., 2018).

The CF airways also have a characteristic mycobiome, which often includes genera such as *Candida, Aspergillus,* or *Malassezia* (Willger et al., 2014). It has been argued that such genera are mostly transient, although *Candida* sp. are now known to be associated with many pwCF (Magee, Louis, Khan, Micalo, & Chaudary, 2021). These fungal species can potentially interact with the bacteriome and/or virome, giving rise to inter-kingdom signalling (Delhaes et al., 2012; Soret et al., 2020; Willger et al., 2014). There is evidence to suggest that archaea can also be found in the CF airways, although current estimates suggest a patchy distribution between patients and low abundance where they are found (Koskinen et al., 2017; Moran Losada et al., 2016).

Lung function and microbial diversity are typically highest in younger pwCF, decreasing with age and plateauing at around age 25 (Cox et al., 2010; Klepac-Ceraj et al., 2010). In long-term follow-up studies, stable respiratory function correlates with maintenance of a stable and diverse microbial population in the airways. Conversely, decreased microbial diversity is linked with declining lung function and the establishment of dominant populations of pathogens such as *P. aeruginosa* (Coburn et al., 2015; Frayman et al., 2017; Jorth et al., 2019; Zemanick et al., 2017). Other potentially pathogenic taxa, such as *Staphylococcus*, *Haemophilus*, and

Burkholderia, are also more prevalent in older pwCF (Boutin et al., 2015; Coburn et al., 2015; Zemanick et al., 2017). Interestingly, sex hormones may also determine (to an extent) predisposition to infection by certain pathogens (Lam, Goodwin, Wilcox, & Quon, 2021).

As noted above, the microbial diversity in the airways of a typical pwCF declines over time. This decline may take decades and may have a lot to do with aggressive and prolonged antibiotic use (Li et al., 2016; Patangia, Anthony Ryan, Dempsey, Paul Ross, & Stanton, 2022). Indeed, the colonising organisms in pwCF often acquire distinct 'resistomes' as a result of these protracted antibiotic regimes. *P. aeruginosa* and *Staphylococcus aureus* infections remain the most challenging CF-associated airway infections to treat, and therapeutic interventions are frequently directed towards managing these (Hatziagorou et al., 2020). However, recent years have seen a shift in the epidemiology of CF lung infections, with a notable increase in the prevalence of *Aspergillus fumigatus* and non-tuberculous mycobacteria (Gannon & Darch, 2021). All of these pathogens have been consistently linked with poorer clinical outcomes (Jhun et al., 2017).

Interspecies interactions within the CF lung are likely to play a significant role in determining the outcome of antibiotic intervention, and in affecting tolerance to host immune defences (O'Brien, Figueroa, & Welch, 2022). Understanding these interactions will therefore be crucial if we are to develop improved, more effective interventions (Gannon & Darch, 2021). However, most studies necessarily currently focus on microbial community composition at the genus level, and ignore intra-genus and even intra-species variability, which can also impact community behaviour. By way of example, the *P. aeruginosa* populations in the CF airways are rarely genomically homogenous, and often include a plethora of genetic variants (Dmitrijeva et al., 2021; Schick, Shewaramani, & Kassen, 2022). This variability might explain differences in disease status that are not apparent at the genus level (Coburn et al., 2015).

1.3 The skin microbiota

Unsurprisingly, given its exposure to the exterior, the skin harbours a diverse microbiota, whose composition changes following injury or insult (Santiago-Rodriguez, Le François, Macklaim, Doukhanine, & Hollister, 2023; Zeeuwen et al., 2012). Moreover, the recorded microbiota can show a strong dependence on the sampling method(s) employed (Kool, Tymchenko, Shetty, & Fuentes, 2023). Skin sampling methods include swabs, tape stripping, and biopsy. Swabs and tape strips primarily collect microbes from the skin surface,

whereas biopsies offer a more comprehensive analysis of the skin, inclusive of the deeper layers (Liang et al., 2022; Santiago-Rodriguez et al., 2023). To explore the skin microbiota, researchers have used both culture-dependent and -independent techniques, with their attendant advantages and limitations (Acosta et al., 2023; Kalan et al., 2019; Schoch et al., 2023). Even following the advent of sequencing methodology, species-level distinction can remain challenging. This is important because in genera such as *Staphylococcus*, pathogenic species such as *S. aureus* are closely related to certain commensals (e.g., *S. epidermidis*) involved in epidermal eubiosis (Lai et al., 2010; Li, Huang, Xu, Li, & Li, 2019; Newstead, Varjonen, Nuttall, & Paterson, 2020). The low microbial biomass and high host DNA content in skin samples can also be limiting factors in the analyses (Rungjang et al., 2022).

1.3.1 The microbiota of healthy skin

Skin may be moist, sebaceous, or dry – making it a potential home for an array of species, including bacteria, fungi, viruses, and archaea. Interestingly, the presence of these commensals often apparently depresses colonisation by pathogenic organisms (Callewaert, Ravard Helffer, & Lebaron, 2020; Chen, Fischbach, & Belkaid, 2018). Protective mechanisms include production of antimicrobial compounds, and indirect competitive exclusion (Nakatsuji et al., 2017; SanMiguel & Grice, 2015).

The healthy skin microbiota are known to be influenced by a variety of host-related factors such as age, genetics, and anatomical site, as well as by environmental factors including climate, country of residence, and urban or rural settings (Gupta, Paul, & Dutta, 2017; Leung, Wilkins, & Lee, 2015; Rungjang et al., 2022; Sachdeva, Satyamoorthy, & Murali, 2022; Wang et al., 2021; Ying et al., 2015). This complexity has led to the concept of the "pan-microbiome", which, like the respiratory microbiome described above, challenges our definition of what a "normal" microbiome should look like (Leung et al., 2015). Using culture-independent methods, Grice et al. (2009) have identified at least 19 phyla and over 1000 bacterial species across 20 different skin sites. Fungal species have received less attention than their bacterial counterparts, but nevertheless, constitute an important contingent of the skin microbiota and often appear to be exquisitely sensitive to the skin microenvironment (Cui, Morris, & Ghedin, 2013; Findley et al., 2013; Paulino, Tseng, Strober, & Blaser, 2006; Roth & James, 1988; Underhill & Iliev, 2014; Zhang et al., 2011). Interestingly, and unlike the respiratory microbiome (where they constitute only a minor fraction of the microbiota), the archaea are more abundant on the skin,

comprising up to 4% of the overall microbial diversity (Moissl-Eichinger et al., 2017; Probst, Auerbach, & Moissl-Eichinger, 2013).

As with the study of gut and lung microbiota, a better understanding of what constitutes a "healthy" skin microbiota offers the potential for artificial reconstitution, enhancing skin health management and wound care.

1.3.2 The wound microbiota

Wounds provide an aperture for microbes to invade and colonise the underlying tissues, potentially leading to infection (Tipton et al., 2019; Tomic-Canic, Burgess, O'Neill, Strbo, & Pastar, 2020). Chronic wounds impact millions of people worldwide, are costly to manage and can be associated with severe complications like pain, immobility, social isolation, morbidity, and mortality (Armstrong, Boulton, & Bus, 2017; Frykberg & Banks, 2015; Järbrink et al., 2017; Nussbaum et al., 2018; Sen, 2021). Not surprisingly, the microorganisms associated with chronic wound infections often resemble those on nearby healthy skin, including both commensals (that may aid in healing) and opportunistic pathogens (which prevent healing) (Kalan & Grice, 2018; Verbanic, Shen, Lee, Deacon, & Chen, 2020). Wolcott et al. (2016) reported a high incidence of *Staphylococcus* and *Pseudomonas* species in chronic wound infection scenarios, alongside commensals and anaerobes (Citron, Goldstein, Merriam, Lipsky, & Abramson, 2007; Malone et al., 2017). The presence of these anaerobes, which include genera such as *Finegoldia*, *Prevotella*, *Peptoniphilus*, *Peptostreptococcus* and *Anaerococcus,* correlates with poorer healing outcomes (Kalan et al., 2019; Min et al., 2020; Sloan et al., 2019; Verbanic et al., 2020). More generally, biofilm formation has been reportedly associated with a majority (60–100%) of chronic wounds in several different studies (James et al., 2008; Johani et al., 2017; Malone et al., 2017; Wolcott et al., 2016). This conclusion appears to be primarily based on the close physical association of microbial cells with wound tissue, and the presence of biofilm-like aggregates of cells in wound exudates, since for most organisms, there are no well-defined biomarkers of "a biofilm".

2. Laboratory endeavours

2.1 Models for the study of polymicrobial infections

As noted above, advances in culture-independent methods have allowed investigators to modestly tap into the estimated 85–99% of microbial life that is "unculturable" and mostly uncharacterised (Lok, 2015; Rinke et al., 2013).

