



Developing a model for cystic fibrosis sociomicrobiology based on antibiotic and environmental stress



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ARTICLE INFO

Keywords:

Antibiotic therapy
Cystic fibrosis
Interspecies interactions
Polymicrobial biofilms
Pseudomonas aeruginosa

ABSTRACT

Cystic fibrosis (CF) infections are invariably biofilm-mediated and polymicrobial, being safe to assume that a myriad of factors affects the sociomicrobiology within the CF infection site and modulate the CF community dynamics, by shaping their social activities, overall functions, virulence, ultimately affecting disease outcome. This work aimed to assess changes in the dynamics (particularly on the microbial composition) of dual-/three-species biofilms involving CF-classical (*Pseudomonas aeruginosa*) and unusual species (*Inquilinus limosus* and *Dolosigranulum pigrum*), according to variable oxygen conditions and antibiotic exposure.

Low fluctuations in biofilm compositions were observed across distinct oxygen environments, with dual-species biofilms exhibiting similar relative proportions and *P. aeruginosa* and/or *D. pigrum* populations dominating three-species consortia. Once exposed to antibiotics, biofilms displayed high resistance profiles, and microbial compositions, distributions, and microbial interactions significantly challenged. The antibiotic/oxygen environment supported such fluctuations, which enhanced for three-species communities.

In conclusion, antibiotic therapy hugely disturbed CF communities' dynamics, inducing significant compositional changes on multispecies consortia. Clearly, multiple perturbations may disturb this dynamic, giving rise to various microbiological scenarios in vivo, and affecting disease phenotype. Therefore, an appreciation of the ecological/evolutionary nature within CF communities will be useful for the optimal use of current therapies and for newer breakthroughs on CF antibiotherapy.

1. Introduction

According to the World Health Organization, infectious respiratory diseases are a major cause of morbidity and mortality worldwide, accounting for 4.18 million premature deaths per year (WHO, 2013). It is believed that most – if not all – of these infections are associated with biofilms, which represent a critical health-care burden (Bjarnsholt et al., 2013) because of their great contribution to the emergence and dissemination of antibiotic resistance. Cystic fibrosis (CF) is a common heritable genetic disorder (100 000 people estimated to be affected) (Davies et al., 2014) with major debilitation in the respiratory tract, where patients are prone to develop severe biofilm-related infections. CF infection was originally characterized as monomicrobial, likely due to the extensive use of culture-dependent isolation techniques. However, the advent of affordable high-resolution molecular technologies has dramatically changed our view on the true diversity of microbes present in the CF airways (Lopes et al., 2015). Indeed, the large variety and concentration of microbes includes not only the key pathogens habitually recovered (e.g. *Pseudomonas aeruginosa*) but also unusual

species (e.g. *Inquilinus limosus*, *Stenotrophomonas maltophilia*, *Dolosigranulum pigrum*, *Pneumocystis jirovecii*, *Scedosporium* spp., adenovirus, rhinovirus, etc.) (Bittar et al., 2008; Coenye et al., 2002; Pederiva et al., 2012). Though that CF aetiology is invariably polymicrobial, most research has focused on specific pathogens, failing to consider the role of unusual microbes in the disease progression (Lopes et al., 2014; , 2015; Magalhães et al., 2016; Waters, 2012) or their social interactions with established pathogens (Lopes et al., 2012). Accordingly, *I. limosus* (a gram-negative aerobe with a very mucoid phenotype) (Coenye et al., 2002) and *D. pigrum* (a gram-positive, facultative anaerobe, arranged in pairs, tetrads, and clusters) (Aguirre et al., 1993) are less typical bacteria reported in expectorated CF patients (Bittar et al., 2008). Whilst the pathogenic potential of *I. limosus* has been already pointed (Chiron et al., 2005), information on *D. pigrum* is still limited and its role in CF infection unclear. The upper respiratory tract is thought to be its natural habitat (Laclaire and Facklam, 2000), but its occurrence in other polymicrobial contexts (Hoedemaekers et al., 2006; Lecuyer et al., 2007) has raised up interest in evaluating its pathogenic potential in CF polymicrobial communities. Interestingly, earlier in vitro studies have

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unravelling the contribute of *I. limosus* and *D. pigrum* for the resilience of CF-associated biofilms to antibiotic treatment (Lopes et al., 2014; Lopes et al., 2012), thereby allowing to hypothesize their influence in the behaviour of coexisting species or even in benefiting the whole community. It is likely that the multifaceted CF microbiome promotes for species-specific relationships, often necessary for the development and structure of the community and other regulatory activities that coordinate the whole community (Magalhães et al., 2016; Tolker-Nielsen and Molin, 2000). In addition, it is now evident that microbial communities can be modulated by a wide variety of other factors that, altogether, give rise to various microbiological scenarios seen in vivo and are likely to affect disease phenotype and the clinical outcome (Guss et al., 2011; Magalhães et al., 2016; Peters et al., 2012a; Stressmann et al., 2011), as has been determined for other pathologies (Azevedo et al., 2014; Azevedo et al., 2016; LiPuma, 2014; Peters et al., 2012b). For instance, the physicochemical characteristics of the airways; the administration of the antibiotic therapy, (maintenance vs. treatment of episodes of acute exacerbation); the nature of the pre-existing microbial community; the concomitant airway inflammation resulting from chronic infections as well as patient- and/or disease-associated issues (increasing age, disease progression, reduced lung function) have been suggested to have a crucial impact on the dynamics of CF polymicrobial communities (Cuthbertson et al., 2016; Klepac-Ceraj et al., 2010; Rogers et al., 2015; Serisier, 2013; van der Gast et al., 2014; Willner et al., 2012; Zhao et al., 2012). Altogether, these factors may drive to social, structural and functional diversification and consequently impact the ecology and evolution in natural species populations (Allen and Banfield, 2005; Schlöter et al., 2000). Although it is still almost impossible to predict biofilm behavior and the dynamics of a community, it is now increasingly recognized that its regulatory networks (e.g. social activities, overall functions, virulence traits, etc.) are often strictly evolved in response to those highly variable stimuli (Allen and Banfield, 2005; Nadell et al., 2016), and improving and directing efforts to better understand how polymicrobial infections behave and respond to surrounding stresses may give insights to better predict disease progression and to define more effective antimicrobial strategies.

In this scope, this study aimed at deeply investigating the behavior of *I. limosus* and *D. pigrum* when associated with the CF-conventional pathogen *P. aeruginosa* in dual-species and even in three-species populations. The response of these multispecies communities to aerobic (AER) microaerophilic (MAER) and anaerobic (ANAER) conditions resembling the CF airways and to subsequent antibiotic interventions was further examined in detail. Thus, changes in the biofilm-formation ability, antibiotic resistance profiles, bacterial killings, microbial composition and distribution within the multispecies biofilms were assessed.

2. Material and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in this work were: *P. aeruginosa* (strain UCBPP- PA14), *I. limosus* (strain M53, isolated from CF sputum), and *D. pigrum* (CIP 104051T, purchased from Institute Pasteur Collection, Paris, France). All strains were stored at -70°C in 20% (v/v) glycerol. Prior to inoculation, bacteria were subcultured twice from the frozen stock preparations onto tryptic soy broth (TSB, Liofilchem, Italy) supplemented with 1.2% (w/v) agar (TSA, Liofilchem) plates and incubated at 37°C for 24–48 h.

2.2. Formation of multispecies biofilms under variable oxygen conditions

The formation of dual- and three-species biofilms formed by *P. aeruginosa*, *I. limosus* and *D. pigrum* under AER, MAER and ANAER conditions was performed as described elsewhere (Lopes et al., 2014).

