



Influence of the lung microbiome on antibiotic susceptibility of cystic fibrosis pathogens

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Interspecies interactions in the lung microbiome may influence the outcome of antibiotic treatment targeted at cystic fibrosis pathogens <http://bit.ly/2WQp0iQ>

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ABSTRACT The lungs of patients with cystic fibrosis (CF) are colonised by a microbial community comprised of pathogenic species, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and microorganisms that are typically not associated with worse clinical outcomes (considered as commensals). Antibiotics directed at CF pathogens are often not effective and a discrepancy is observed between activity of these agents *in vitro* and in the patient. This review describes how interspecies interactions within the lung microbiome might influence the outcome of antibiotic treatment targeted at common CF pathogens. Protective mechanisms by members of the microbiome such as antibiotic degradation (indirect pathogenicity), alterations of the cell wall, production of matrix components decreasing antibiotic penetration, and changes in metabolism are discussed. Interspecies interactions that increase bacterial susceptibility are also addressed. Furthermore, we discuss how experimental conditions, such as culture media, oxygen levels, incorporation of host-pathogen interactions, and microbial community composition may influence the outcome of microbial interaction studies related to antibiotic activity. Hereby, the importance to create *in vitro* conditions reflective of the CF lung microenvironment is highlighted. Understanding the role of the CF lung microbiome in antibiotic efficacy may help find novel therapeutic and diagnostic approaches to better tackle chronic lung infections in this patient population.

Introduction

In patients with cystic fibrosis (CF), mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene create a multifactorial syndrome, with pulmonary disease representing the largest contributor to morbidity and mortality [1]. The CFTR gene, which encodes a cAMP-regulated chloride and bicarbonate transport channel, regulates water and ion homeostasis in epithelial tissues [2]. Mutations in this gene affect an array of physiological processes ranging from mucosal hydration and mucociliary clearance to (innate) immunity. In the lung epithelium, this co-occurrence of defective pathways generates a favourable niche for (chronic) bacterial infection, which results in progressive and irreversible lung damage [3, 4]. Up to 80% of adult CF patients become chronically infected with the conventional pathogen *Pseudomonas aeruginosa*. In addition, *P. aeruginosa* is the dominant bacterium in ~40% of adult CF patients [5]. The prevalence of *Staphylococcus aureus* is ~50%, with the prevalence of methicillin-resistant *Staphylococcus aureus* rapidly increasing, predominantly in the USA [6]. Another

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conventional CF pathogen is *Burkholderia cenocepacia*, which is only found in ~3–4% of patients but is associated with a drastic decrease in lung function [6]. Pathogens also include *Streptococcus* spp., which have been identified in over 25% of patient samples, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* and non-tuberculous mycobacteria such as *Mycobacterium abscessus*, which are found in ~5–10% of patients [6–8]. *Haemophilus influenzae* frequently colonises the respiratory tract of children with CF (up to 32% for children aged 2–5 years), and <10% of adults with CF are carriers of this pathogen [6]. Most of these pathogenic species form biofilms in the CF lung mucus, which are clusters of microorganisms encased in a self-produced matrix attached to biotic/abiotic surfaces and/or host components (such as mucins in the case of CF) [9, 10]. The biofilm mode of growth is associated with reduced susceptibility to antimicrobials [11].

In case of an exacerbation or respiratory symptoms, the above-listed pathogens are targeted through antibiotic therapy. In general, the choice of antibiotics is based on the susceptibility profile determined using routine clinical microbiology assays, such as minimal inhibitory concentration tests. Nevertheless, a discrepancy between the antibiotic efficacy in these laboratory tests and in CF patients is observed [12]. Microenvironmental factors present in the CF lung that are not mimicked in conventional antibiotic susceptibility assays and which may contribute to the gap between antibiotic efficacy *in vitro* and *in vivo* include low oxygen levels, increased iron levels, host cells (such as lung epithelial and immune cells) and their secreted products (such as mucins and metabolites), acidic pH, and the microbiome [13, 14]. This review focuses on the influence of microorganisms present in the CF lung environment, collectively termed the lung microbiome, and the antibiotic efficacy against pathogenic species.