Even within the estimated 1–15% contingent of microbes that are culturable, experimental regimes that allow stable co-cultivation alongside other species are few-and-far between. The problem is that simple inoculation of a flask or microplate with a mixed microbial inoculum almost always fails to capture the stability and diversity associated with chronic infections. Such unstable co-cultures display considerable dynamic change (usually, over the course of hours rather than days or weeks) and therefore exaggerate the significance of inter-microbial competition and dominance. Despite their obvious flaws, these in vitro setups are still widely reported, even though they can sometimes have only limited scientific value. One reason for this may be that such models are usually cheap and well-suited to high throughput, and despite their limitations, many laboratories continue to use them (Bernardy, Raghuram, & Goldberg, 2022; Filkins et al., 2015; Kvich et al., 2022; Luján et al., 2022; Magalhães, Jorge, & Pereira, 2019; Mitchell et al., 2010; Pajon et al., 2023; Price, Brown, Limoli, Phelan, & O'Toole, 2020; Tognon et al., 2017; Vasiljevs, Gupta, & Baines, 2023). By contrast, models that better capture long-term polymicrobial stability and infection-associated characteristics are usually more expensive to set up, and much lower in throughput. In essence, you get what you pay for.

Before going into details, it is worth noting that a set of guidelines have been suggested by O'Toole et al. (2021) for modelling CF-related polymicrobial infections. They recommend defining the research goal clearly and ensuring that this goal is matched with an appropriate model, albeit in the spirit of the adage that "all models are wrong, but some can be useful". This is because reliance on a single model system is likely to exaggerate outcomes that emerge from peculiarities of that particular experimental system. Simple *in vitro* and *ex vivo* systems are more manipulable and conducive to hypothesis-driven work but may perhaps be best used in synergy with more complex and representative *in vivo* studies (although *in vivo* polymicrobial disease models are still in their infancy).

2.2 Models of CF lung microbiology

A fundamental criterion for *in vitro* CF infection models should the ability to maintain a stable steady state, although recent research has raised the question of just how "stable" this steady state is *in vivo* (Raghuvanshi et al., 2020). [Mitigating, and as those authors point out, the daily fluctuations in CF microbial diversity that they observe may also be a reflection of the nature and origin of the samples being assayed (sputum) rather than genuine fluctuations in the steady-state dynamics of the CF microbiota.].

In CF research, the current medium of choice is Artificial Sputum Media (ASM) or Synthetic Cystic Fibrosis Sputum (SCFM), which closely replicate the chemistry of CF patient sputum (Aiyer & Manos, 2022; Kirchner et al., 2012). Common laboratory media such as lysogeny broth (LB) should be avoided. The transcriptome of *P. aeruginosa* in SCFM and in human sputum is essentially identical (On et al., 2023; Turner, Wessel, Palmer, Murray, & Whiteley, 2015). However, Neve, Carrillo, and Phelan (2021) caution that differences in ASM formulations, originating from the more complex ingredients like mucin, can influence bacterial physiology and virulence, and impede reproducibility. Moreover, it should be noted that, although ASM/SCFM have been optimised to capture the physiological characteristics of *P. aeruginosa* in the CF environment, the medium is not optimised for the growth of other CF-associated pathogens, and indeed, not all of these grow in ASM.

Quinn et al. (2015) developed the *in vitro* Winogradsky-based culture model to study how the physiology of the CF lung contributes to pulmonary exacerbations. They made Winogradsky columns by filling narrow- gauge capillary tubes with ASM to simulate the physicochemical gradients in CF bronchioles. After inoculating columns with CF patient sputum expectorated during pulmonary exacerbations, they noted changes in pH, gas production, and community composition (by 16S rDNA analyses). These authors concluded that fluctuations in fermentative anaerobes likely play a role in pulmonary exacerbations.

In the interest of studying the effect of perturbations on a stable, CF-relevant polymicrobial ecosystem, of O'Brien and Welch (2019) established an *in vitro* continuous flow system using ASM. They inoculated the setup with a Gram-negative CF pathogen (*P. aeruginosa*), a Gram-positive CF pathogen (*S. aureus*) and a fungal CF pathogen (*C. albicans*). The optimal flow rate turned out to closely approximate with the estimated fluid replacement rate in human airways, and remarkably, irrespective of the ratio of inoculating microbes, the steady state achieved after 24 h was always the same. This indicated a "hardwired" ecological relationship between the species. Transcriptomic analyses revealed that the microbial population was maintained in slow exponential growth (just as in the CF airways) (Bartell et al., 2019; La Rosa, Rossi, Feist, Johansen, & Molin, 2021; O'Brien et al., 2022; Yang et al., 2008). A similar flow-chamber system has been used by Tolker-Nielsen and Sternberg (2011) and Yang et al. (2011) for co-culturing biofilms of *S. aureus* and *P. aeruginosa*.

Models have also been developed to capture the spatial and metabolic heterogeneity of the CF lung environment (Bragonzi et al., 2012). For example, Lopes, Azevedo, and Pereira (2017) explored how polymicrobial biofilm formation is influenced by oxygen availability, and more recently, Kasetty, Mould, Hogan, and Nadell (2021) used microfluidics to quantitatively assess the relationship between flow and biomass production in biofilms of *P. aeruginosa* and *C. albicans*. These diverse approaches underscore the increasing sophistication being applied to polymicrobial cultures, particularly in the context of CF airway infections.

One obvious feature missing from the approaches described above are host cells and a host immune input. On the one hand, the highly-reproducible polymicrobial cultures described by O'Brien and Welch (2019) appear to faithfully mimic the species titres seen in CF sputum, suggesting that such host influences may play little role in airway microbiology. On the other hand, it seems inconceivable that the host immune response plays no major role in shaping the microbial community architecture. Human cell-culture and cellular microbiology infection models, including chips, organ-on-chips, and organoids, present an innovative approach by blending *in vitro* and *in vivo* characteristics (López-Jiménez & Mostowy, 2021). These models are proving increasingly valuable in exploring how human bronchial epithelial cells interact with microbes (and *vice versa*), especially those carrying the ΔF508 CFTR mutation (Filkins et al., 2015).

So, what does the future hold for such models? A key gold standard will be to culture polymicrobial communities direct from CF sputum or BAL samples. This approach, of creating "personalised infection models" would capture not only the species present (and ideally, in the same ratios as found in the patient's airway secretions), but also the specific lineages of each species found in the patient. Such intra-species diversity has received scant attention in the context of CF polymicrobial infection, yet it has been known for years now that the *P. aeruginosa* population in pwCF is usually clonally derived but heterogenous. A personalised microbiome would capture all of these variants for each of the species present. However, and as noted above, current ASM formulations do not permit the growth of many CF-associated species, and we still have much to learn about what additives will be required to overcome this. Nevertheless, a captured personal CF microbiome would enable facile testing of drug combinations that might target the key pathogens but maintain microbial diversity, thereby benefitting the patient. By comparison, at present, clinical treatment choices are still largely empirical.

2.3 Chronic wound models

Recent years have also seen the development of several *in vitro* chronic wound models. Perhaps the most widely used "base model" is the Lubbock chronic wound biofilm model (Diban et al., 2023). Here, a blood/plasma-based medium is employed that readily coagulates and enables facile polymicrobial growth of several wound-associated species, such as *P. aeruginosa*, *S. aureus*, and *Enterococcus faecalis* (Diban et al., 2023). Recently, the recipe has been improved, to yield chronic wound medium (CWM). This medium uses human blood ingredients and is enriched in keratinocyte debris – designed to optimally simulate the biochemical environment of wounds (Pouget et al., 2022). But having the right chemical environment (through judicious choice of growth medium) is only part of the solution; the wound microenvironment is also spatially heterogenous. To address this, Thaarup et al. (2023) employed a layered collagen-based scaffold that structurally simulates mammalian wound tissue. The model was able to stably maintain *P. aeruginosa* and *S. aureus* over many days, and enabled testing of potential therapeutic interventions.

2.4 Ex vivo models

In the realm of chronic wound and CF research, *ex vivo* models, particularly those using porcine tissue, have gained increasing prominence. These models have been instrumental for studying infection dynamics, albeit predominantly in mono-species experiments. An *ex vivo* porcine lung model has been developed to examine the growth, virulence, and signalling mechanisms of *P. aeruginosa* and *S. aureus* in a CF context (Harrison et al., 2021; Sweeney et al., 2021). However, and although the use of lung tissue is anthropocentrically appealing in the context of CF, we do wonder whether there is anything special about this particular tissue; would any cut of meat do? After all, *P. aeruginosa* is known to have a predilection for soft tissues generally. In a different application of porcine models, porcine skin explants have been used to model biofilms in chronic skin wounds, and Regan, Taylor, and Karunakaran (2022) have used *ex vivo* ovine skin for similar purposes (Lorenz et al., 2023). Bringing this general approach to its inevitable and logical conclusion, Yoon et al. (2019) have described how human abdominal skin (excised during cosmetic procedures) can be used as a chronic wound model, although in their case, this was a monospecies model employing only *S. aureus*. Nevertheless, they noted that biofilm formation on the skin was accompanied by increased expression of pro-inflammatory genes, exactly as in chronic wound infections.