Briefly, for preparing biofilm inocula, several colonies from a fresh subculture of each strain were suspended in TSB to match a 1.0 McFarland standard. This was further diluted in broth medium (TSB) to obtain pure cultures with $\sim 10^7$ cells/mL. For dual-species cultures, the suspended inoculum of each species was combined in a 1:1 ratio, whereas the three-species cultures were obtained by mixing the three pure bacterial suspensions in equal proportions. Bacterial suspensions were dispensed in standard 96-well microtiter plate wells and incubated at 37°C and 120 rpm until achieving the threshold the cell concentration interval of 2×10^5 to 2×10^6 CFU/cm² (determined by previous established biofilm growth curves) (Lopes et al., 2014). To resemble the oxygen environments of CF airways, plates were incubated at different conditions: AER (n-biotek, Model NB-205Q, Korea incubator), MAER (Thermo Scientific, Forma 311, USA incubator, calibrated with 5% v/v CO₂) and ANAER (AnaeroGen Atmosphere Generation system, Oxoid, Cambridge, UK). The ANAER atmosphere was created by sealing the plates containing the cells suspensions in plastic boxes with AnaeroGen sachets (Oxoid), which reduces oxygen concentration to below 1% (v/v). This oxygen reduction was confirmed by using anaerobic indicator strips (Oxoid) (see Fig. S1 in Supplementary Material).

2.3. Quantification of biomass and metabolic activity of biofilms

After biofilm formation, the content of microtiter plate wells (planktonic cell fraction) was discarded, the wells washed once with sterile 0.9% (w/v) saline solution to remove non-adherent and weakly adherent bacteria, and biofilms were quantified for biomass and metabolic activity. Biofilm mass quantification was based on staining biofilms with crystal violet (CV), with protocol adapted from Stepanovic et al. (Stepanovic et al., 2000). Briefly, the wells were air dried for 10 min before fixing attached bacteria with pure methanol (Fisher Scientific, Leicestershire, UK) for 15 min. Then, biofilms were stained for 1 min with 1% (v/v) CV (Merck, Germany). The excess stain was removed by washing twice the wells with sterile distilled water. Lastly, the bound dye was dissolved in pure methanol (200 μL /well) and the optical density (OD) was read at 550 nm (OD_{550nm}) in a microtiter plate reader (Model Sunrise-basic Tecan, Austria).

The metabolic activity of biofilms was measured using the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2Htetrazolium-5-carboxanilide sodium salt (XTT) colorimetric method, initially described by Stevens and Olsen (Stevens and Olsen, 1993), with some modifications. Basically, after biofilm growth and washing procedures, 200 μL of a combined solution of XTT (Sigma) and phenazine methosulfate (PMS) (Sigma) were added to each well in order to obtain a final concentration of 150 mg/L of XTT and 10 mg/L of PMS. After that, plates were incubated at 37°C for 3 h in the dark. Biofilm metabolic activity was determined by reading the OD at 490 nm (OD_{490nm}) in each well.

2.4. Determination of the competitive index (CI) and the relative increase ratio (RIR)

In order to determine whether the species could compete with each other in multispecies biofilms, the Competitive Index (CI) was defined as the X/Y ratio within the output sample (immediately after biofilm formation) divided by the corresponding ratio in the inoculum (input):

$$\text{CI} [X \text{ vs } Y] = \frac{[N (X/Y)_{\text{output}}/N (X/Y)_{\text{input}}]}$$

where N is the value of CFU/cm² obtained for the species X or Y in the biofilm, in the output (at fixed times) and input ($t = 0$ or the inoculum) sample (Macho et al., 2007). For statistical analyses, CI values were first subjected to a Log transformation for normal distribution, then interpreted as follows: a CI value equal to 0 indicates equal competition of species X and Y in the consortium; a positive CI value indicates a competitive advantage for the species X; a negative CI value indicates a competitive advantage for the species Y. Similarly, the

Relative Increase Ratio (RIR) was calculated and based on the growth results obtained from the single-species biofilms formed by each strain. Calculations were adapted from (Macho et al., 2007). Statistical significant differences between CI and RIR means for each case were suggestive of a meaningful competition between the species in the consortia.

2.5. Biofilm resistance profiles

Antibiotics susceptibilities of biofilms were determined by adapting the protocol described by Ceri et al. (Ceri et al., 1999). For that, pre-established biofilms (containing 2×10^5 to 2×10^6 CFU/cm²) formed on the microtiter plate wells were exposed to increasing 2-fold concentrations (ranging from 2 to 1024 mg/L) of 9 clinically-relevant antibiotics: tobramycin (TOB), gentamicin (GEN), levofloxacin (LVF), ciprofloxacin (CIP), clindamycin (CLI), cefotaxime (CTX), chloramphenicol (CAM), rifampicin (RIF) and aztreonam (ATM, all from Sigma), prepared in cation-adjusted Mueller-Hinton broth (C-AMHB). Plates were then incubated at 37 °C, under AER, MAER and ANAER environments, as described above. Following 24 h of antibiotic exposure, planktonic cell fractions (supernatant) released for treated-biofilms were transferred to new microtiter plates and the minimum inhibitory concentrations (MICs) were obtained by reading the OD at 650 nm (OD_{650 nm}), where OD_{650 nm} < 0.1 was evidence of bacterial growth inhibition. Biofilms attached to the wells were rinsed twice with saline solution and disrupted (by sonication with an ultrasound bath) into TSB supplemented with 1% (v/v) tween® 20 (Fisher Scientific, NJ, USA). The minimum biofilm eradication concentration (MBEC), corresponding to the lowest antibiotic concentration able to inhibit biofilm growth, was determined after plating serial dilutions of disrupted biofilms samples onto TSA.

2.6. Assessment of bacterial killings within biofilms after antibiotic exposure

The efficacy of the aforementioned antibiotics at concentrations equal to 2, 128 and 1024 mg/L was evaluated for bacterial killings within biofilms, which was expressed in terms of percentage reduction in biofilm-cell numbers. After biofilms being exposed to antibiotics (as previously described), total cultivable cell numbers were enumerated by plating 10-fold serially diluted biofilm-detached samples onto TSA plates. Biofilm-cell killing was reported as the percentage of log reduction calculated to the relative cell count at the time of initiation of antibiotic exposure, according to the following:

Biofilm-cell reduction (%) = $100 - [N \text{ (after exposure to the antibiotic)} / N \text{ (prior to antibiotic exposure)} \times 100]$

where N is the value of CFU/cm² present in the biofilm determined before or after being exposed to the antibiotic at the desired concentration.

2.7. Determination of biofilms composition

In this work, the relative species composition within polymicrobial biofilms was estimated before and after antibiotic exposure. Basically, after the biofilms rinse step, the wells were filled with saline solution (200 µL/well) and biofilms formed in the wells were detached by sonication using an ultrasound bath (Sonicor, model SC-52, UK) operating at 50 kHz, during 10 min and then resuspended by pipetting up and down. The sonication step was previously optimized to ensure that all cells were detached from the wells of the microtiter plate, while avoiding cell disruption (data not shown). The estimation of total biofilms-cells was enumerated by using TSA whereas selective agar media was used to discriminate each bacterial population in the consortia: *Pseudomonas* isolation agar (PIA; Sigma) and *Burkholderia cepacia* selective agar, (BCSA; Oxoid Ltd, Hampshire, UK) supplemented with 300

000 IU/L polymyxin B (Biochrom, Berlin, Germany) and 100 mg/L ticarcillin (Sigma) for *P. aeruginosa* and *I. limosus* isolation, respectively. Due to the lack of a specific selective medium for *D. pigrum*, this species was estimated by the difference between the average total cell number (in TSA) and the average of other bacteria presented in the consortia.