The lung microbiome of patients with CF

Besides the pathogens associated with worse clinical outcomes described above, a large number of other microorganisms are present in the CF lung environment, whose role in pathogenesis (if any) is poorly understood. Genera that are frequently identified in CF lungs include *Rothia*, *Gemella*, *Actinomyces*, *Neisseria*, *Atopobium* and *Granulicatella* [8, 15–17]. Obligate anaerobic bacteria such as *Prevotella*, *Veillonella*, *Fusobacterium* and *Porphyromonas* spp are also present in CF airways [18–20]. Besides bacterial microbiome members, the CF lung microbiome also includes a fungal community, primarily consisting of *Aspergillus fumigatus* and *Candida albicans*, but other species of *Candida* or *Aspergillus*, *Scedosporium* spp and *Malassezia* spp have also been detected [21, 22]. A collection of respiratory viruses and bacteriophages are present as well [23–25]. The composition of the CF lung microbiome gradually changes over time. Highest microbiome diversity is observed during childhood, and diversity decreases from adolescence to adulthood [26]. This decline in diversity mostly leads to communities dominated by members of the Pseudomonadaceae, or even by one pathogen (*i.e.* *P. aeruginosa*) [27]. The decreased microbial diversity is typically associated with antibiotic use [28] and with worsened lung function [27, 29]. Importantly, significant heterogeneity in community composition is observed between patients [19, 29], and spatial variability in bacterial populations is found in the lungs of paediatric CF patients [30].

Interspecies interactions influence efficacy of antibiotics

Given that a complex bacterial community is present in the CF airways, the behaviour and antibiotic susceptibility of CF pathogens is increasingly studied in the presence of other species (referred to as multispecies cultures). Microorganisms in a multispecies community may establish interspecies interactions that can lead to synergistic or antagonistic effects on specific microbiome members and eventually influence the overall behaviour of the community in the host. Specifically, interspecies interactions can induce changes in spatial organisation of the community, biofilm formation, metabolic pathways, production of virulence factors (*e.g.* siderophores, enzymes, toxins, adhesins and surfactants) and quorum sensing molecules, microbial viability or antibiotic susceptibility [31–36]; the latter being the focus of the present review. An overview of reported mechanisms through which members of the microbiome can protect or sensitise CF pathogens to antibiotics is presented in figure 1. While some of the cited literature is not CF-specific and may be presented in the context of other polymicrobial infections (such as bronchiectasis, chronic bronchitis and otitis media), all discussed studies involve microbial and fungal species which are often part of the CF lung microbiome.

Interspecies interactions that protect pathogens from antibiotics

Through enzymatic antibiotic degradation

An important form of antibiotic protection in multispecies communities is “indirect pathogenicity” (also termed “passive resistance”), which is achieved through antibiotic degradation by a resistant species. Antibiotic degradation by a resistant species may enable survival/growth of a susceptible pathogen in the community, the latter being the intended target of the antimicrobial treatment [37, 38]. Several examples

