



Insights into Cystic Fibrosis Polymicrobial Consortia: The Role of Species Interactions in Biofilm Development, Phenotype, and Response to In-Use Antibiotics

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Cystic Fibrosis (CF) airways disease involves complex polymicrobial infections where different bacterial species can interact and influence each other and/or even interfere with the whole community. To gain insights into the role that interactions between *Pseudomonas aeruginosa* in co-culture with *Staphylococcus aureus*, *Inquilinus limosus*, and *Stenotrophomonas maltophilia* may play in infection, the reciprocal effect during biofilm formation and the response of dual biofilms toward ciprofloxacin under *in vitro* atmospheres with different oxygen availabilities were evaluated. Biofilm formation kinetics showed that the growth of *S. aureus*, *I. limosus*, and *S. maltophilia* was disturbed in the presence of *P. aeruginosa*, under both aerobic and anaerobic environments. On the other hand, under aerobic conditions, *I. limosus* led to a decrease in biofilm mass production by *P. aeruginosa*, although biofilm-cells viability remains unaltered. The interaction between *S. maltophilia* and *P. aeruginosa* positively influenced dual biofilm development by increasing its biomass. Compared with monocultures, biomass of *P. aeruginosa*+ *S. aureus* biofilms was significantly reduced by reciprocal interference. When grown in dual biofilms with *P. aeruginosa*, ciprofloxacin was less effective against *S. aureus*, *I. limosus*, and *S. maltophilia*, with increasing antibiotic doses leading to drastic inhibitions of *P. aeruginosa* cultivability. Therefore, *P. aeruginosa* might be responsible for the protection of the whole dual consortia against ciprofloxacin activity. Based on the overall data, it can be speculated that reciprocal interferences occur between the different bacterial species in CF lung, regardless the level of oxygen. The findings also suggest that alterations of bacterial behavior due to species interplay may be important for disease progression in CF infection.

Keywords: polymicrobial interaction, cystic fibrosis, antibiotic therapy, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Inquilinus limosus*, *Stenotrophomonas maltophilia*

INTRODUCTION

Cystic Fibrosis (CF) is a common lethal disease affecting nearly 70000 people around the world. It is characterized by the build-up of thick mucus overlying lung epithelial cells, wherein persistent cycles of chronic infection and inflammation occur (Gibson et al., 2003; Goss and Burns, 2007). The CF airways provide heterogeneous microenvironments containing variable levels of oxygen,

pH, nutrients, and antibiotics. This heterogeneity contributes largely for the proliferation of a phylogenetically diverse ecosystem, influencing the consortia of microbes able to occupy it (Yang et al., 2011). Several key microbial species contribute to CF lung infection and disease progression, beginning early in life with *Staphylococcus aureus* and *Haemophilus influenzae* and culminating in chronic infections caused by *Pseudomonas aeruginosa* or *Burkholderia cepacia* complex species (Razvi et al., 2009; Price et al., 2013).

It is now recognized that the different bacteria coexisting in CF airways have mutual interactions and contribute to the pathogenesis of the disease (Hibbing et al., 2010; Rogers et al., 2010). Nonetheless, the precise ways under which the many different organisms interact within the CF airways, and how these interactions influence the behavior of the individual species, the activity of the polymicrobial communities, and the relationship between host and microbes are poorly understood. Some studies have highlighted the potentially important roles of such interspecies interactions in disease phenotype and clinical outcome of CF infections (Amin et al., 2010; Chatteraj et al., 2010; Bragonzi et al., 2012; Lopes et al., 2012; Twomey et al., 2012).

Because CF infection is no longer viewed as being caused by a single pathogen, antibiotics, often used to target a small group of species recognized as key CF pathogens, are generally ineffective when other atypical species are present (Lopes et al., 2012, 2014) failing in many cases (Leekha et al., 2011).

In this study, the reciprocal influence of *P. aeruginosa* with *S. aureus*, *S. maltophilia*, and *Inquilinus limosus* was assessed. The first three species are important opportunistic pathogens that are often multidrug resistant and contribute significantly to the disease progression (Döring and Hoiby, 2004; Hauser et al., 2011; Ciofu et al., 2013). *S. aureus* and *S. maltophilia* are commonly co-isolated with *P. aeruginosa* from CF respiratory cultures (Hoffman et al., 2006; Blau et al., 2014; Zemanick et al., 2015). It is, therefore, plausible to hypothesize that these species interact and that this could theoretically affect their virulence and persistence. *I. limosus* has been pointed as a potential threat for CF patients, mainly due to the mucoid physiology, the multidrug resistance pattern, and the ability to persist in the respiratory tract (Chiron et al., 2005). Therefore, it is aimed to evaluate the contribution of species interactions under variable-oxygen atmospheres, particularly among *P. aeruginosa* and the referred CF-associated species, in biofilm formation, phenotype, and in its response to antibiotherapy often used for CF lung infections treatment.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Pseudomonas aeruginosa (wild-type strain UCBPP-PA14), *S. aureus* (wild-type strain ATCC 25923), *I. limosus* (strain M53, isolated from CF sputum), and *S. maltophilia* (isolated from CF sputum) were used throughout this work. *I. limosus* M53 was gently provided by Dr. Michael Surette (University of Calgary, Calgary, AB, Canada) (Sibley et al., 2011) and *S. maltophilia* by Dr. Joerg Steinmann (Institute of Medical Microbiology,