2.8. Multiplex PNA FISH assay applied to biofilms

In order to discriminate the different populations within the polymicrobial biofilms and to confirm (qualitatively) the results obtained from culture, fluorescence in situ hybridization (FISH) using peptide-nucleic acid (PNA) probes followed by an additional staining step with 4', 6-diamidino-2-phenylindole (DAPI; Sigma) was performed directly on polymicrobial consortia. Firstly, biofilms were formed on the surface of Nunc™ Lab-Tek™ II Chamber Slide™ System (Nalge Nunc International; Naperville, IL, USA). After achieving the threshold concentration, biofilms were washed twice with 1 mL of sterilized deionized water and air dried at ~60 °C for 15 min. Biofilms were then fixed with methanol (100% v/v) for 20 min, followed by 4% (w/v) paraformaldehyde and 50% (v/v) ethanol (10 min each), at room temperature. After the fixation step, the surfaces of the chamber slide were covered with 20 µL of hybridization solution with a mixture of two probes at 200 nM and covered with coverslips. These PNA probes, designated Paer565 and Ilim569, were previously developed, optimized and validated (Lopes et al., 2017) in order to specifically detect *P. aeruginosa* and *I. limosus* within polymicrobial communities. Hybridization was performed in the dark, for 1 h at 65 °C, as determined elsewhere (Lopes et al., 2017). For washing (65 °C for 30 min), a fresh solution composed of 5 mM Tris Base, 15 mM NaCl and 1% (v/v) Triton X-100 (all from Sigma) was prepared less than 24 h before use. Finally, the chamber slides were allowed to air dry in the dark. An additional staining step with DAPI was performed at the end of the hybridization procedure, covering the surfaces with 20 µL of DAPI (40 µg/mL) for 5 min at room temperature in the dark. Then, immediate observation was elaborated in the fluorescence microscope. Negative controls were performed for each experiment, with no probes added to the hybridization solution. For microscopic visualization, a fluorescence microscope (Olympus BX51, Perafita, Portugal) equipped with the filters sensitive to DAPI (BP 365-370, FT 400, LP 421) and to the signaling molecule of the PNA probes (BP 470–490, FT 500, LP 516 and BP 530–550, FT 570, LP 591, for Alexa 488 and 594, respectively) was used.

2.9. Statistical analysis

Data were analyzed using the Prism software package (GraphPad Software version 6.0 for Macintosh). Results were compared by one-way analysis of variance (ANOVA) and applying the Tukey multi-comparison post-test. Differences were considered statistically significant at *P* values < 0.05.

3. Results

3.1. Biofilm mass and metabolic activity under different oxygen conditions

In order to inspect for changes in biofilm phenotype under variable oxygen conditions, dual- and three-species biofilms encompassing the CF-associated species *I. limosus*, *D. pigrum* and *P. aeruginosa* were grown under AER, MAER and ANAER environments, being further analyzed in terms of biomass and metabolic activity (Fig. 1). An incubation period of 6 h was required for biofilms to achieve the threshold interval (2×10^5 to 2×10^6 CFU/cm²) as was previously determined by biofilm adhesion kinetics under the selected oxygen conditions (Fig. S2). Regarding biofilm mass (Fig. 1a), dual-species biofilms encompassing *P. aeruginosa* and *I. limosus* produced the lowest values in comparison with dual-species biofilms of *P. aeruginosa* and *D. pigrum* and the consortia

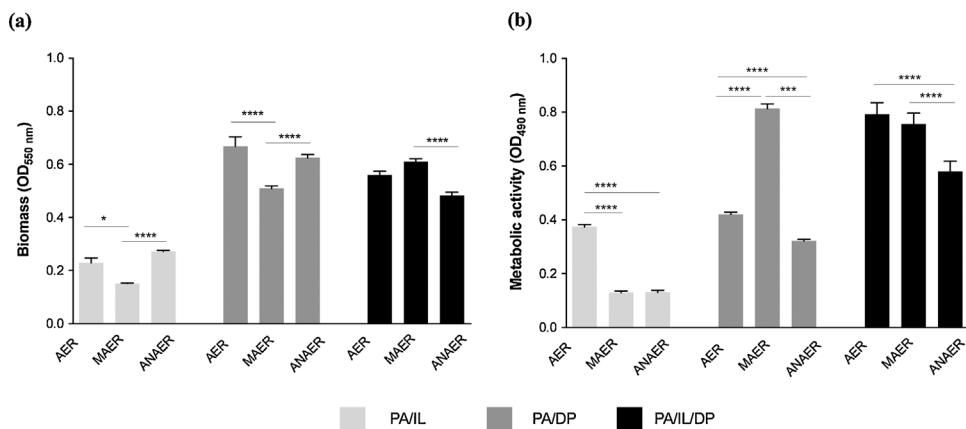


Fig. 1. Characterization of polymicrobial biofilms formed by *P. aeruginosa* (PA), *I. limosus* (IL) and *D. pigrum* (DP) in terms of (a) biomass and (b) metabolic activity. Biofilms were grown in microtiter plate wells under aerobic (AER), microaerophilic (MAER) and anaerobic (ANAER) atmospheres until achieving the threshold concentration of 2×10^5 to 2×10^6 CFU/cm² (6 h). The means \pm SDs for three independent assays are presented. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, AER vs. MAER vs. ANAER, one-way ANOVA with Tukey’s post-hoc test.

involving the three species. The same tendency was observed for the metabolic activity of these biofilms (Fig. 1b). Overall, biofilms tend to develop significantly less biomass and present lower metabolic activity for limited oxygen conditions, in particular under ANAER ($P < 0.05$). A particular increase in the metabolic activity was observed for *P. aeruginosa* and *D. pigrum* dual-species consortia developed under a MAER environment.

3.2. Microbial composition of biofilms under variable oxygen conditions

In order to investigate which bacterial species were prevailing in the biofilms under AER, MAER and ANAER conditions, the relative population distributions were determined by culture (Fig. 2a). Previously to antibiotic exposure, dual-species biofilms generally presented similar bacterial proportions for all oxygen environments, with *P. aeruginosa* slightly dominating the overall consortia with *I. limosus* and occupying similar extents with *D. pigrum*. Likewise, the three-species biofilms were

clearly dominated by *P. aeruginosa* and *D. pigrum*, whereas *I. limosus* was the smallest representative population in the overall biofilm. In order to better discriminate and to infer about changes in the distribution and prevalence of the bacterial populations within the multispecies consortia, a multiplex PNA-FISH counterstained with DAPI was employed directly on biofilms (Fig. 2b). Overall, FISH images seemed to qualitatively corroborate culture results. In regard to dual-species biofilms, both populations were detected, with *P. aeruginosa* (red cells) and *D. pigrum* (blue cells) occupying similar extensions in the consortia, but with *P. aeruginosa* slightly dominating the dual-species biofilms encompassing *I. limosus*. Together with *D. pigrum*, *P. aeruginosa* still prevailed in the three-species consortia, whereas *I. limosus* populations were sparsely detected under fluorescence microscopy.