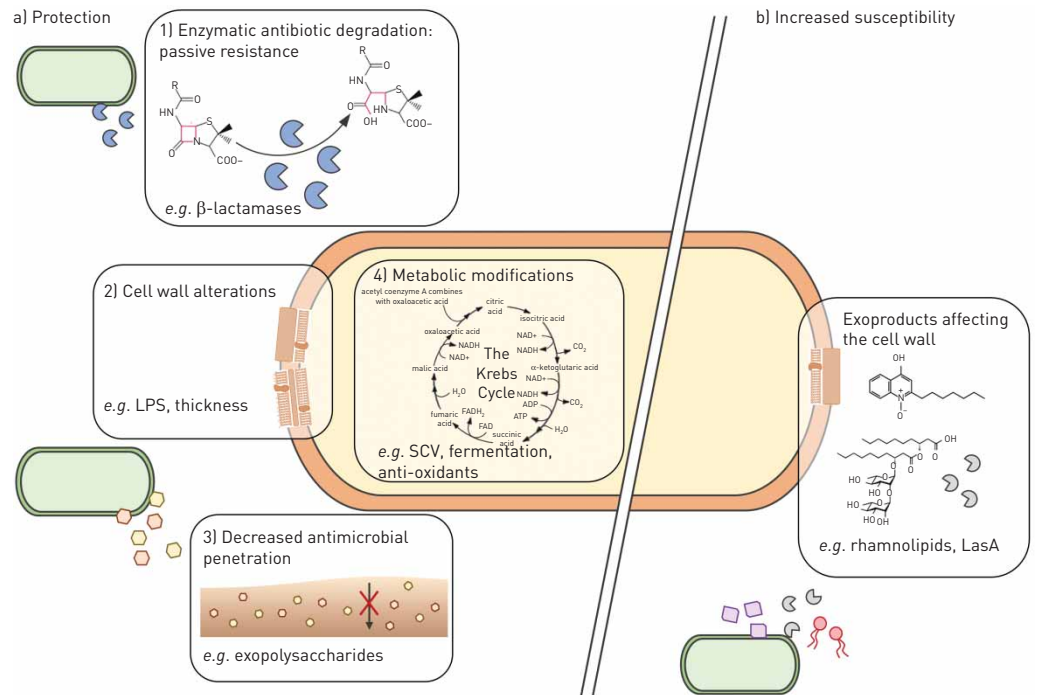


FIGURE 1 Overview of possible mechanisms through which members of the cystic fibrosis microbiome can influence antibiotic efficacy against pathogenic species. a) Protection of pathogens by microbiome members from antibiotics through: 1) enzymatic degradation of antibiotics (indirect pathogenicity); 2) alterations in the composition or thickness of the cell wall; 3) decreased antimicrobial penetration; and 4) metabolic modifications. b) Increased antibiotic susceptibility of pathogens by microbiome members through production of exoproducts compromising the cell wall. LPS: lipopolysaccharide; SCV: small colony variant; LasA: elastase.

of indirect pathogenicity are described for bacterial members of the CF lung microbiome, and are shown in table 1. One of the best known examples of indirect pathogenicity is the protection of one species from killing by β -lactam antibiotics through β -lactamases produced by another species. MADDOCKS *et al.* [39] were the first to describe this phenomenon. Indirect pathogenicity is also described for other antimicrobial agents. For example, chloramphenicol resistant *S. pneumoniae* and *S. aureus* strains expressing chloramphenicol acetyltransferase (CAT) were able to protect susceptible *S. pneumoniae* cells by intracellular antibiotic degradation of chloramphenicol [38]. Strikingly, susceptible cells were able to outcompete resistant bacteria during antibiotic therapy when the fitness cost (such as reduced growth rates or a reduced maximum cell density) of CAT expression became too high. Therefore, passive resistance is only sustainable when the expression comes at a modest fitness cost. Certain anaerobic species that are part of the CF lung microbiome, such as *Prevotella*, have also been reported to produce β -lactamases, thus protecting *P. aeruginosa* from ceftazidime [46]. Similarly, *M. catarrhalis* provided protection to *H. influenzae* through secreted β -lactamases in a polymicrobial biofilm [40]. Interestingly, *H. influenzae* also protected *M. catarrhalis* from antibiotics which were mediated through another mechanism, *i.e.* increased biofilm formation by *M. catarrhalis* induced by quorum sensing molecules (dihydroxypentanedione) produced by *H. influenzae*.

Through alterations of the cell wall

Another mechanism that may enable CF pathogens to be protected from antibiotic treatment is through effects of members of the lung microbiome on the cell wall. *Streptococcus anginosus* grown in a biofilm with *P. aeruginosa* and *S. aureus* became more tolerant to various cell wall-acting antibiotics [49]. The enhanced tolerance of *S. anginosus* to vancomycin was due to an increased cell wall thickness, as a result of co-culturing with these CF pathogens [50]. Furthermore, it was found that soluble factors produced by *S. aureus* were mediating the enhanced antibiotic tolerance in the described multispecies community, in a strain-independent manner [49]. Another example of antibiotic protection through alterations of cell wall components was described by TOGNON *et al.* [51]. Experimental evolution of *P. aeruginosa* in the presence of *S. aureus* resulted in lipopolysaccharide alterations in *P. aeruginosa* which in turn led to increased β -lactam resistance [51].