University Hospital Essen, University of Duisburg-Essen, Germany) (Vidigal et al., 2013).

All strains were stored at $-70 \pm 2^\circ\text{C}$ in tryptic soy broth (TSB, Liofilchem, Italy) supplemented with 20% of glycerol. Prior to each assay, bacteria were subcultured twice from frozen stock preparations onto TSB supplemented with 12% (w/v) of agar and incubated aerobically at 37°C for 24–48 h.

All assays were carried out by using a standardized bacterial inoculum. Briefly, an overnight culture was grown aerobically in TSB under agitation (120 rpm) at 37°C , being then adjusted with sterile broth medium to an OD_{640} corresponding to 1×10^7 CFU/mL for all strains. For dual-species cultures, the suspended inoculums of each bacterial species were combined in a 1:1 ratio.

Biofilm Formation under Aerobic and Anaerobic Environments

Each well of a 96-well polystyrene microtiter plate (Orange Scientific, Braine L'Alleud, Belgium) was seeded with 200 μL of standardized inoculum (single or dual cultures) and incubated at 37°C , 120 rpm, under aerobic and anaerobic environments. For aerobic assays, microtiter plates were placed in a standard incubator (n-biotek, Model NB-205Q, Korea). The anaerobic atmosphere was created in plastic boxes with AnaeroGen (Oxoid Limited, Hampshire, England). At different sampling time points, the liquid content of the microtiter plates was discarded and the wells were washed once with distilled sterile water. Biofilm formation was then assessed by plate counts and crystal violet (CV) assay as described below.

This experiment was performed in three independent assays for each one of the species used and conditions, and for a total of three combinations between species (*P. aeruginosa*+*S. aureus*; *P. aeruginosa*+*I. limosus*; and *P. aeruginosa*+*S. maltophilia*).

Kinetics of Biofilm Formation

The kinetics of biofilm growth were performed every 2 h, until 24 h, through biofilm-cells cultivability.

Briefly, biofilm cells were detached by sonication using an ultrasound bath (Sonic model SC-52, UK), operating at 50 kHz, for 10 min, and then resuspended by pipetting up and down three times. This sonication step was previously optimized to ensure that all biofilm cells were detached from the wells of the microtiter plate, without cell disruption, and biofilms aggregates dispersed into single bacteria (Supplementary Figures 1 and 2).

Subsequently, the disrupted biofilms were serially diluted (1:10) in sterile water, streaked onto tryptic soy agar (TSA) plates and incubated at 37°C for 24 h, for total CFU counting. For dual-species biofilms, different selective agar media were used for better discrimination between the two species. *Pseudomonas* isolation agar (PIA) was used to assess *P. aeruginosa* counts. Mannitol Salt Agar (MSA) and *Burkholderia cepacia* selective agar (BCSA) supplemented with 300 000 IU/L polymyxin B and 100 mg/L ticarcillin are selective media commonly used to discriminate *S. aureus* and *I. limosus*, respectively. In dual biofilms, *S. maltophilia* viable cell counting was estimated by the difference between the average of total counts on TSA and the

average of *P. aeruginosa* (planted on PIA). Afterward, selective agar plates were incubated at 37°C for 24 h. The specificity of the agar media was previously tested by growing each bacteria on the selective media of the other species and no growth was observed.

The number of cultivable bacterial cells in biofilms was determined and expressed per area of well (log CFU cm⁻²).

Phenotype of 24 h-Old Biofilms

Single and dual species biofilms were allowed to grow for 24 h and, afterward, characterized in terms of their biomass and number of culturable cells, as follows:

Biomass

Biomass was quantified by CV staining method (Stepanovic et al., 2000). Briefly, wells were allowed air-drying for 10 min after washing. Attached bacteria were then fixed with methanol (Fisher Scientific, Leicestershire, UK) for 15 min and stained with 1% (vol/vol) CV (Merk, Germany) for 1 min. The excess stain was removed by aspirating the content of each well and washed twice with distilled sterile water. Lastly, wells were decolorized with 33% (vol/vol) of acetic acid (Fisher Scientific, UK) and the optical density (OD) of the obtained solution was measured at 570 nm using a microtiter plate reader (Model Sunrise-basic Tecan, Austria).