3.3. Interspecies competition within biofilms

For a clear understanding of the competition between the species

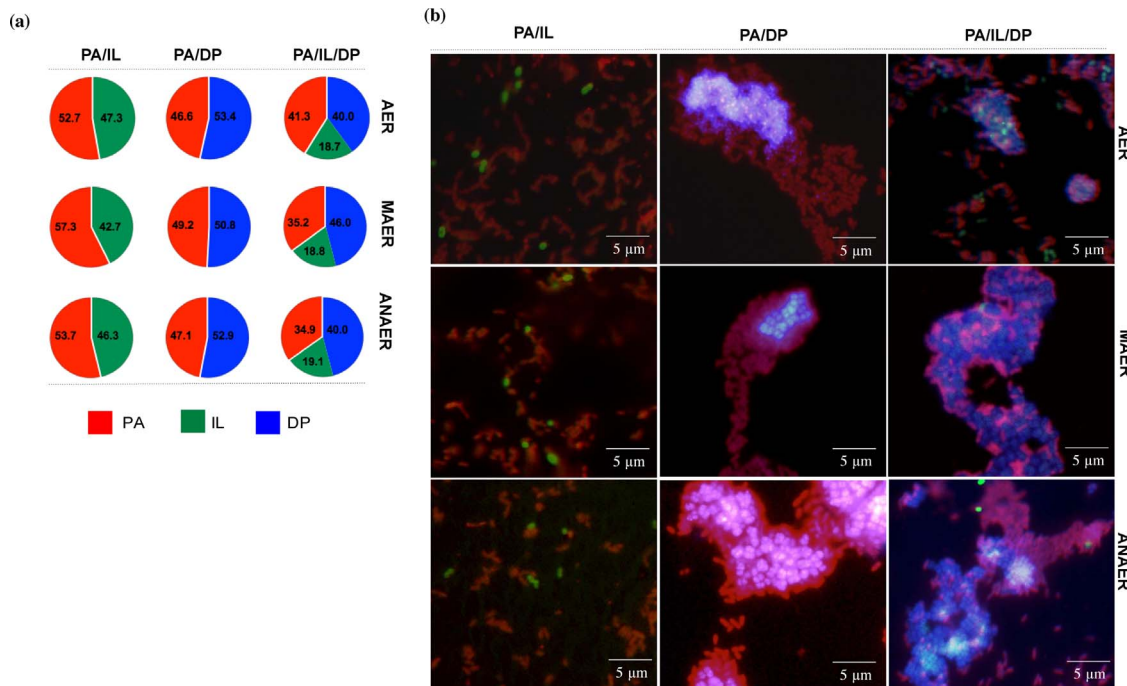


Fig. 2. Characterization of polymicrobial biofilms formed by *P. aeruginosa* (PA), *I. limosus* (IL) and *D. pigrum* (DP) in terms of (a) microbial compositions and (b) location and distribution of the populations. Biofilms were grown in microtiter plate wells under aerobic (AER), microaerophilic (MAER) and anaerobic (ANAER) atmospheres until achieving the threshold concentration of 2×10^5 to 2×10^6 CFU/cm² (6 h). In (a), the microbial compositions were determined by culture and were expressed as the average of the relative species proportions in the consortia; in (b) a multiplex PNA-FISH assay followed by DAPI staining was applied to the polymicrobial biofilms, in order to discriminate between the populations in the consortia. Images resulted from the bands superposition of the three channels used to visualize the fluorochromes used (Alexa fluor 594, red: PA; Alexa Fluor 488, green: IL; DAPI, blue: DP). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

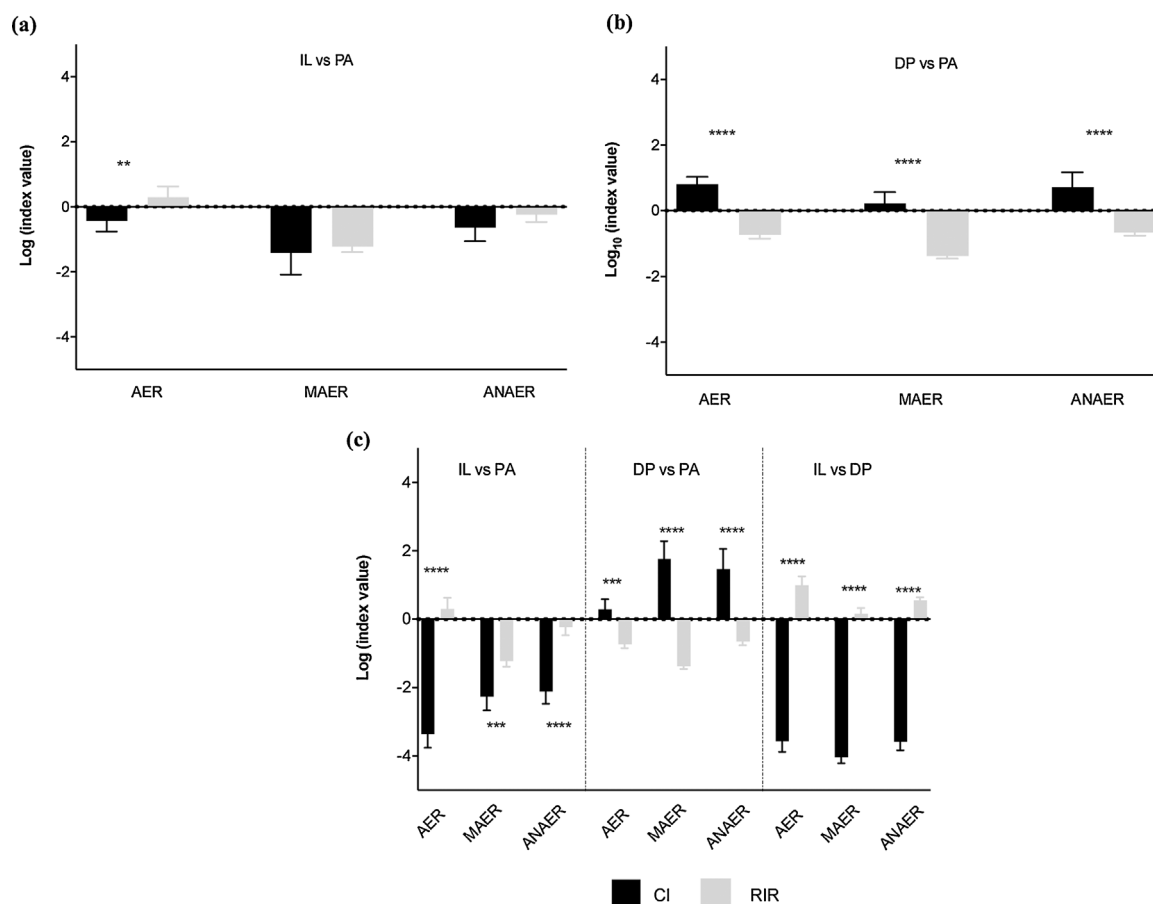


Fig. 3. Competitive index (CI; black bars) and Relative Increase Ratio (RIR; grey bars) calculated from 6h-old biofilms formed by: (a) *P. aeruginosa* (PA) and *I. limosus* (IL); (b) PA and *D. pigrum* (DP) and (c) PA, IL and DP. Biofilms were formed under aerobic (AER), microaerophilic (MAER) and anaerobic (ANAER) conditions. Statistical significant differences between CI and RIR is suggestive of a meaningful competition between the species in the consortia. Competition of species X and species Y in a consortium [CI (X vs Y)] is interpreted as follows: CI = 0 means similar competition for both X and Y species; CI > 0 means competitive advantage for the species X; CI < 0 means competitive advantage for the species Y. The results are shown as mean \pm SDs. ** P < 0.01, *** P < 0.001, **** P < 0.0001, CI vs. RIR, one-way ANOVA with Tukey⁴ post-hoc test.

during biofilm growth under AER, MAER and ANAER environments, the CI and RIR indexes were estimated for dual- and three-species biofilms (Fig. 3). Whilst CI allows comparing the differences among the growth of each species in mixed cultures, the RIR index compares the growth of species within pure cultures. As shown, a negative CI index was observed for the *P. aeruginosa*/*I. limosus* dual-species consortia (Fig. 3a), meaning a competitive advantage for *P. aeruginosa* over *I. limosus*. However, this competition was only significant for AER conditions (CI vs. RIR, P < 0.01). By the contrary, for dual-species consortia involving *P. aeruginosa*/*D. pigrum*, the unusual species clearly presented competitive advantage over *P. aeruginosa* for all oxygen environments (CI > 0; CI vs. RIR, P < 0.0001) (Fig. 3b). Similarly, when both unusual species were in co-culture with *P. aeruginosa* (Fig. 3c), *I. limosus* was outcompeted by *P. aeruginosa* (CI < 0), whereas *D. pigrum* still outcompeted the conventional CF-associated species in the consortia for all oxygen conditions (CI > 0; CI vs. RIR, P < 0.05). Equally, negative CI indexes indicated *D. pigrum* significantly dominating over *I. limosus* growth within the three-species consortia under all environments (CI < 0; CI vs. RIR, P < 0.0001). Other approach to represent these latest results consisted in calculating the relative fitness of *P. aeruginosa* when co-cultured with the unusual species (Fig. S3). This demonstrated enhanced *P. aeruginosa* fitness when co-cultured with *I. limosus* and a decline in its performance when *D. pigrum* is present in the polymicrobial consortia.