TABLE 1 Examples of indirect pathogenicity for bacteria that are part of the cystic fibrosis lung microbiome

Species protected	Protected against	Protected by	Produced by	[Ref.]
<i>Haemophilus influenzae</i>	Ampicillin	Penicillinases	Enterobacteriaceae	[39]
	β -lactam antibiotics	β -lactamases	<i>M. catarrhalis</i>	[40]
Streptococci	Penicillin	Penicillinases	Staphylococci	[41]
Ampicillin-susceptible <i>Staphylococcus aureus</i>	Ampicillin	Penicillinases	<i>P. aeruginosa</i>	[42]
<i>Streptococcus pneumoniae</i>	β -lactam antibiotics	β -lactamases	<i>M. catarrhalis</i>	[43]
	Amoxicillin	β -lactamases	<i>H. influenzae</i>	[44]
	Chloramphenicol	Chloramphenicol acetyltransferase	Resistant <i>S. aureus</i> / <i>S. pneumoniae</i>	[38]
<i>Pseudomonas aeruginosa</i>	Imipenem and ceftazidime	β -lactamases	<i>S. maltophilia</i>	[45]
	Ceftazidime	β -lactamases	<i>Prevotella</i> sp.	[46]
<i>Escherichia coli</i>, <i>Klebsiella pneumoniae</i> and <i>Pseudomonas aeruginosa</i>	Carbapenems	Carbapenem-hydrolysing class D β -lactamases	<i>A. baumannii</i>	[47]
<i>Staphylococcus aureus</i>	Gentamicin	Aminoglycoside-modifying enzymes	<i>P. aeruginosa</i>	[48]

Through decreased antimicrobial penetration

Exopolysaccharides produced by *B. cenocepacia* can interact with those produced by *P. aeruginosa* and result in a firmer multispecies biofilm, which in turn leads to a decrease in diffusion of antibiotics, and to an increased tolerance [52]. Similarly, the production of Psl polysaccharides by *P. aeruginosa* biofilms protects non-Psl producers, such as *S. aureus*, against antibiotics when grown as multispecies biofilm communities [53]. Specifically, Psl protects members of the biofilm from cationic antimicrobial peptides (colistin and polymyxin B), tobramycin and, to some extent, ciprofloxacin; this effect is only observed for early, and not later, stages of biofilm development. However, it is important to consider that *S. aureus* is often killed by *P. aeruginosa*, due to the production of multiple antimicrobial and anti-staphylococcal factors by the latter [54]. Hence, only if *S. aureus* is able to survive in a mixed species community with *P. aeruginosa*, might it benefit from the *P. aeruginosa*-produced extracellular matrix.

Protective interactions between fungal and bacterial species of the CF microbiome have also been reported. Indeed, *S. aureus* was shown to be preferentially associated with the hyphae of *Candida albicans* through binding to surface-associated adhesins [55]. More specifically, the agglutinin-like sequence 3 (Als3p), a *C. albicans* hyphae-specific adhesin, is involved in the co-adherence process [56]. Co-colonisation with *C. albicans* hyphae enables *S. aureus* to invade epithelial cells, as *C. albicans* adheres to and invades tissues through its invasive hyphae. This phenomenon is described as “microbial hitchhiking” [57]. During this process, *S. aureus* becomes coated by extracellular matrix (and more particularly β -1,3-glucan) secreted by *C. albicans*, which results in an enhanced tolerance of *S. aureus* to vancomycin [58].

Through changes in metabolism

Bacterial metabolism and antibiotic tolerance are closely connected [59]. Exoproducts of *P. aeruginosa*, such as 2-heptyl-4-hydroxyquinoline (HQNO) and pyocyanin, inhibit respiration in *S. aureus*, which can result in the formation of small colony variants; phenotypic variants that are highly tolerant to antibiotics [60, 61]. Also, HQNO and siderophores of *P. aeruginosa* were found to induce fermentation pathways in *S. aureus* [62]. As many antibiotics, including aminoglycosides, rely on energy generated by the tricarboxylic acid cycle for their uptake and downstream activity [63, 64], switching to a fermentative metabolism could affect antibiotic efficacy. In a follow-up study, ORAZI *et al.* [65] found that *P. aeruginosa* supernatant (containing HQNO and siderophores) protected *S. aureus* against antibiotics acting on the cell wall and protein synthesis. The authors hypothesised that the observed slower growth and/or shift to a fermentative growth mode of *S. aureus* in response to *P. aeruginosa* supernatant were at the origin of the antibiotic tolerance. A separate team further provided mechanistic evidence that HQNO produced by *P. aeruginosa* resulted in inhibition of respiration and intracellular ATP depletion in *S. aureus*, which in turn conferred antibiotic tolerance in the latter [66]. Another example of metabolic modifications induced by interspecies interactions is the protection of *A. baumannii* by pyocyanin produced by *P. aeruginosa* against killing by amikacin and carbenicillin [67]. Pyocyanin leads to the generation of reactive oxygen species in *A. baumannii*, subsequently inducing the expression of catalase and superoxide dismutase, as a protective mechanism against oxidative stress. Since the generation of reactive oxygen species is part of the antimicrobial activity of bactericidal antibiotics [68], the production of anti-oxidative molecules can result in enhanced bacterial tolerance.