Cell Cultivability

The number of adhering bacteria was determined after biofilm cell detachment by sonication for 10 min, using an ultrasound bath, and then the viable cell count was carried out, as described above.

Ciprofloxacin Activity against Single and Dual Biofilms

The antimicrobial action of ciprofloxacin was assessed against single and dual biofilms analysing cell cultivability. Briefly, each well of a 96-well polystyrene microtiter plate was seeded with 100 µL of bacterial culture (single or dual cultures), at 1×10^6 CFU/mL, + 100 µL of ciprofloxacin at 1/4; \times MIC, MIC or $4 \times$ MIC determined for *P. aeruginosa*. The minimum inhibitory concentration (MIC) of ciprofloxacin (Sigma-Aldrich) for *P. aeruginosa* was 0.125 mg/L, as assessed by microdilution technique according to the EUCAST guidelines (EUCAST, 2003). Negative controls (CTRL) were also performed by adding 100 µL of TSB instead of the antibiotic. Microtiter plates were then incubated at 37°C, 120 rpm, under aerobic and anaerobic environments for 24 h.

The number of viable bacteria within biofilms was determined by CFU counting following the procedure abovementioned. Three independent assays were performed for each species and condition.

Determination of the Competitive Index (CI) and the Relative Increase Ratio (RIR)

In dual cultures, the Competitive Index (CI) was defined as the *P. aeruginosa*/*S. aureus* or *P. aeruginosa*/*I. limosus* or *P. aeruginosa*/*S. maltophilia* ratio within the output

sample divided by the corresponding ratio in the inoculum (input): $CI = (P. aeruginosa/S. aureus \text{ or } I. limosus \text{ or } S. maltophilia)_{\text{output}} / (P. aeruginosa/S. aureus \text{ or } I. limosus \text{ or } S. maltophilia)_{\text{input}}$, where output and input samples were assessed after plating onto selective media serial dilutions of the sample taken at fixed times or the inoculum ($t = 0$), respectively, (Macho et al., 2007). For statistical purposes, 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 the two species; a positive CI value indicates a competitive advantage for *P. aeruginosa*; a negative CI value indicates a competitive advantage for *S. aureus* or *I. limosus* or *S. maltophilia*. Similarly to CI, the Relative Increase Ratio (RIR) was calculated based on the growth results obtained from monocultures of each strain (Macho et al., 2007).

Statistical Analysis

Means and standard deviations are shown for each graph, derived from three independent assays. All statistical analyses were performed using Prism Software (GraphPad version 6.0 for Macintosh), considering as statistically significant a *p*-value less than 0.05.

Differences were assessed by ANOVA-test followed by Tukey multiple-comparison test for biofilm formation (aerobic *versus* anaerobic conditions and single *versus* mixed consortia single) and for ciprofloxacin activity (single *versus* mixed consortia). Regarding the kinetics of biofilm growth, statistical analysis was provided using Student's *t*-test and the null hypothesis (single *versus* mixed consortia single). The latter was also used to inspect each CI and RIR indexes: the mean index was not significantly different from 1.0. When appropriate, CI and RIR from a given experiment were compared using unpaired Student's *t*-test, and significant differences are suggestive of a meaningful competition between the species (Macho et al., 2007). Statistical significance is represented in figures by asterisks.

RESULTS

Single and Dual-Species Biofilm Experiments under Variable Oxygen Conditions

It is well known that, in multispecies biofilms, the interactions established may encourage the coexistence (synergism) or, contrarily, confer advantage to one species, inhibiting the growth of other species (antagonism) (Harrison, 2007). In order to explain the possible interactions occurring between four CF-related pathogens in dual-species biofilms, several features were analyzed: the kinetic of biofilm formation in single and dual-species, the competitive indexes (CI and RIR), the phenotypic diversity (through cultivability assessment and biomass quantification) and the antimicrobial effect of ciprofloxacin on biofilm formation of single and dual-species.