3.4. Biofilm resistance profiles

The biofilms in the required threshold concentration were exposed to increasing 2-fold concentrations, ranging from 2 to 1024 mg/L, of nine antibiotics. The MICs, obtained from planktonic populations, and the MBECs obtained for biofilm consortia are summarized on Table S1. Results obtained for mixed-species populations were compared to data obtained from single-species cultures, determined in a previous report of Lopes et al. (Lopes et al., 2014), where an increase in antibiotic resistance was observed for *I. limosus* and *D. pigrum* biofilms. In order to better visualize the tendency for antibiotic tolerance for those populations, two heat maps representing the MIC and MBEC data were generated (Fig. 4a and b, respectively). Biofilm-entrapped bacteria showed predictable greater tolerance towards antibiotics than planktonic counterparts, with MBECs (in general \geq 1024 mg/L) higher than MICs, independently from the oxygen concentration. For planktonic and biofilm cultures, the activity of most antibiotics was preserved under environments defective in oxygen. Specifically, as aminoglycoside uptake is severely limited in Gram-negative and Gram-positive bacteria under oxygen-restricted environments (Hancock, 1962; Kogut et al., 1965; Kohanski et al., 2010), is therefore indicative that oxygen in the environment (used as the electron acceptor) is the motive force available to assist the aminoglycoside uptake and maintain sensitive under anaerobic conditions (it was the case of *P. aeruginosa*).

Comparing with monospecies planktonic cultures, polymicrobial populations remained sensitive for most antibiotic agents, with exception for CLI and RIF, which were ineffective in inhibiting those

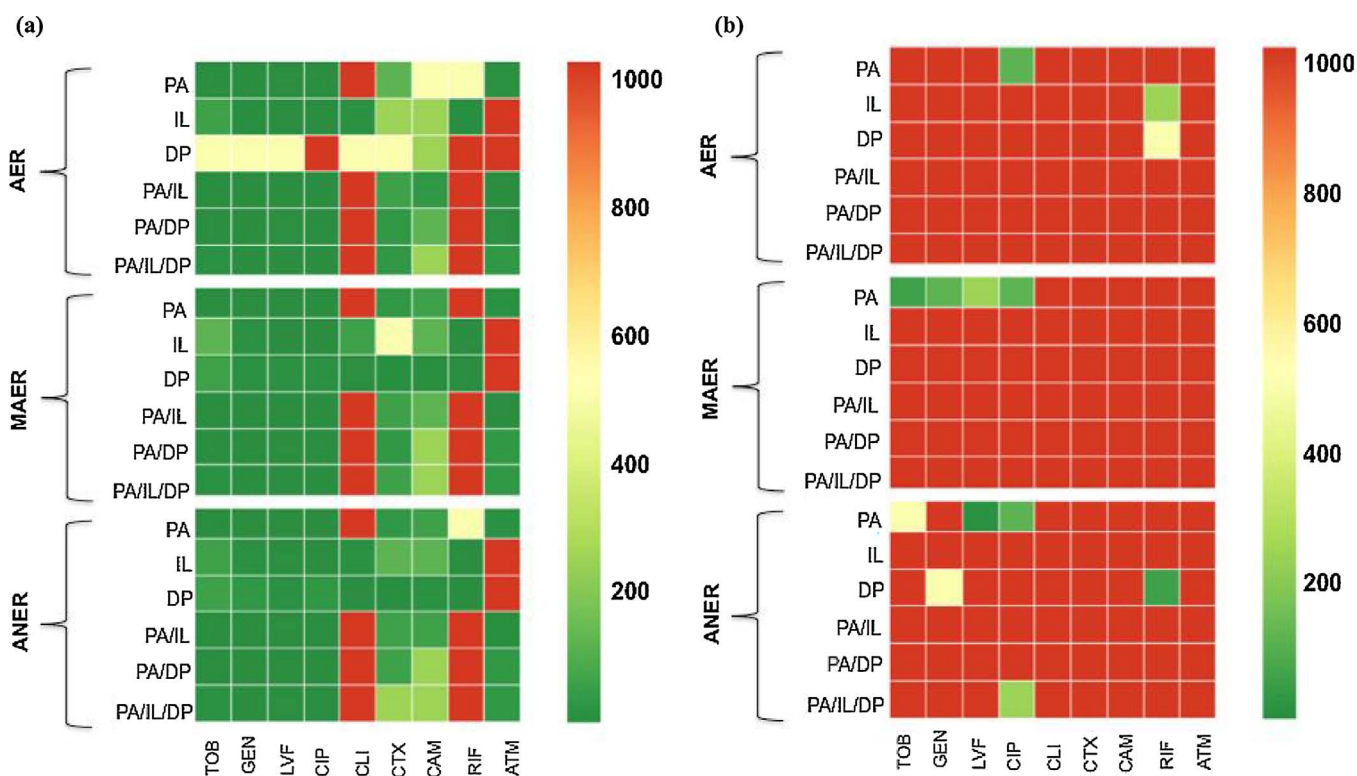


Fig. 4. Antimicrobial tolerance of polymicrobial planktonic (a) and biofilm cultures (b) formed by *P. aeruginosa* (PA), *I. limosus* (IL) and *D. pigrum* (DP) grown for 6 h, under aerobic (AER), microaerophilic (MAER) and anaerobic (ANAER) conditions. The vertical scale bars represent the range values for MICs (in (a)) and MBECs (in (b)), expressed as mg/L. Legend for antibiotics: TOB = tobramycin; GEN = gentamicin; LVF = levofloxacin; CIP = ciprofloxacin; CLI = clindamycin; CTX = cefotaxime; CAM = chloramphenicol; RIF = rifampicin; ATM = aztreonam. Data from single-species populations were retrieved from an earlier publication (Lopes et al., 2014). The heatmaps were created by using *Seaborn*, a Python visualization library.

populations (MBEC > 1024 mg/L). When encased in biofilms, the unusual species showed high resistance for most antibiotics, which endured when these species were co-cultured with *P. aeruginosa*, independently of the oxygen condition.

3.5. Effect of antibiotics in reducing biofilm cells

Since MBECs were generally high, it was interesting to address how increasing antibiotic concentrations (at 2, 128 and 1024 mg/L) could affect the viability of bacterial populations within the polymicrobial biofilms (Fig. 5). For this, the percentage reduction in total biofilm-cells was estimated by counting bacteria on TSA after exposing biofilms to antibiotics and the results compared from those of un-treated biofilms. Overall, biofilm-cells declined gradually with increasing doses of antibiotics, with the highest concentrations leading to the highest reductions. A clear loss of activity for many antibiotics under low-oxygen atmospheres was also observed, with no visible cell reductions. This was particularly true for *P. aeruginosa/I. limosus* dual-species biofilms, which showed higher antibiotic resistance under ANAER conditions, with biofilm-cell reduction percentages not exceeding 10% (achieved for GEN). Biofilms encompassing *D. pigrum* were more sensitive than the previous ones, being markedly noticeable for the consortia involving the three-species, where antibiotics could achieve great bacterial reductions within the biofilms and even with fluoroquinolones (LVF and CIP) leading to the complete eradication for MAER and ANAER environments.

3.6. Microbial composition of polymicrobial biofilms after antibiotic treatment

The relative populations proportions of the polymicrobial biofilms after antibiotic exposure was monitored by culture (Fig. 6). After the

antibiotic treatment, substantial changes in the composition of the polymicrobial communities occurred compared with non-treated biofilms (i.e. biofilms pre-exposed to antibiotics), with fluctuations depending on the type and on the concentration of the antibiotic and even on the oxygen conditions. *P. aeruginosa/I. limosus* dual-species biofilms were still predominately governed by the CF-classical species, particularly under AER conditions (Fig. 6a). However, *I. limosus* populations started to occupy a significant proportion, together with *P. aeruginosa*, for environments of low oxygen tension (MAER and ANAER). Similarly, *P. aeruginosa* prevailed within the *P. aeruginosa/D. pigrum* dual-species consortia (Fig. 6b), persisting alone for many cases. For a vast number of situations, *D. pigrum* was able to persist and inhabit nearly half part of the whole consortia, together with *P. aeruginosa*. Changes in the microbial composition of three-species biofilms showed to be quite complex and variable compared with dual-species consortia (Fig. 6c). A clear dominance of the three-species consortia was typically obtained for *D. pigrum* and/or *P. aeruginosa*, whilst *I. limosus* populations rapidly declining.