Interspecies interactions that render pathogens more susceptible to antibiotics

Some studies have reported interspecies interactions that lead to enhanced susceptibility to antibiotics. In contrast to the previously described reports that showed protection of *S. aureus* by *P. aeruginosa*, several studies found an enhanced susceptibility of *S. aureus* when cultured with this CF pathogen. An elegant study by RADLINSKI *et al.* found that the antagonising or potentiating effect of *P. aeruginosa* towards *S. aureus* antibiotic susceptibility when grown as planktonic cultures strongly depended on the strain of *P. aeruginosa* used. More specifically, changes in antibiotic susceptibility of *S. aureus* induced by *P. aeruginosa* depended on the production of HQNO, rhamnolipids and LasA endopeptidase by the latter. Rhamnolipids, amphiphilic molecules that interact with the cell membrane thereby increasing permeability [69], were found to stimulate tobramycin uptake and potentiate its activity. Hence, *P. aeruginosa* strains producing high levels of rhamnolipids and no HQNO strongly enhanced tobramycin activity against *S. aureus*. In addition, production of the staphylolytic enzyme LasA by *P. aeruginosa*, which cleaves pentaglycine cross bridges in *S. aureus* peptidoglycan, strongly potentiated vancomycin activity. Furthermore, using an *in vivo* burn wound co-infection model of *P. aeruginosa* and *S. aureus*, RADLINSKI *et al.* [66] found that the antibiotic-potentiating effect of LasA dominates over the antibiotic-antagonising effect of HQNO, resulting in a net increased susceptibility of *S. aureus* in the presence of *P. aeruginosa*. These data are in agreement with recent findings by TAVERNIER *et al.* [49], who reported a decreased tolerance of *S. aureus* to different antibiotic classes when grown in a multispecies biofilm with *P. aeruginosa* and *S. anginosus*. Co-culturing of *S. aureus* with these two CF community members prevented vancomycin-induced thickening of the cell wall (a protective mechanism during antibiotic exposure), which might be explained by the production of LasA by *P. aeruginosa* [66]. Increased antibiotic susceptibility of *Mycobacterium abscessus* was observed when co-cultured with *P. aeruginosa* on three-dimensional *in vivo*-like lung epithelial cells [70], yet the mechanism behind this observation remains to be elucidated.

Importance of experimental set-up in investigating interspecies interactions

Finding an optimal balance between an achievable experimental set-up and physiological relevance is challenging when studying the influence of microbial communities on antibiotic efficacy. Indeed, a multiplicity of experimental variables need to be considered, which may influence experimental outcomes, even though comparative studies are scarce. This section highlights studies that have demonstrated an influence of experimental conditions on the outcome of interspecies interaction studies related to antibiotic activity.

Culture conditions

The choice of culture media, ranging from minimal media to rich and viscous sputum-like media, may shape the composition, organisation and susceptibility of the community. BERNIER *et al.* [71] showed that *B. cenocepacia* became less susceptible to cyanide produced by *P. aeruginosa* in the complex synthetic CF medium (SCFM) compared to in an undefined rich medium. Physiological levels of serum albumin can decrease *P. aeruginosa* virulence by sequestering acyl homoserine lactone (AHL) quorum sensing molecules [72]. Given the role of these AHLs in the production of virulence factors such as the previously described LasA [66], this might influence the potentiating activity of *P. aeruginosa* towards antibiotics targeted at *S. aureus*. Another important parameter influencing research outcomes is the oxygen level. In CF airways, viscous CF sputum creates a steep oxygen gradient and anoxic niches [73, 74]. Interspecies interactions can be altered in response to varying oxygen concentrations. In a study by SCHERTZER *et al.* [75], the Pseudomonas Quinolone Signal (PQS) and PQS-controlled factors were not produced during anaerobic growth of *P. aeruginosa* due to the oxygen-dependency of PqsH, hence limiting virulence and the antimicrobial effect of PQS on other species. Furthermore, host cells such as lung epithelial cells and innate immune cells may influence interspecies interactions that result in altered antibiotic efficacy. Only a few studies have included host cells when studying interspecies interactions [62, 76, 77]. A recent study demonstrated that *P. aeruginosa* influences the antibiotic susceptibility of *M. abscessus* differently when biofilms of both species are grown on plastic or on a three-dimensional lung epithelial cell culture model [70].