The current landscape of *ex vivo* models, while advanced, has not been specifically tailored for polymicrobial studies. There are some exceptions to this, albeit not explicitly focused on the microbiology of the system (Dumigan et al., 2019; Oates, Lindsay, Mistry, Ortega, & McBain, 2018; Phan et al., 2020). We suspect that there is a significant opportunity to adapt these *ex vivo* models to investigate the polymicrobial communities derived from CF or wound samples.

2.5 Invertebrate models

Caenorhabditis elegans is a widely used laboratory organism that has been developed as a model host organism for pathogenic bacteria and fungi (Powell & Ausubel, 2008). *C. elegans*-based models have been employed to study *P. aeruginosa, C. albicans* and *S. epidermidis*, both in isolation and in combination (Holt, Houston, Adams, Edwards, & Kjellerup, 2017). A major benefit of *C. elegans* is its apparent potential to be understood at a 'phenomic' level such that physiological processes can be interrogated holistically and understood deterministically (Rossi, Falcone, Molin, & Johansen, 2018).

Galleria mellonella larvae have equally gained popularity as a model due to their accessibility, cost-effectiveness, and compatibility with a range of temperatures including 37 °C – an option that is not available for *Drosophila melanogaster* or *C. elegans* models (Andrea, Krogfelt, & Jenssen, 2019; Fedhila et al., 2010). The *Galleria* innate immune system shares similarities with that of mammals, offering insights into conserved immune responses (Lange et al., 2018). The model is also a flexible one; for example, Maslova et al. (2020) have introduced a *G. mellonella* burn wound model. However, a limitation with *G. mellonella* is the comparative paucity of genetic tools, large-scale databases and standardised procedures (Serrano, Verdial, Tavares, & Oliveira, 2023).

D. melanogaster emerges as the most promising invertebrate model for polymicrobial infection research, thanks to the extensive genetic tools available for creating disease-mimicking mutations, and the conservation of physiology and cell biology between *Drosophila* and humans (Apidianakis & Rahme, 2009; O'Brien & Welch, 2019). Reports of successful *S. aureus* and *P. aeruginosa* co-infection models in *Drosophila* highlight its potential for shedding light on interspecies interactions and their underlying mechanisms (Korgaonkar, Trivedi, Rumbaugh, & Whiteley, 2013; Lee et al., 2020; Sibley et al., 2008). Leveraging the conservation of immune signalling processes may allow insights to be gleaned as to how the human immune system responds to polymicrobial infection, and perhaps even to

uncover phenomena as opaque as the apparently "spontaneous" acute episodes (exacerbations) which punctuate the life of many chronically-infected patients.

2.6 Vertebrate models

Vertebrate CF models tend to be used to study colonisation and infection of the lung, with inoculation typically taking intratracheal or intravenous routes (Lebeaux, Chauhan, Rendueles, & Beloin, 2013). Although mouse models are commonly-used to study pulmonary infections, including those pertinent to pwCF, they are limited insofar as mice carrying *CFTR* mutations do not exhibit a lung phenotype (McCarron, Parsons, & Donnelley, 2021). However, β-ENaC mice – those with deficient epithelial sodium channels – can be used as an alternative, as they do display a CF-like lung phenotype (McCarron et al., 2021). Alternatively, animal models such as CF ferrets, rabbits, pigs, sheep, and rats may offer pulmonary phenotypes more comparable to humans (Birket et al., 2018; Cho et al., 2018; Fan et al., 2018; Stoltz, Meyerholz, & Welsh, 2015; Sun et al., 2014). The simple confounding fact remains, however, that animal microbiomes are naturally distinct from those of humans, suggesting that the lung environment in these models is intrinsically different. Humanised microbiome mouse models may bridge this gap, though their utility in CF research remains to be seen (Fiorotto et al., 2019).

Despite the numerous animal models available, very few have been adapted/applied to investigate polymicrobial infections; the primary emphasis to date has always been to investigate wound healing. Dorsett-Martin (2004) developed an *in vivo* polymicrobial, biofilm-related infected wound model, which entailed transplantation of diverse multispecies aggregates grown *in vitro* onto fresh wounds. Those wound infections sustained diversity across four of the bacterial species examined throughout the experimental time-frame. Crucially, the data indicate multispecies biofilm infections impair wound healing compared with mono-species infections (Dalton et al., 2011).

Despite these and other advances, the challenge of developing an ideal animal model for studying human chronic wounds persists (Tan, Chin, Madden, & Becker, 2023). A key issue is the simple fact that most non-human animals do not apparently suffer from chronic wounds themselves. Consequently, a large inoculum of bacteria is required to establish an infection, thereby risking systemic infection and death (Pletzer, Mansour, Wuerth, Rahanjam, & Hancock, 2017). In cases where localised wound infections are attained, they only last for days to weeks – a time-frame that

does not capture the complex evolution of host-microbe interactions associated with human chronic infections (Burmølle et al., 2010; Ganesh et al., 2015).

Future developments in these models, particularly those incorporating humanised microbiomes and humanised hosts, hold promise for replicating human disease states more accurately, thereby enhancing our understanding of chronic infections.

3. Imaging: direct observation and heterogeneity

From its foundation at the hand of Antonie Van Leeuwenhoek in the 17th century to the most modern discoveries unlocked by fluorescence, electron beams and nanoscopic scanning probes, microscopy has been a key tool in the investigation of microbiology (Binnig, Quate, & Gerber, 1986; Chalfie, Tu, Euskirchen, Ward, & Prasher, 1994; Heim, Prasher, & Tsien, 1994; Mulvey, 1962; Shimomura, Johnson, & Saiga, 1962). Nevertheless, the direct observation of *in situ* and *in vivo* infections is an extremely challenging task due to the inaccessibility and opacity of the human body. Although new techniques, reviewed elsewhere, are attempting to enhance the visual contrast between microbes within the body, most of our direct observations come from *ex vivo* samples, animal models, or lab infection models (Ordonez et al., 2019). *Ex vivo* samples of both human and animals have revealed the intimate relationship between microbes and host cells, often highlighting a high degree of spatial organisation, indicative of a rich ecology (Barbosa, Miranda, Azevedo, Cerqueira, & Azevedo, 2023; Bergström et al., 2016; Hasegawa, Welch, Rossetti, & Borisy, 2017; Johani et al., 2019; Kim et al., 2020; Lin, Du, Song, Wang, & Yang, 2021; López-Álvarez et al., 2022; Mark Welch, Rossetti, Rieken, Dewhirst, & Borisy, 2016; Mark Welch, Hasegawa, McNulty, Gordon, & Borisy, 2017; Shi et al., 2020; Viana, O'Kane, & Schroeder, 2022). In many of these studies, the different microbial species in *ex vivo* samples are typically identified through fluorescence in situ hybridisation, a technique that uses small fluorescent oligonucleotide probes that uniquely tag specific sequences in the target organism (Moter & Göbel, 2000). However, these approaches require fixation steps, which potentially alter the sample and limit observations to single temporal snapshots (Tropini, Earle, Huang, & Sonnenburg, 2017).

Animal models enable the use of synthetic infective communities, which can be engineered to express fluorescent proteins. An advantage of

these models over the *ex vivo* ones is that infections can be followed through both in time and space throughout their lifecycle, both on the surface of the animal, and, with the introduction of transparent animal models, within the animal (Benard et al., 2012; Hattab, Dagher, & Wheeler Robert, 2022; Jim et al., 2016; Lopez et al., 2021; Sibley et al., 2008). However, in both *ex vivo* and animal model approaches, the experimental environment is complex and variable-rich, such that teasing apart contributions from single factors can become difficult. By contrast, the simpler laboratory models of infection may or may not involve the host, although that simplicity inevitably means that some of the "real" contributions to infection are sacrificed in order to gain easier access to hypothesis testing.

Recent advancements in the field of organoids are positioning organ-on-chip approaches at the forefront of developments in microscopy-based infection studies (Feaugas & Sauvonnet, 2021; Ingber, 2022; Leung et al., 2022; Shahabipour et al., 2023; Zhao et al., 2022). Albeit still in development, these platforms are starting to shed light on topics such as the role of peristalsis and mechanical forces in gut infections, of surfactant in early lung infections, and on the protective function of commensals in the colon, all of which would otherwise be hard to test with other methods (Gazzaniga et al., 2021; Grassart et al., 2019; Thacker et al., 2020).

4. The extracellular interactome: *P. aeruginosa* as micro-architect of the lung

Over much of the period since the advent of axenic culture technique, microbes have been predominantly considered free-swimming individuals (despite the fact that some of the earliest descriptions of single-celled organisms depicted them as multicellular aggregates). Indeed, and as van Leeuwenhoek noted upon observing a plaque scraped from his own tooth, "Animals in the scurf of a man's Teeth are so many that I believe they exceed the number of Men in a kingdom" (Fred, 1933). Moreover, evidence of "biofilm-like" aggregates of microbial cells can be observed in fossils dating from as far back as 3.4 billion years (Cavalazzi et al., 2021). These examples are demonstrative of just how ubiquitous and ancient the microbial tendency is to grow together in multicellular communities.