4. Discussion

The recent explosion of large-scale studies has significantly enhanced our appreciation on the true complexity and diversity of the CF multispecies communities. Indeed, the airways and lungs of CF patients are often heterogeneous systems enclosing gradients of antibiotics, nutrients, and oxygen (Yang et al., 2011), which have been documented as significant factors driving to the diversification at the community level (Cuthbertson et al., 2016; Goddard et al., 2012; Lee et al., 2014; Quinn et al., 2016; Rogers et al., 2015; van der Gast et al., 2014; van der Gast et al., 2011; Zhao et al., 2012).

Our findings demonstrated that CF polymicrobial communities were not static populations, with biofilm dynamics evolving in response to

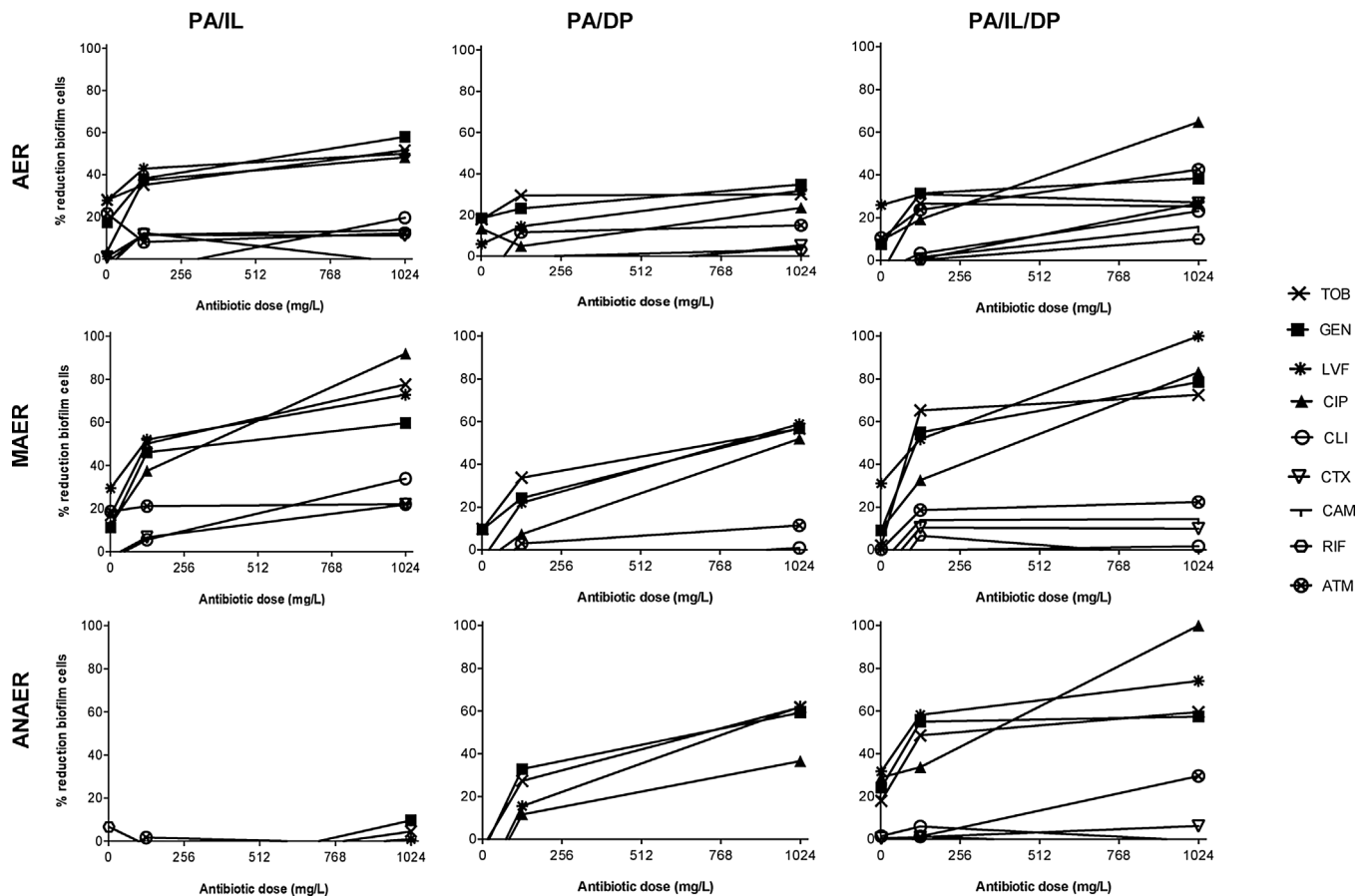


Fig. 5. Average percentage reductions in total cultivable cells within polymicrobial biofilms formed by *P. aeruginosa* (PA), *I. limosus* (IL) and *D. pigrum* (DP) after exposure to increasing doses (2, 128 and 1024 mg/L) of antibiotics under aerobic (AER), microaerophilic (MAER) and anaerobic (ANAER) environments. The means for three independent assays are illustrated. Legend for antibiotics: TOB = tobramycin; GEN = gentamicin; LVF = levofloxacin; CIP = ciprofloxacin; CLI = clindamycin; CTX = cefotaxime; CAM = chloramphenicol; RIF = rifampicin; ATM = aztreonam.

environments with distinct oxygen availabilities and to antibiotic therapy. Minimal fluctuations were detected before antibiotic exposure, particularly in terms of biofilm composition. In fact, *I. limosus* and *D. pigrum* were proficient to establish biofilms together with the CF emblematic pathogen *P. aeruginosa* under AER, MAER and ANAER environments with slight variations in biomass and metabolic activity. Earlier studies (Lopes et al., 2014) have shown that both unusual species were able to develop single-species biofilms under these oxygen environments, even showing resistance to multiple antibiotics under those conditions. Several reasons were pointed out to substantiate the underlying antibiotic resistance for both species, which was particularly attributed to the mucoid physiology of *I. limosus* and to the high biomass achieved for *D. pigrum* biofilms, associated to the dense extracellular polymeric matrix.

It is important to note that co-infections with equal ratios of *P. aeruginosa* and the unusual organisms selected may never occur in a CF lung, but it is possible that all strains might reside in the airways of patients for identical lengths in time. Strains of *P. aeruginosa*, *I. limosus* and *D. pigrum* were recovered from CF exacerbations from one geographical region over a period of just 2 months, which suggests that they may encounter each other in their natural microhabitat (Bittar et al., 2008), eventually not as a stable but as a transient community consequently derived from shifts within the natural environment (Conrad et al., 2013). While applying PNA-FISH assay to discriminate in situ the bacterial populations within the biofilms, culture results were corroborated by microscopy. An exception was observed for *P. aeruginosa*/*I. limosus* dual biofilms, where the sparse distribution of *I. limosus* indicated a lower prevalence in the consortium. Only an ex situ

quantitative monitoring of bacterial populations should better clarify this issue. Polymicrobial biofilms were minimally challenged by variable oxygen conditions. Whilst dual-species biofilms (in particular *P. aeruginosa* and *D. pigrum*) presented similar relative proportions for both populations, the three-species biofilms were dominated by *P. aeruginosa*, with *D. pigrum* also presenting a great extension together with the CF-traditional species. PNA-FISH and culture assays, complemented with CI and RIR data seems to indicate that *D. pigrum*/*I. limosus* and *D. pigrum*/*P. aeruginosa* interplay was likely negative, with *D. pigrum* taking competitive advantage against the other species, thus eventually suggesting competition for the same niche or for nutrients (Hibbing et al., 2010).