Microbial community members

The choice of microorganisms to include in synthetic communities is important. Thus far, most studies have used synthetic communities comprised of two to three species, hereby focusing on interactions between pathogenic microorganisms (*P. aeruginosa* and *S. aureus*) [54, 78]. While this approach provides initial insights into microbial interactions and downstream effects on antibiotic activity, the reduced complexity is not reflective of the *in vivo* situation, as an average of 30 species are present in the CF airways [28]. To compose multispecies communities, strains can be selected *via* several approaches: 1) strains from established strain collections (*e.g.* ATCC, BCCM/LMG and DSMZ); 2) (co-evolved) strains

isolated from the same environment (such as from CF sputum samples); or 3) a complex, unprocessed, environmental or clinical sample containing the entire microbiome [79].

Although studies aim to create an environment that is as (clinically) relevant as possible, there has to be a balance between complexity and practical feasibility to create a controlled environment. In this regard, RØDER *et al.* [79] speculated that, at the level of interspecies interactions, the entire bacterial community might not need to be represented, as bacterial species presumably only come into contact with a subset of other species. However, as secreted signalling molecules and virulence factors can migrate over short (~100 µm) to long (blood stream) distances, it could still be expected that distant community members may influence local interspecies interactions [80, 81]. Co-adaptation of species has been observed, leading to mutations and metabolic dependencies, which can influence subsequent interactions [82]. Also, phenotypic and genotypic adaptations and resulting diversity of individual bacterial species has been reported both for CF pathogens (*P. aeruginosa* and *S. aureus*) and non-pathogens (*Rothia mucilaginosa*) [83–85]. An additional consideration when selecting microbiome members to design synthetic communities, is that patient samples may contain culturable and non-culturable bacterial species. Yet, for *in vitro* studies, only culturable species are eligible for inclusion in synthetic communities. However, SIBLEY *et al.* [86] determined that in the CF microbiome, most species are culturable. This includes fungal species, which, compared to bacterial species, have received limited attention in the context of interspecies interactions leading to effects on antibiotic activity. In addition to the above-described influence of *C. albicans* on *S. aureus* [58], bacterial pathogens may also modify the susceptibility of fungal species to antifungal agents [87], enforcing the importance to include fungal species in synthetic communities.

Finally, while many studies have used commonly isolated and/or abundant members of the lung microbiome for studying interspecies interactions [78], species with lower abundance and/or prevalence in the respiratory tract of CF patients are also included in synthetic communities [70]. In this regard, it remains to be defined whether a threshold abundance of microbiome members needs to be reached for downstream effects on antibiotic susceptibility of CF pathogens. This brings forward the importance of incorporating physiologically relevant absolute and relative abundances of individual microbiome members in synthetic communities. Herewith, using the entire microbiome for *in vitro* experiments can increase the experimental relevance. QUINN *et al.* [88] cultured complete sputum samples and homogenised explant lung tissue in glass tubes with artificial sputum medium (based on the Winogradsky column) to generate relevant microbial consortia. Nevertheless, challenges may arise using this type of approach, including changes in microbiome composition with culture time and high antibiotic concentrations in the microbiome samples, which may influence downstream antibiotic susceptibility assays.

Quantification of individual species in a consortium following antibiotic treatment

A common method to quantify different bacterial species in synthetic bacterial consortia is through plating on selective growth media. These media rely on the addition of selective compounds (*e.g.* antimicrobial agents and high salt concentrations), or incubation parameters (*e.g.* low oxygen conditions) to allow growth of a single species [89–91]. While this approach does not allow to quantify viable but non-culturable bacteria, it provides information on the community composition. Nevertheless, antimicrobial agents present in selective media were found to influence recovery of specific lung microbiome members following antibiotic exposure [92]. Hence, prior evaluation and/or optimisation of selective media is needed to ensure quantitative recovery of individual members of a multispecies community. Furthermore, with increasing community richness, the availability and technical challenges associated with developing selective media might limit their wide application.