A biofilm is often described as a community of sessile cells, with altered metabolic and phenotypic properties, congregated within a matrix of their own production. There exists no definitive biofilm, which presents a

methodological problem for researchers. Rather, the word serves as an umbrella term to describe a collection of related phenomena and physiological states – biofilms are part of a landscape of growth modes demarcated by somewhat fuzzy boundaries. Perhaps more pertinently, we should appreciate more the rarity of a monospecies biofilm outside the laboratory environment. Indeed, biofilms 'in the wild' are now considered ecosystems in their own right, displaying heterogeneity and complexity, at all levels (Dar, Dar, Cai, & Newman, 2021; Jo, Price-Whelan, & Dietrich, 2022; Wimpenny, Manz, & Szewzyk, 2000; Wolcott, Costerton, Raoult, & Cutler, 2013).

Advances in our understanding of biofilms have come thick and fast in the investigation of *P. aeruginosa* and its co-habitants in the CF lung environment. Developments in microscopy have allowed a more sophisticated model of the '*in vivo* biofilm' to be developed, which eschews the substratum-bound description of biofilms in favour of aggregated microcolonies suspended in mucus (Bjarnsholt et al., 2013). However, even with these improvements, it is still not clear what a biofilm *in situ* in the lung actually looks like. Without this understanding, clinical problems associated with such would-be 'polyspecies *in pulmone* biofilms' may be hard to tackle.

What is clear is that as a key pathogen and active denizen of the diseased lung, *P. aeruginosa* interferes with its neighbours, and 'terraforms' the host environment, driving a shift in the virulence profile of the entire microbial ecosystem. Commensurate with this notion, *P. aeruginosa* has evolved to secrete a wide range of extracellular substances that modify its environment. For example, some of *P. aeruginosa*'s most characteristic biochemistry lies in its pigmentation. Phenazines (mobile electron carriers) are deployed by the organism to move electrons (a necessary byproduct of oxidative metabolism) from environments where electron acceptors are scarce – such as the interior of a biofilm – to the more oxygenated periphery (Wang, Kern, & Newman, 2010). The same small molecules can also serve as virulence factors, which aggravate host tissue inflammation and inhibit competing microbes (Grahl, Kern, Newman, & Hogan, 2013; Hunter et al., 2012). Intriguingly, these redox-active molecules also appear to be involved in inter-cellular signalling. Indeed, phenazine-dependent stimulation of oxidative-stress-related pathways in co-habiting *Aspergillus* species has been shown to influence the morphology of those fungi (Zheng et al., 2015). Consequently, secreted factors represent not only specific contributors towards *P. aeruginosa*'s fitness in the lung (and other) environment(s); they are also directly involved in the microbial melée that characterises the airway mucosa (Kang, Xu, & Kirienko, 2024; Meyer, Neely, Stintzi, Georges, & Holder, 1996; Recinos et al., 2012; Schiessl et al., 2019).

P. aeruginosa also produces a welter of pigmented iron-chelating siderophores. These not only control iron availability – they also affect the efficacy of clinical interventions. A nice example of this is that antifungal drugs are more efficacious in conditions where siderophore activity has scrubbed the airway environment clean of iron (Hattab et al., 2022). *P. aeruginosa*'s engagement in the secretion economy therefore has unanticipated consequences.

We define the extracellular interactome of *P. aeruginosa* as the collection of secreted molecules that influences the interaction of the bacterium with other species around it. In terms of understanding, we are only just scratching the surface of the CF airway-associated extracellular interactome and often only in snapshots (Nazeer, Wang, & Welch, 2023). The extracellular interactome likely evolves over the course of infection, driven by shifting transcriptional programs and genotypic changes, the unfolding of which culminates in 'pathoadaptation' (Diaz Caballero et al., 2015; Jurado-Martín, Sainz-Mejías, & McClean, 2021; Rossi et al., 2018). In this regard, and although there are now large lists of pathoadaptive genes for *P. aeruginosa*, we know much less about genetic pathoadapation in the co-habiting species.

P. aeruginosa has been demonstrated to acquire a number of mutations during longitudinal infection of the lung. For example, mutations affecting the LPS O-antigen have been found, *in vitro*, to alter cell aggregation in such a manner that *P. aeruginosa* appears to physicochemically separate itself from other species (Azimi et al., 2021). The organism has also been observed to dynamically modify its secreted proteome. Such modification includes post-translational modifications on secreted proteins, as well as more irreversible alterations, such as proteolysis, with wide-ranging effects on inter-microbial relations, host-pathogen interactions and biofilm structure (reviewed recently by Forrest & Welch, 2020). These modifications to the secretome have functional consequences; along with many other bacteria, *P. aeruginosa* methylates EF-Tu, a ribosomal elongation factor, in a manner that has no discernible effect on translation but which improves adhesion of the biofilm to respiratory epithelia and consequently augments virulence (N'Diaye et al., 2019). This example serves to demonstrate that subtle chemical changes made to *intracellular* proteins can lead to a 'moonlighting' *extracellular* role. Presumably, such nominally "cytoplasmic" proteins escape and populate the cell matrix through explosive cell lysis (Turnbull et al., 2016). Post-translational modification has also been proposed as a means to protect the secreted bacterial proteome against the wealth of lytic enzymes that occupies the extracellular

space (Forrest & Welch, 2020). A similar strategy can be seen with the biofilm cohesion protein CdrA, which is stabilised against proteolysis by its interaction with exopolysaccharides (Reichhardt, Wong, Passos da Silva, Wozniak, & Parsek, 2018).

The secreted proteome itself shifts in composition over time – a shift which alters the metabolism of neighbouring microbes (Margalit, Sheehan, Carolan, & Kavanagh, 2022). The majority of extracellular virulence factors of *P. aeruginosa* are understood to be secreted by Type II secretion, through one or both of the main terminal branches, termed the Xcp and Hxc secretons (Ball, Durand, Lazdunski, & Filloux, 2002; Bally et al., 1992). Interestingly, there exists a pair of orphan accessory proteins, XphA and XqhA, which can form a chimeric secreton assemblage with the Xcp proteins (Michel, Durand, & Filloux, 2007). Expression studies show that, early in the growth cycle, these two orphan genes are expressed synchronously with most of the Xcp genes. However, later in growth, expression of two homologs (XcpQ and XcpP) is turned on, displacing the orphan proteins from the secretion complex. The main apparent change that accompanies this is one of substrate selectivity – significantly that of PaAP, an aminopeptidase and virulence factor (Zhao et al., 2018). PaAP, it turns out, seems to be involved in biofilm remodelling, nutrient cycling and antibiotic tolerance – suggesting that this shift in the secretome over the course of the infection could have significant effects on the pathophysiology of *P. aeruginosa* (Harding, Bischoff, Bergkessel, & Czekster, 2023). Clearly, there are many levels at which the secreted proteome can be dynamically and spatially modified by *P. aeruginosa*.

As noted above, *P. aeruginosa* can be considered to 'terraform' its environment. Another way in which it does this is by using exopolymers to establish a microenvironment conducive to survival (Greenwald & Wolfgang, 2022). *P. aeruginosa*'s functional amyloid protein, FapC, has a number of functions, including increasing surface stiffness, hydrophobicity and cell aggregation (Dueholm et al., 2010; Zeng et al., 2015). The *fap* operon is up-regulated in chronic wound and burn infection models, and a deletion of *fapC* substantially diminishes virulence in *C. elegans*, suggesting importance in infection (Turner, Everett, Trivedi, Rumbaugh, & Whiteley, 2014; Wiehlmann et al., 2007). Intriguingly, FapC seems not only to play a structural role per se, but also to stimulate a wholesale shift in cellular physiology, perhaps constituting a form of pathoadaptation. The proteome of a *fap* mutant is significantly different to that of its wild-type progenitor, with large alterations in the expression of proteins related to

attachment, biofilm formation, anaerobic metabolism and quorum sensing (Beg et al., 2023; Herbst et al., 2015). The mechanism by which this functional amyloid might drive these major proteomic and physiological shifts towards an infection lifestyle is yet to be determined, although it may have something to do with the observed interaction between FapC and quorum sensing molecules, trapping (and therefore intensifying), these QS signals (Seviour et al., 2015). A more prosaic explanation is simply that the *fap* mutants display aberrant aggregation, affecting their global physiology. Nevertheless, the possibility that production of amyloids by other co-habiting species may affect the virulence of *P. aeruginosa* remains an interesting one (Melnik et al., 2019).

5. Interspecies relations: social and antisocial microbes in infection

The last few decades have revealed remarkable insights into how microbes communicate with one another, and how certain 'shibboleth molecules' facilitate communication that bridges microbial 'tribes'. A common theme in the relations of most microorganisms that co-habit the human body is that they have the capacity to do one another good and harm in equal measure. It is hardly surprising that, faced with the existential threats of the immune system and antibiotic intervention, organisms have co-evolved to band together when times are tough but, otherwise, will seek to gain an advantage when an opportunity arises. In this section, we trace the frontiers of our understanding of these ambivalent microbial relations.