If biofilm dynamics was minimally disturbed with oxygen conditions, treatment with antibiotics had huge impact on multispecies diversification. First, biofilms demonstrated high resistance towards most antibiotics compared with respective planktonic cells, which is consistent with the commonly accepted notion that biofilm-grown bacteria display enhanced resistance to antibiotics, compared to their planktonic counterparts (Costerton et al., 1999; Hoiby et al., 2010; Lopes et al., 2012; Mah and O'Toole, 2001). This work, thus, supports that the antibiotic resistance previously demonstrated for *I. limosus* and *D. pigrum* (Lopes et al., 2014) endured even in co-culture with *P. aeruginosa*, thus strongly emphasizing the impact that those species may have on CF antibiotic treatment and pathogenesis. Second, even presenting higher MBECs, the antibiotics could lead to significant reductions in the viability of bacterial populations within the polymicrobial biofilms. The decline in the cell viability was less noticed for treatments challenged under ANAER conditions, with a great number of antibiotics losing

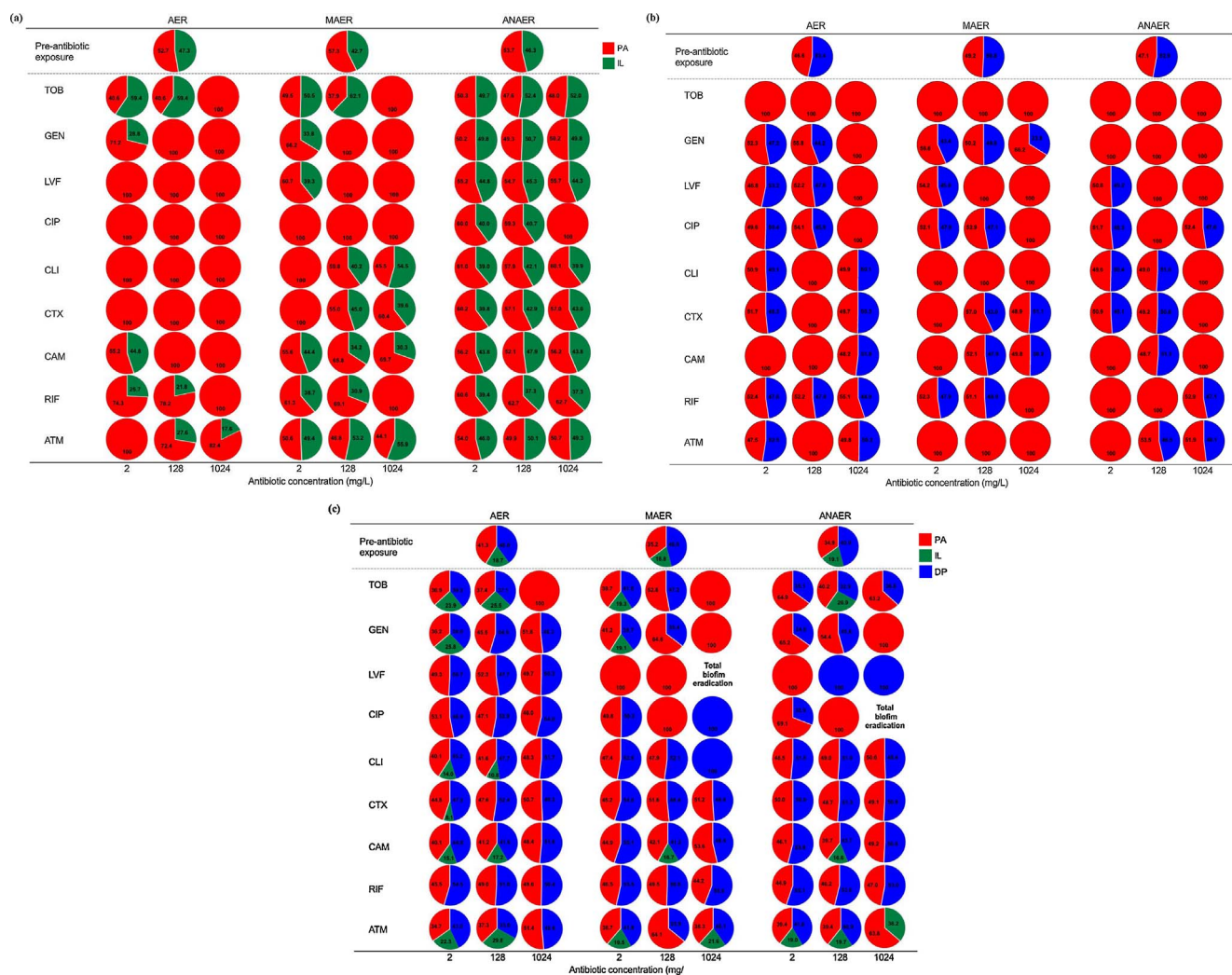


Fig. 6. Relative bacterial proportions, in average, of *P. aeruginosa* (PA; red), *I. limosus* (IL; green) and *D. pigrum* (DP; blue) cells within polymicrobial biofilms formed by PA and IL (a), PA and DP (b) and PA, IL and DP (c) before and after exposure to increasing doses (2, 128 and 1024 mg/L) of antibiotics under aerobic (AER), microaerophilic (MAER) and anaerobic (ANAER) environments. Legend for antibiotics: TOB = tobramycin; GEN = gentamicin; LVF = levofloxacin; CIP = ciprofloxacin; CLI = clindamycin; CTX = cefotaxime; CAM = chloramphenicol; RIF = rifampicin; ATM = aztreonam. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

their effectiveness under oxygen-limited environments. Third, the composition of polymicrobial biofilms was dramatically challenged by antibiotic therapy, with fluctuations depending on the type and concentration of the antibiotic applied and on the oxygen environment. But whatever the antibiotic and concentration applied, it tends to select for *P. aeruginosa* rather than for the unusual species. Changes were in general mainly reflected on the relative proportions of *I. limosus* and/or *D. pigrum*, with *P. aeruginosa* as the eminent organism for most cases. In turn, changes on biofilms composition instigated by antibiotic therapy could shape the overall function of the polymicrobial consortia. As an example, *P. aeruginosa*/*I. limosus* dual-species consortia were equally distributed within the biofilm (as showed by culture), mainly under ANAER conditions. The demonstrated endurance of *I. limosus* populations may indicate that this species was potentially the organism that conferred resistance to the overall biofilm when challenged under low oxygen tensions (observed by bacterial killing assays). We therefore suggest that the enhanced sensitiveness of the three-species consortia can be related with the clear decline in the *I. limosus* population size, with the overall consortia being dominated by *P. aeruginosa* and *D. pigrum*. This remarks the competition between both unusual species, with *D. pigrum* likely disturbing the numbers of the organism that originally had conferred resistance to the dual-biofilm (mainly under depleted

oxygen conditions) and leading to more susceptible mixed-species biofilms for a great number of antibiotics, as demonstrated by killing kinetics assays. Taken together, these results can open novel ways for CF treatment, by disturbing one factor that regulates the microbial community (in terms of stability, function and/or antibiotic-resistance) to facilitate subsequent control of the overall community by antibiotics.

Aware that rather than a single process, a myriad of variants is responsible for impacting the dynamics of polymicrobial communities in CF natural settings, leading to different scenarios seen in vivo, we were able to reach a model representing how oxygen environments and antibiotic therapy affect the composition of polymicrobial biofilms in CF airways (Fig. 7). For this, we focused our model in the three-species consortia due to their higher complexity comparable with dual-species biofilms. Prior to antibiotic exposure (Fig. 7a), minor fluctuations occur across oxygen gradients, with the three-species consortia being dominated by *P. aeruginosa* (red cells) and *D. pigrum* (blue) and with *I. limosus* (green) as the lowest representative population. When biofilms are exposed to antibiotics commonly used to treat *P. aeruginosa* infections in CF (Doring et al., 2012), diversification among biofilms is evident (Fig. 7b), with variable fluctuations occurring in the relative proportions of the resident species depending on the antibiotic and on the oxygen conditions.