Quantitative real-time (qRT)-PCR is a culture-independent approach which can be combined with propidium monoazide (PMA) pre-treatment to exclude dead cells and extracellular DNA from the quantification [93]. However, it should be considered that antimicrobial therapies that kill bacteria without sufficiently compromising the cell membrane can result in overestimation of viable bacteria using PMA-qRT-PCR [94]. An alternative method for evaluating microbial community composition is PNA-FISH (peptide nucleic acid-fluorescence *in situ* hybridisation) which can be combined with imaging, hence providing information on spatial organisation of communities [95]. LOPES *et al.* [96] recently quantified bacteria in a multispecies biofilm consisting of *P. aeruginosa*, *Inquilinus limosus* and *Dolosigranulum pigrum* comparing plating on selective media, qRT-PCR and PNA-FISH. Higher bacterial counts were obtained with qPCR and PNA-FISH compared to culture, and discrepancies between PNA-FISH and qRT-PCR data for certain species were observed, showing the impact of the quantification method on study outcomes. The composition of complex synthetic consortia can also be determined using the methodology adopted for microbiome analysis of patient samples, *i.e.* 16S rRNA sequencing (16S rRNA amplicon sequencing of all microorganisms) and metagenomics sequencing (total genomic content of the sample). Nevertheless, these approaches are not common for studying interspecies interactions

in vitro given the use of synthetic communities with low richness for most CF-related research thus far. NELSON *et al.* [97] recently subjected synthetic communities of seven common CF microbiome members to 16S rRNA or metagenomics sequencing, and compared the phylogenetic composition to viable cell counts obtained through plating. Prior to sequencing, various DNA extraction protocols were compared aiming to diminish interference of extracellular DNA (from dead bacteria and/or biofilms) in the experimental outcome. DNA extraction protocols included, among others, benzonase (nuclease) and PMA treatment prior to standard phenylchloroform-based extraction. The authors concluded that metagenomics sequencing in combination with a modified benzonase treatment protocol resulted in a phylogenetic profile that most closely resembled the culture-based input [97]. While sequencing-based approaches provide information on the relative abundance of microorganisms in a community, they do not provide quantitative data on the bacterial load of individual species (absolute abundance). To this end, 16S rRNA sequencing has been combined with absolute bacterial cell quantification by flow cytometry, as a proxy for quantification of individual microbiome members in fecal or environmental samples [98, 99]. Whether this approach would be feasible and provide a comprehensive picture of community composition in respiratory samples remains to be determined.

Finally, other non-culture based omics approaches such as metabolomics and metatranscriptomics are receiving increasing attention in the CF microbiome field [100–103], and may represent interesting tools to monitor microbial community composition and viability *in vitro* as well. To this end, in-depth comparative studies between these non-culture based and culture-based approaches will be essential to determine their suitability to accurately reflect microbial community content and viability before and after antibiotic treatment.

Conclusions

With the expanding knowledge on microbial community composition in the lungs of patients with CF, researchers have started deciphering whether interspecies interactions within this community influence the efficacy of antibiotics against CF pathogens. To address this question, most studies have used synthetic communities comprised of two to three microbial species. These studies often report pathogen protection from antibiotics by microbiome members, though several reports also identified interactions that enhance antibiotic susceptibility of CF pathogens. Nevertheless, it remains to be determined whether microbial interactions take place in the highly complex milieu of the CF lung. In this regard, the adopted experimental set-up is of essence and should incorporate physiologically relevant factors in order to obtain an *in vitro* response that is reflective of the *in vivo* scenario. This includes environmental conditions that mimic the physicochemical properties of mucus (including composition and viscosity), low oxygen levels, host cells (*e.g.* lung epithelial cells and neutrophils), and a representative microbial richness and evenness. In addition, including phenotypic and genotypic diversity of specific microbial species, observed for CF pathogens such as *P. aeruginosa*, when studying interspecies interactions *in vitro* could allow generating an experimental outcome relevant for the patient. Mapping microbial interactions and downstream effects on the efficacy of antibiotics may help understand the limited antibiotic activity in CF patients, as well as the discrepancy between antibiotic activity *in vitro* and in the patient. Furthermore, identifying microbiome members that protect key CF pathogens from antibiotics could represent an indirect treatment target, while microorganisms that enhance antibiotic efficacy could provide novel microbiome-based approaches for treatment.

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