5.1 Bacterial interactions

5.1.1 P. aeruginosa and S. aureus

Investigation of the relationship between *P. aeruginosa* and *S. aureus* continues to reveal an extensive and complex network of interactions, associated with both peaceful coexistence and fierce rivalry. These divergences highlight the polyvalent character of the relationship between the two pathogens, sensitive to factors such as population diversity, clinical conditions, and environmental challenges. However, we also note that the interactions between *S. aureus* and *P. aeruginosa,* in both the CF airways and in chronic wounds, can be strain-specific, predominantly driven by inter-strain variation in the *P. aeruginosa* (Bernardy et al., 2022; Ibberson & Whiteley, 2020).

Gram-positive bacteria, including *S. aureus*, stimulate *P. aeruginosa* to up-regulate virulence gene expression (Korgaonkar et al., 2013; Rickard et al., 2006). In mixed cultures, *P. aeruginosa* detects *N*-acetyl glucosamine (GlcNAc) derived from the peptidoglycan of *S. aureus* cell walls, prompting the production of the *Pseudomonas* Quinolone Signal (PQS). The latter controls the expression of virulence factors such as pyocyanin, elastase, and 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) (Korgaonkar et al., 2013). Intriguingly, *P. aeruginosa* mutants lacking the peptidoglycan-sensing gene, *agtR*, are less effective at outcompeting *S. aureus*, so this ecological story likely has more to offer (Korgaonkar et al., 2013).

HQNO, in turn, is known to interfere with oxidative respiration in *S. aureus* by inhibiting its cytochrome bc_1 complex (Hazan et al., 2016). Sufficient concentrations of HQNO can lead to cell lysis, whereas lower, sub-lytic levels trigger a shift towards fermentative metabolism and lead to a number of changes in the *S. aureus* phenotype (Barraza & Whiteley, 2021; Hazan et al., 2016). HQNO can therefore both stymie biofilm development and spatial ordering, and inhibit cell growth in biofilms (Barraza & Whiteley, 2021; Gomes-Fernandes et al., 2022; Ibberson & Whiteley, 2020; Oluyombo, Penfold, & Diggle, 2019; Orazi & O'Toole, 2017). HQNO – whose production is strongly strain-dependent – promotes formation of the small-colony variants (SCVs) that are commonly associated with clinical samples (Hoffman et al., 2006; Mitchell et al., 2010). These SCV strains, characterised by defects in their electron transport systems, are less susceptible to *P. aeruginosa*-mediated killing compared with their non-SCV counterparts, suggesting a potential survival strategy during co-habitation (Filkins et al., 2015). Interestingly, Yang et al. (2011) found that mutations in *P. aeruginosa*'s *mucA* and *rpoN* genes diminish *S. aureus* microcolony formation, effectively cloaking *P. aeruginosa* to *S. aureus* and conferring a competitive advantage in two-species biofilms.

HQNO "signalling" between *P. aeruginosa* and *S. aureus* can also lead to altered antibiotic tolerance (DeLeon et al., 2014; O'Brien et al., 2022). It has been found that secretion of HQNO by *P. aeruginosa*, bolsters *S. aureus* resistance to tobramycin by preventing uptake of the antibiotic (Hoffman et al., 2006; Radlinski et al., 2017). This enhanced phenotypic resistance to antibiotics of SCVs is noted by several other studies (Biswas & Götz, 2022; Hammer Neal et al., 2014; Kahl, Becker, & Löffler, 2016). Conversely, *S. aureus* can make *P. aeruginosa* more susceptible to ciprofloxacin and aminoglycosides (Trizna et al., 2020).

In parallel developments, Mashburn, Jett, Akins, and Whiteley (2005) showed that *S. aureus* can act as an iron donor to *P. aeruginosa* during co-culture, evidenced by reduced expression of the iron regulon in *P. aeruginosa*. Another dimension of the iron economy was illuminated by Bisht, Baishya, and Wakeman, who show that under iron scarcity, *P. aeruginosa* ramps up the production of quinolones, which, in turn, lyse *S. aureus* cells, releasing iron for *P. aeruginosa's* use (Bisht, Baishya, & Wakeman, 2020). Additional strategies by which *P. aeruginosa* suppresses *S. aureus* growth have been reviewed elsewhere (Al-Wrafy, Alariqi, Noman, Al-Gheethi, & Mutahar, 2023; Biswas, Biswas, Schlag, Bertram, & Götz, 2009).

The host can also mediate the *P. aeruginosa* – *S. aureus* relationship, directly or indirectly. For example, *P. aeruginosa* can indirectly impact *S. aureus* by modulating the human immune response, stimulating production of the phospholipase sPLA2-IIA by bronchial epithelia cells (Pernet et al., 2014). This phospholipase kills *S. aureus*. Remarkably, a Type VI secretion system (T6SS), typically associated with targeting Gram-negative and eukaryotic cells, has been shown to give *P. aeruginosa* a competitive edge over *S. aureus*, with the potential to inadvertently harm the host during co-infection (Wang et al., 2023).

However, *P. aeruginosa* does not always get everything its own way. For example, exogenous *P. aeruginosa*-derived alginate has been shown to promote the survival of *S. aureus*, suggesting that mucoid *P. aeruginosa* is more tempered in its engagements with its neighbour (Limoli et al., 2017; Price et al., 2020). Moreover, when exposed to glucose, *S. aureus* secretes compounds capable of effectively eliminating *P. aeruginosa* in a dose-dependent manner. These compounds include acetoin, acetic acid, and possibly small peptides (Kvich et al., 2022; Vasiljevs, Gupta, & Baines, 2023). Interestingly, recent work also suggests that *P. aeruginosa* and *S. aureus* coevolve within the CF lung, since *S. aureus* isolates show greater in-host survival when *P. aeruginosa* is present (Bernardy et al., 2022).

The above notwithstanding, the ongoing and nuanced conversation between *P. aeruginosa* and *S. aureus* during co-infections allows sustained microbial colonisation and has a profound impact on the host (Alves et al., 2018; Wang et al., 2023). The polyvalence of *P. aeruginosa*'s attitude towards *S. aureus* seems largely contingent on the specific particularities of genetic backgrounds and environmental conditions, although it is clear that cooperation and antagonism between these two species is common during coinfection, and has implications for antibiotic susceptibility, bacterial burden and clinical outcome (Fischer et al., 2021; Hotterbeekx, Kumar-Singh, Goossens, & Malhotra-Kumar, 2017; Suryaletha, John, Radhakrishnan, George, & Thomas, 2018).

5.1.2 The other neighbours of *P. aeruginosa*

Interactions between *P. aeruginosa* and emerging pathogens like *Achromobacter* spp. present a complex dynamic of both competition and coexistence in chronic co-infection scenarios (Sahl, Baumgarten, Shannon, & Påhlman, 2023; Sandri et al., 2021). *Achromobacter* spp. can secrete exoproducts that interfere with the adhesion ability and biofilm formation of *P. aeruginosa* strains. These interactions are not static but can shift and evolve over the course of a chronic infection (Menetrey, Dupont, Chiron, Jumas-Bilak, & Marchandin, 2020; Sandri et al., 2021). *Achromobacter xylosoxidans* (and other species, such as *P. aeruginosa* and *S. aureus*) have been shown to affect the growth, motility, and antibiotic susceptibility of *Stenotrophomonas maltophilia* (McDaniel, Schoeb, & Swords, 2020; Menetrey et al., 2020; Pompilio et al., 2015). Interestingly, this growth inhibition is not observed with *P. aeruginosa* supernatants alone, indicating a contact-dependent inhibitory mechanism (Tashiro, Yawata, Toyofuku, Uchiyama, & Nomura, 2013). Co-culturing of *S. maltophilia* with *P. aeruginosa* also leads to changes in the expression of several virulence genes in the latter, with an increase in protease and alginate production and a decrease in quorum sensing activity (Menetrey et al., 2020; Pompilio et al., 2015). Perhaps more surprisingly, *S. maltophilia* can mitigate the effects of toxic *P. aeruginosa*-derived metabolites such as hydrogen cyanide on *Burkholderia cenocepacia*. This further reinforces the notion that there is much to learn about the complex network of interactions in polymicrobial infections scenarios (Bernier et al., 2016). In this regard, a recent *in vivo* study suggests that *S. maltophilia* and *P. aeruginosa* co-localise in a mouse pulmonary infection model, and that the two species can readily form mixed biofilms, indicative of a synergistic interaction (McDaniel et al., 2020).