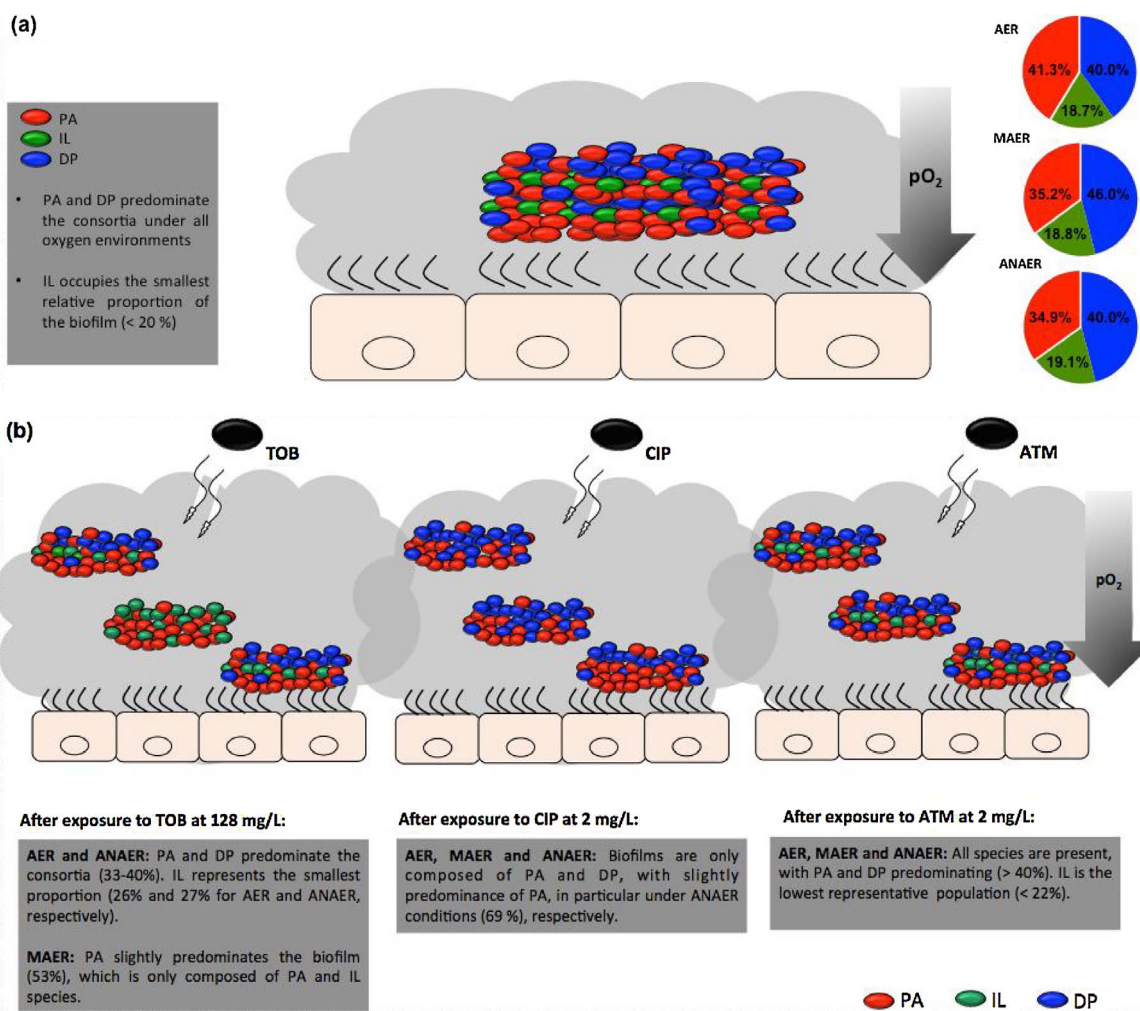


Fig. 7. Proposed model for multispecies dynamics of polymicrobial biofilms in CF. (a) Three-species biofilms of *P. aeruginosa* (PA), *I. limosus* (IL) and *D. pigrum* (DP) developed in the CF airways before antibiotic exposure; (b) Bacterial composition dynamics of three-species biofilms of PA, IL and DP, when exposed to TOB (at 128 mg/L), CIP (at 2 mg/L) and ATM (at 2 mg/L) under aerobiose (AER), microaerophilia (MAER) and anaerobiose (ANAER). Legend: pO_2 = oxygen tension; TOB = tobramycin; CIP = ciprofloxacin; ATM = aztreonam.

We are aware that this biofilm model embraces a few limitations (e.g. it is an in vitro model, based on culture experiments complemented with PNA-FISH; it embodies a mixture of three species rather than the full diversity and abundance of microbes in CF and hence dismisses other species with potential huge impact for CF infection). Nonetheless, it reflects how spatially heterogeneous and distributed are CF communities within the site of infection, likely affecting the virulence of the encased pathogens and being a great predictor of disease progression and outcome (Stacy et al., 2016). It is therefore plausible that discerning the mechanisms that govern CF sociomicrobiology will impair, for instance, that treatment regimens often applied to specific populations may be ineffective against infections occurring in particular regions or microenvironments of the lung/airways. Additionally, it may lead to the development of rationales for the selection of suitable and personalized therapeutic strategies (targeting microbiomes unique to each individual), hence increasing antibiotic therapy and reducing socioeconomic repercussion often associated to inadequate treatments.

5. Conclusion

This study expanded the understanding about how the dynamics of microbial communities involving CF classical and unusual organisms is collectively influenced by perturbations often common in the CF natural environments. In fact, our results led to conclude that chemical stress is more “aggressive” than environmental stress in modulating the

dynamics of CF polymicrobial communities. While the benefits of antibiotic therapy are indisputable, antibiotics may exert dramatic fluctuations on biofilms, thereby shaping resident species composition and affecting overall function of the consortia. In parallel, this study highlights that the presence of unusual species in the CF airways communities should not be disregarded, since they are able to mediate social interactions, affecting the whole community and ultimately modifying the clinical course of the disease. As such, a more deep appreciation of the ecological and evolutionary nature that shape the airways communities as well as their effects on lung will be certainly useful for the optimal use of current therapies and the development of newer breakthroughs on CF antibiotic therapy.

Funding

This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit and COMPETE 2020 (POCI-01-0145-FEDER-006684). The authors also acknowledge the financial support provided by FCT through the projects: PTDC/SAU-ESA/646091/2006/FCOMP-01-0124-FEDER-007480FCT; strategic project PEst-OE/EQB/LA0023/2013; “BioHealth – Biotechnology and Bioengineering approaches to improve health quality”, Ref. NORTE-07-0124-FEDER-000027, co-funded by the Programa Operacional Regional do Norte (ON.2–O Novo Norte), QREN, FEDER; RECI/BBB-EBI/0179/2012 – Consolidating

Research Expertise and Resources on Cellular and Molecular Biotechnology at CEB/IBB, FCOMP-01-0124-FEDER-027462, FEDER; and the DNA mimics project PIC/IC/82815/2007. The FCT BPD fellowship of Susana P. Lopes SFRH/BPD/95616/2013 and the support of the COST-Action TD1004: Theragnostics for imaging and therapy is also acknowledged.

Conflicts of interest

None.

Acknowledgements

The authors would like to thanks to Dr Michael Surette (Farncombe Family Digestive Health Research Institute, McMaster University, Hamilton, ON, Canada) for kindly provide the *I. limosus* strain and to Fábio Ramalho (Department of Bioinformatics, University of Minho, Braga, Portugal) for creating the heatmaps.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijmm.2017.09.018>.

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