Anaerobes like *Porphyromonas* and *Prevotella* are also being increasingly recognised for their potential roles in chronic infections, including those associated with CF and bronchiectasis (Sherrard et al., 2016). The hypothesis that certain anaerobes may foster environments conducive to colonisation by more "notorious" CF pathogens has gained traction, and is indicative of a more complex microbial interplay within the CF lung than previously appreciated (Quinn et al., 2015). For instance, co-infections involving *P. aeruginosa* and *Veillonella parvula* in a murine model have revealed a marked increase in *P. aeruginosa* loads, highlighting the synergistic potential of anaerobic bacteria in promoting pathogenicity (Pustelny et al., 2015). Similarly, the virulence of obligate anaerobes such as *Porphyromonas gingivalis* appears to be heightened in the presence of *P. aeruginosa*-derived pyocyanin (Benedyk et al., 2015). Interestingly, the lung "anaerobiome" has been

linked with elevated resistance to certain antibiotics (Lamoureux et al., 2021). This points to underexplored community relations in the lung microbiota, where anaerobes impact the course of chronic lung infections and the efficacy of treatment strategies.

5.2 Interkingdom interactions: bacteria and fungi

Interkingdom interactions, particularly between bacteria and fungi, also exhibit contact-dependent communication, extracellular signalling, and metabolic inter-dependencies (Rapala-Kozik et al., 2023). Such relations can profoundly influence biofilm architecture, and therefore virulence and antibiotic susceptibility (Du, Ren, Zhou, Zhang, & Xu, 2022). A significant portion of research in this area has focused on the interplay between fungi such as *C. albicans* or *A. fumigatus* and prevalent bacterial pathogens such as *P. aeruginosa* or *S. aureus*.

5.2.1 Interactions between C. albicans and P. aeruginosa

The dynamics between *C. albicans* and *P. aeruginosa* showcase both synergistic and antagonistic effects (Fourie et al., 2017; Fourie, Cason, Albertyn, & Pohl, 2021; Nogueira, Sharghi, Kuchler, & Lion, 2019). Several studies have demonstrated that the transition between yeast and hyphal forms of *C. albicans* is significantly influenced by *P. aeruginosa*. As Hogan and Kolter (2002) observed, *P. aeruginosa* targets and eradicates the hyphal cells of *C. albicans*, sparing the yeast form. Among the molecular signals involved in this, 3-oxo-C12 homoserine lactone, a QS molecule produced by *P. aeruginosa,* stands out for its ability to inhibit *C. albicans* filamentation without impeding fungal growth (Hogan, Vik, & Kolter, 2004; Ovchinnikova, Krom, Van Der Mei, & Busscher, 2012). Critically, this inhibition of hyphae formation reduces the capacity of *C. albicans* for tissue adhesion and invasion (Maza et al., 2017).

P. aeruginosa also suppresses biofilm formation by *C. albicans*, in a manner dependent on the Pseudomonas quinolone signal (PQS) and its precursor, HHQ. PQS prompts the release of phenazines, which trigger ROS formation, thereby also disrupting fungal biofilm integrity and hyphal development (Kaleli, Cevahir, Demir, Yildirim, & Sahin, 2007; Phelan et al., 2014; Reen et al., 2011). Interestingly, phenazines also synergise the activity of azole-based antifungals (Nishanth Kumar et al., 2014). Conversely, secreted pseudomonal proteases such as LasB unexpectedly stimulate fungal virulence (Peleg, Hogan, & Mylonakis, 2010).

Two-species biofilms containing *C. albicans* and *P. aeruginosa* are thicker and alginate-rich (Kasetty et al., 2021; Phuengmaung et al., 2020). Furthermore,

proteomic analysis of these mixed biofilms revealed altered expression of proteins related to virulence, multidrug resistance, and stress response (Trejo-Hernández, Andrade-Domínguez, Hernández, & Encarnación, 2014).

C. albicans' secretions, including farnesol, tyrosol and eicosanoids, influence both growth and biofilm formation by *P. aeruginosa*. Farnesol not only hampers pyocyanin production and rhamnolipid-mediated swarming, but also broadly dampens the virulence of *P. aeruginosa* (Cugini et al., 2007; Hassan Abdel-Rhman, Mostafa El-Mahdy, & El-Mowafy, 2015; McAlester, O'Gara, & Morrissey, 2008). Similarly, tyrosol curtails the secretion of *P. aeruginosa* haemolysin and protease(s) (Hassan Abdel-Rhman et al., 2015). Eicosanoids, such as prostaglandin E2 (PGE2), are secreted by *Candida spp*. And may serve as immunomodulatory agents in bacterial-fungal dialogues, influencing the progression and outcome of mixed microbial infections (Fourie et al., 2016; Fourie et al., 2017).

Research outcomes significantly hinge on the choice of model systems and experimental conditions. A recent meta-analysis indicated that subtle differences in the timing of sampling, media composition, and experimental setup can drive divergent results, which needs to be borne in mind (Grainha, Jorge, Alves, Lopes, & Pereira, 2020; Kahl et al., 2023; Santos-Fernandez et al., 2023).

5.2.2 Interactions between C. albicans and S. aureus

Compared with *P. aeruginosa*, the interplay between *S. aureus* and *Candida spp*. tends towards a more synergistic relationship (Durand et al., 2022; Short et al., 2023). In mixed biofilms, *S. aureus* adheres to *C. albicans* hyphae *via* the fungal adhesin, Als3p, and uses fungal structures as a scaffold for growth and deeper tissue invasion (Kean et al., 2017; Peters et al., 2012; Peters, Ward, Rane, Lee, & Noverr, 2013). This symbiosis not only increases the biofilm biomass but also enhances the biofilm's antimicrobial tolerance – perhaps due to an enrichment of extracellular matrix (Harriott & Noverr, 2011; Kean et al., 2017; Pammi, Liang, Hicks, Mistretta, & Versalovic, 2013; Peters et al., 2019; Vila et al., 2021). Moreover, *C. albicans* stimulates the *S. aureus agr* QS system, boosting toxin production and amplifying virulence (Todd et al., 2019). However, *C. albicans*-derived farnesol can also disrupt *S. aureus* biofilm development, once again illustrating that these interactons are not always one-way (Jabra-Rizk, Meiller, James, & Shirtliff, 2006).

5.2.3 Interactions between C. albicans and Streptococcus species

The chronic wound microbiome features a notable abundance of *Streptococcus* species, especially *Streptococcus agalactiae*, which interfaces with *C. albicans*

(Smith et al., 2016). A spectrum of behaviours has been attributed to the interaction between *S. agalactiae* and *C. albicans*, again, suggesting a multi-dimensional relationship (Short et al., 2023). Some studies indicate that *S. agalactiae* can suppress *C. albicans* hyphal growth (Yu et al., 2018). Given that the yeast form of *C. albicans* displays reduced immunogenicity (*cf.* the hyphae) this might favour chronic wound colonisation. By contrast, other studies have shown that *C. albicans* can promote *S. agalactiae* colonisation, suggesting that a more symbiotic relationship is involved in enhancing colonisation and virulence (Pidwill, Rego, Jenkinson, Lamont, & Nobbs, 2018; Shing et al., 2020).

5.2.4 Interactions between A. fumigatus and P. aeruginosa

In the CF lung, *P. aeruginosa* often plays a dominant role in its relationship with *A. fumigatus*, employing an array of secretions, including phenazines, pyoverdine and rhamnolipids to suppress fungal growth (Briard et al., 2015, 2017). Again though, not all studies are consistent on this point, and phenazines have also been shown to mobilise environmental iron, thereby nourishing *A. fumigatus* (Nazik, Sass, Déziel, & Stevens, 2020). Moreover, the exoproducts of *P. aeruginosa* can influence *A. fumigatus* protein expression, affecting the synthesis of secondary metabolites like gliotoxin and the fungal response to oxidative stress (Margalit et al., 2022). Remarkably (given the inter-kingdom nature of the interaction), Penner et al. (2016) have suggested that *P. aeruginosa* also employs phage-mediated strategies to curb metabolic activity in established biofilms of *A. fumigatus*.

5.2.5 Interactions between A. fumigatus and others

Co-cultivation studies between *S. aureus* and *A. fumigatus* conidia have revealed a predominantly antagonistic relationship, with *S. aureus* demonstrating a competitive advantage over the fungus. In these interactions, the *S. aureus* cells not only adhere to the fungal conidia but also facilitate aggregation of additional bacterial cells to the site, effectively inhibiting fungal growth. The presence of *S. aureus* on the fungal surface prior to conidial germination is crucial for this conidial lysis and disruption of hyphal development (Ramírez Granillo et al., 2015).

Streptococcus pneumoniae (a Gram-positive species primarily known for causing pneumonia and sepsis among the elderly and children) also appears in the airways of pwCF, and has been linked with pulmonary exacerbation episodes (Askim et al., 2016; Asner et al., 2019; Bhatt, 2013; Maeda et al., 2011; Paganin et al., 2015). *S. pneumoniae* has been shown to inhibit the

growth of *A. fumigatus* and to dismantle pre-formed fungal biofilms *in vitro*; a process mediated by the production of bacterial pneumolysin and hydrogen peroxide (Iwahashi, Kamei, & Watanabe, 2020).

Mixed biofilms of *Stenotrophomonas maltophilia* and *A. fumigatus* have been reported in the airways of pwCF, although again, we caution the reader that the definition of "biofilms" in these samples is largely an observational interpretation. Nevertheless, the fungal morphology in these aggregates is notably affected (Margalit, Carolan, Sheehan, & Kavanagh, 2020; Melloul et al., 2016). Moreover, the antimicrobial susceptibility of both organisms is affected in these samples; *A. fumigatus* shows increased sensitivity to amphotericin B, whereas *S. maltophilia* exhibits enhanced tolerance to levofloxacin (Melloul et al., 2018; Roisin et al., 2020).

5.3 The diverse forms of interspecies communication

Cell-to-cell signalling is a major requirement for social behaviour in the microbial world, and recent discoveries have raised an awareness of the extent and importance of interspecies communication (Banerji, Kanojiya, & Saroj, 2020; He et al., 2023).

QS in Gram-negative proteobacteria employs small diffusible signalling molecules, such as *N*-acylated homoserine-lactones (HSL), alkylquinolones, fatty acid-like compounds (also known as diffusible signal factor (DSF), and α-hydroxyketones) (Hawver, Jung, & Ng, 2016; Ng & Bassler, 2009; Tiaden, Spirig, & Hilbi, 2010). These freely-diffusible molecules bind directly to cognate receptor molecules (transcription factors) in the cytoplasm which, in turn, bind to target DNA. On the other hand, Gram-positive bacteria primarily use modified oligopeptides that are impermeable to biological membranes and are recognised by a two-component membrane-bound receptor (Ng & Bassler, 2009).

For some species, it has been shown that "orphan" receptors can bind and engender a response to exogenous signalling molecules in a process referred to as "eavesdropping" (Banerji et al., 2020; Case, Labbate, & Kjelleberg, 2008). For example, eavesdropping has been observed for the DSF quorum sensing system. DSF signals include a diverse group of *cis*-2-unsaturated fatty acids with varying lengths and branching patterns (Ryan, An, Allan, McCarthy, & Dow, 2015). Until now, the genes related to DSF biosynthesis have been found only in Gram-negative bacteria from the γ- and β-proteobacteria groups (He et al., 2023). However, these molecules appear to be sensed by a much wider range of microbes, even participating in inter-kingdom signalling (reviewed recently by He et al., 2023). In a notable

example of DSF-mediated communication, *cis*-2-decenoic acid produced by *P. aeruginosa* induces not only its own biofilm dispersal, but also that of other bacteria and of *C. albicans* (Davies & Marques, 2009; Jennings, Courtney, & Haggard, 2012). Additionally, it has also been shown that *cis*-2-decenoic acid inhibits *S. aureus* growth (Jennings et al., 2012).

Perhaps the best studied example of inter-species eavesdropping is associated with autoinducer 2 (AI-2), a furanosyl borate diester synthesised by LuxS in both Gram-positive and Gram-negative bacteria (Cao & Meighen, 1989; Miller & Bassler, 2001; Schauder, Shokat, Surette, & Bassler, 2001). The involvement of AI-2 in inter-species communication was first suggested by Duan, Dammel, Stein, Rabin, and Surette (2003), who showed that in a murine "chronic lung infection" model, lung damage by *P. aeruginosa* infection was enhanced by the presence of avirulent microflora. These microflora stimulated the expression of a similar set of genes as AI-2 (Duan et al., 2003). Notably, *P. aeruginosa* does not encode *luxS*, and consequently, does not itself produce AI-2, yet these data indicate reception of exogenously-synthesised AI-2 (Pereira, Thompson, & Xavier, 2013; Stover et al., 2000). It was subsequently shown that AI-2 signalling integrates into the *P. aeruginosa las* signalling system, and thus encourages expression of a wide range of virulence factors (Li et al., 2017). Interestingly, there are two well-characterised receptors of AI-2, LuxP and LsrB, yet *P. aeruginosa* does not encode homologues of these either. Recently, two additional potential AI-2 receptors have been identified (Zhang et al., 2020). These periplasmic proteins are widespread in nature and may indicate that AI-2 signalling is essentially universal (Zhang et al., 2020). Interestingly, AI-2 signalling seems to promote biofilm formation by a certain 'tribe' of colonising microbes (*E. coli*, *Porphyromonas gingivalis*, *Streptococcus spp.*, *P. aeruginosa*), but inhibits biofilm formation by others (*Candida albicans*, *Bacillus cereus*). AI-2 is a good example of a potential 'shibboleth' signal, in whose decoding the fate of the lung environment is determined (Auger, Krin, Aymerich, & Gohar, 2006; Bachtiar et al., 2014; Cuadra-Saenz et al., 2012; González Barrios et al., 2006; Yoshida, Ansai, Takehara, & Kuramitsu, 2005).

Given the overwhelmingly complex and varied experimental findings concerning inter-microbial relations, two things are clear: (i) that proper interpretation of these data requires an awareness of the peccadillos associated with specific experimental regimes, and (ii) that theoretical models describing these data must accommodate the polyvalence of microbial politics.

6. Understanding the ecology of inter-species interactions: computational approaches and perspectives

Experimental investigation remains the gold standard approach for understanding the mechanistic basis for inter-species interactions. However, in order to truly understand ecosystems, computational models are required. Such computational models can capture not only the ecological interactions between species, but also allow modelling of more complex properties of polymicrobial infections. For example, it is now becoming clear that polyspecies interactions in one organ can influence other systems in the body. A good example of this is the so-called gut-lung axis (O'Toole et al., 2021). Computational approaches are well-suited to analysing problems of this sort.

Ho et al. (2023) recently used computational approaches to tease apart the impact of key medications on the ecology of the CF airway microbiota. In a similar vein, McKay et al. (2023) have used computational approaches to segregate the microbiota associated with pwCF and healthy control subjects (McKay et al., 2023). Both of these analyses started off with the "messy" real-world clinical records of several thousand individuals, yet demonstrated not only that patterns could be extracted, but also generated a number of experimentally-testable hypotheses. With finer data granularity, such models can only improve. Indeed, with the wealth of metagenomic data available now, we anticipate that such modelling will become routine (or even, *de rigueur*) in future analyses.

6.1 The joys and miseries of 'curated' databases

There exist a number of microbiological and clinical databases which the infection modeller can mine for data. These open access biological databases include the National Center for Biotechnology Information (NCBI), Human Microbiome Project, Integrated Microbial Genomes and Microbiomes, European Nucleotide Archive, and EnsemblBacteria (Harrison, 2007; Nayfach et al., 2021; Sayers et al., 2022; Turnbaugh et al., 2007; Yates Andrew et al., 2022). The data hosted are predominantly sequencing data and their accompanying metadata.

In the specific case of CF, most developed countries maintain a centralised, curated database of all consenting pwCF in that country. For example, the UK CF Registry maintains records of the CFTR genotype, sex, annual microbiota culture records, and (anonymized) medication

history of each patient (Taylor-Robinson et al., 2018). Such a resource is only possible due to the generosity of the CF community who have given their consent to contribute to science.

However, these databases are often plagued by curation errors; typographical errors, inconsistencies in spelling during transcription, and the plethora of digital artefacts which inevitably accompany the assembly of very large datasets. Moreover, even among good quality data, there exist potential biases. For instance, in clinical microbiology, the use of selective media means that often, we only "see" what the investigators were looking for. And even then, fast-growing/easily cultured species/strains may be disproportionately represented (Goelz et al., 2021; Sondo, Wonni, Klonowska, Koïta, & Moulin, 2023; Wang et al., 2020). This is why small colony variants escaped investigation for so long. We also note that mucoid variants often swamp the plate, making enumeration difficult (if not impossible). Data cleaning, even in "well-curated" databases, is therefore something of a necessity (Ho et al., 2023).

6.2 Mathematical models

Mathematical models describing ecological interactions (Fig. 2) have a long history, with a classical example being the Lotka-Volterra predator-prey model (Ho et al., 2023). This model was developed in the mid-19th to

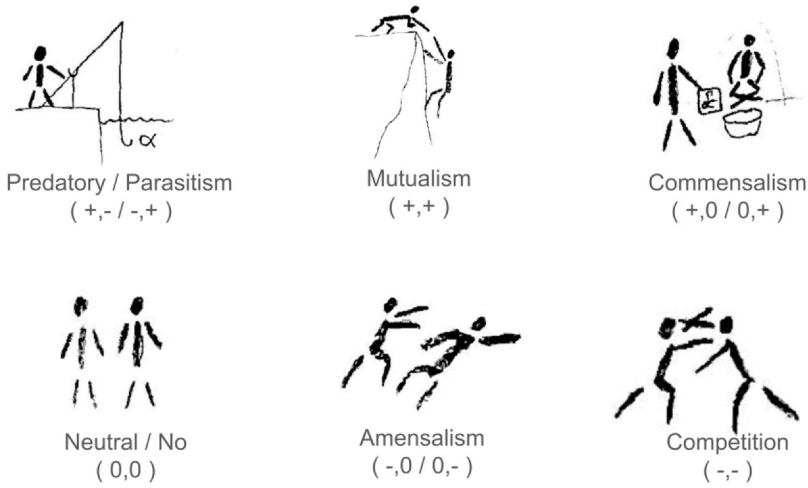

Fig. 2 The polyvalence of ecological relations. Ecological relationships can be simplified to positive, negative and neutral valences, representing an increase or decrease in population size in the presence of a co-habiting species. Here we show the many relationships that can potentially arise from the combinations of those valences, which manifest in both macro- and micro-organisms.

mid-20th century to model lynx-hare dynamics in Canada. [The source data for the model were sales of lynx and hare pelts by the Hudson's Bay Company.] Since that time, a number of other ecological models have been developed, which have been broadly categorised by Van Den Berg et al. (2022) into five families: the Lotka-Volterra type, resource-consumer type, the trait-based type, the individual-based type, and the genome-scale metabolic model type. Choosing the appropriate type of ecological model is important, not only because it affects the number and the type of parameters needed to construct the model, but also because the subject of the model needs to fit its assumptions.

In the context of CF airway infections, exacerbations have been modelled by the Climax-Attack Model (CAM) (Conrad et al., 2013). CAM employs two explicitly defined communities, the "attack" (representing virulent, exacerbation-associated microbiota) and "climax" (representing the stable microbiota) communities, and implements a generalised LV model to predict airway microbiota composition following exacerbation. In this model, each interaction between two microbial categories is explicitly quantified. That means the type and the intensity of each ecological relationship is assumed to be known and is kept constant throughout the airway microbiota succession following exacerbation. Another model, the Island Biogeography Model (IBM), has been proposed for modelling colonisation by airway-associated species (Whiteson et al., 2014). This trait-based model relies on assumptions about both the structure of the airway and the method of microbial colonisation. The model assumes the airway microbiota are always sourced from the trachea (upper respiratory tract) and spread towards the terminal bronchioles. Along the way lie airway "islands", whose species richness is determined by (i) the interconnectedness of "islands", (ii) their distance from the trachea, and (iii) the valence of species interactions. This model was found to be an appropriate model to explain airway microbiota dynamics in young pwCF, but not of pwCF with more progressed disease (Boutin & Dalpke, 2017). Finally, the "neutral model" was proposed as a null model (Huang et al., 2011). This individual-based model is purely based on a probabilistic account of species migration over a grid scenario. The model assumes that the airway is a large surface of grids initially colonised by a random microbial species. Following a series of extinction events on random grid cells, each emptied grid is then immediately recolonised by another microbe (of the same or different species). The recolonisation event is based on the distance of the invading microbe from the emptied grid cell, and the success rate is based on a probability distribution function. However,

the neutral model has proven to be a poor model, as the predictions are drastically different from clinical observations (Huang et al., 2011). Nevertheless, the CAM, IBM and neutral models all share one critical similarity: they assume ecological interactions between two microbial sub-populations are constant and unchanging in character. This is now known to be incorrect; even pairwise interactions between a single pair of microorganisms, such as *P. aeruginosa* and *S. aureus*, can be mutualistic, predatory, amensalistic, or competitive, depending on the experimental model being examined (Reece, Bettio, & Renwick, 2021). Consequently, another approach is required.

6.3 The current state of the art: rethinking levels of organisation

There is a growing realisation that microbial interactions can be far more malleable than previously appreciated. Moreover, that our models need to be somewhat more agnostic and less assumption-based. For example, the computational study of CF- and wound-associated infections often focus on the individual ecologies of a few key pathogens, determined to be important *a priori* (Frost, Nazareth, Charman, Winstanley, & Walshaw, 2019; Hatziagorou et al., 2020; Kalan et al., 2019). On the plus-side, the enormous explosion of available genomic/metagenomic data in recent years is providing the modeller with unparalleled access to fine-grained, and often temporally- and spatially-resolved data. Indeed, this wealth of resource presents investigators with a problem of where to look first, and many approaches likely lose something by dividing up the data. This notwithstanding, several recent analyses have started to look holistically at treatment and microbiota in the context of CF and wounds, so progress is being made (Chen, Burgess, Verpile, Tomic-Canic, & Pastar, 2022; Keogh, Seaman, Barrett, Taylor-Robinson, & Szczesniak, 2019; Tang et al., 2023). However, in doing so, they tend also to eschew causative explanations (remaining agnostic to the mechanistics). This lack of mechanistic insight tends to frustrate foundational scientists, further cementing the psychological divide between experimentalists and modellers. Our view is that this unhealthy situation needs to be reversed.

One modelling approach that has gained significant traction across disciplines over the last decade is metabolic modelling (Henson, Orazi, Phalak, & O'Toole, 2019; Lee et al., 2016; Phalak & Henson, 2019). Although this remains grounded in certain key assumptions (e.g., that the microbial communities being modelled are constrained to certain dominant taxa), the main elements of the model (shared metabolites) are considered

to be "public goods" available to all species in the niche (Henson, Orazi, Phalak, & O'Toole, 2019; Mould et al., 2024). In addition, these models appeal to experimentalists because they explicitly consider genes and defined metabolic frameworks. Consequently, such models integrate a variety of 'omic and microbiological data.

On a final note, and as noted above, data quality is often limiting. However, we recently introduced a Bayesian-like simulation system for parameter optimisation based on the generalised Lotka-Volterra model (Ho et al., 2023). This approach took a purely ecological approach to interpreting the interactions between microbes, antimicrobial agents, and other medications, treating all of these interventions as equivalent ecological agents that contribute to the sum of the microbial interactome. Using this agnosticism as a point of departure, we grouped organisms and medical regimes, not according to prior knowledge *per se*, but rather according to the patterns observable in the data. This confirmed the fluidity of microbial interactions, and further, revealed the important role played by certain therapeutic interventions in remodelling these interactions (Ho et al., 2023).

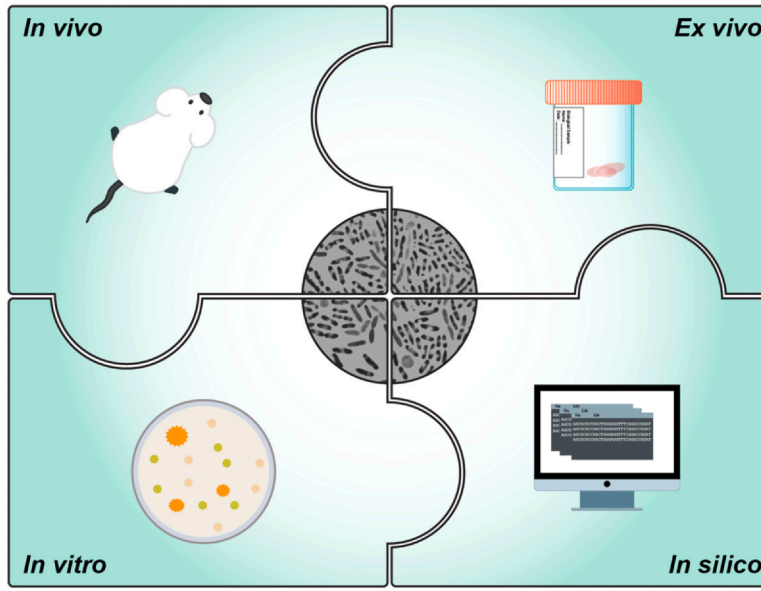

Fig. 3 The jigsaw puzzle of experimental microbiology. Across the many schools of approach in microbiology, advances are being made in both understanding and methodological power. However, there remains a lag in marrying those advances together. This illustration points to the necessity of fitting together findings made *in vivo*, *in vitro*, *ex vivo* and *in silico*.

7. Conclusions

With the advent of culture-independent analyses, it is now clear that microbial ecosystems are more complex and inter-linked than previously imagined, and that a good deal of "new biology" awaits the next generation of researchers (Fig. 3). Consequently, this recent ecological turn of mainstream microbiology looks to be a positive one, and is driving new conceptual tools and resources. This will be crucial if we are to better understand how antimicrobials work (at a community level) and how biofilm formation, virulence and AMR are linked in such highly-networked populations.

A promising direction is the development of comprehensive, continuously updated databases that can serve as central repositories for information on polymicrobial interactions. A notable example of such a database is the Inter-Species Crosstalk Database by Magalhães et al. (2022), which offers expertly curated data on the molecular basis of interspecies interactions in co-infection scenarios. In our view, and although currently underused, such resources hold great promise for better understanding polymicrobial infections. The only lament of the senior author of this review is that he did not engage in this area of research when he was first embarking on his career; mitigating, at that time, polymicrobial interactions were seen as something of a "backwater" of microbiology, associated mainly with understanding sewerage sludge bioreactors, ruminants and so on – not exactly either "molecular" or inspiring, but how things have changed! We are entering a fascinating new era of microbiology, and the next generation of researchers are lucky indeed.

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