The biofilm growth under variable oxygen atmospheres for *S. aureus*, *I. limosus* and *S. maltophilia*, growing individually or

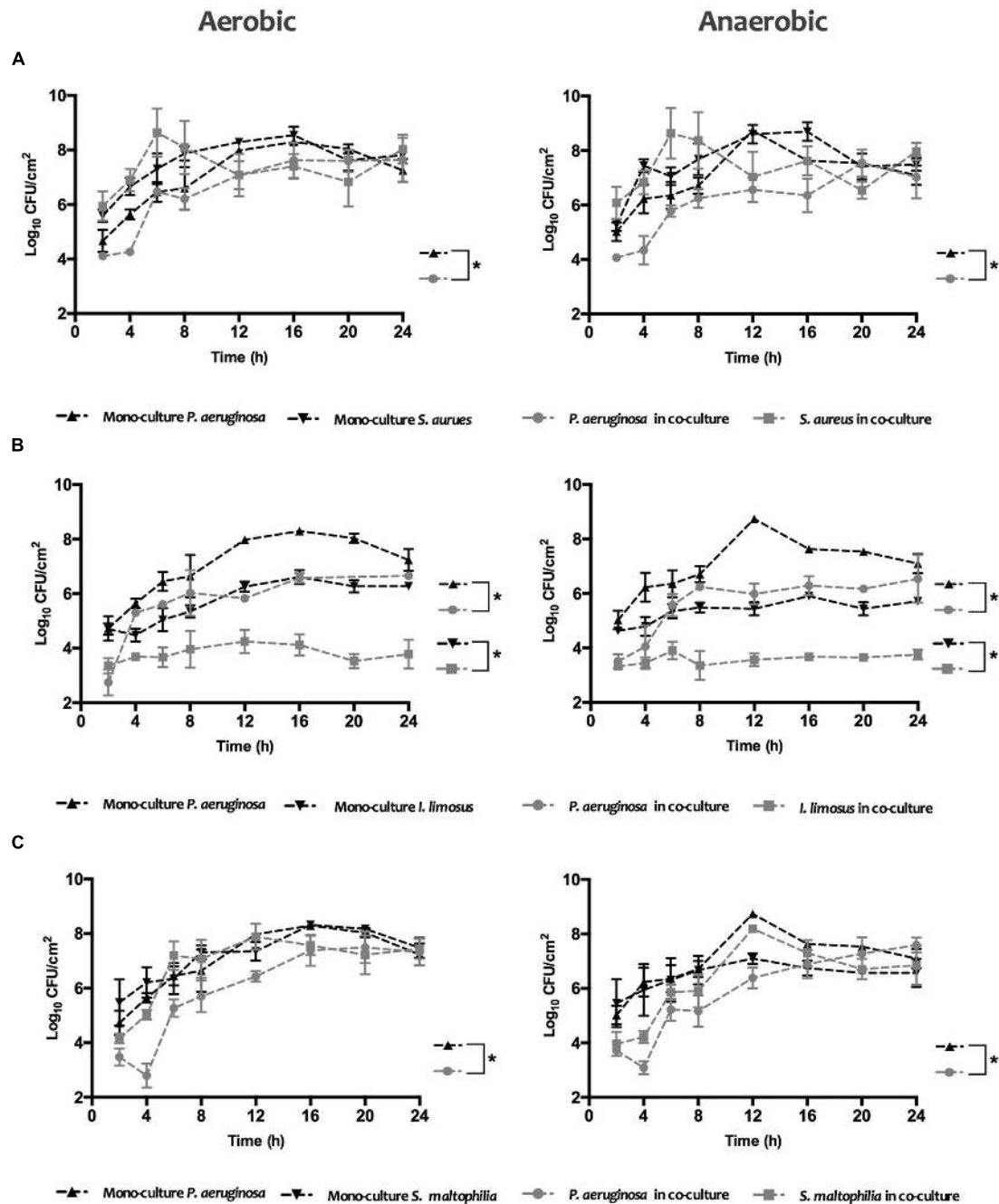


FIGURE 1 | Biofilm formation kinetics of (A) *P. aeruginosa* and *S. aureus* (B) *P. aeruginosa* and *I. limosus*, and (C) *P. aeruginosa* and *S. maltophilia*, alone and in combination, under aerobic and anaerobic conditions. Data for each time point are shown as mean \pm SD.

in combination with *P. aeruginosa*, was assessed by CFU counts over 24 h (Figure 1). Concerning single cultures, similar growth trends were observed for aerobic and anaerobic environments with *P. aeruginosa* dominating for most cases (maximum of \sim Log 8.6 achieved). In dual cultures, the viability of each species in the consortium decreased in comparison with monocultures, being that *P. aeruginosa* prevailed over *I. limosus* in co-culture.

For a clear comprehension of the differences in the growth curves of each species in single *versus* dual cultures under the different O₂ environments, CI and RIR indexes were estimated (Figure 2). Whilst CI allows comparing the differences among the growth curves of dual cultures, RIR index compares the growth curves of both species within pure cultures. As shown in Figure 2, a positive CI index is always observed, meaning a clear competitive advantage for *P. aeruginosa* over the other

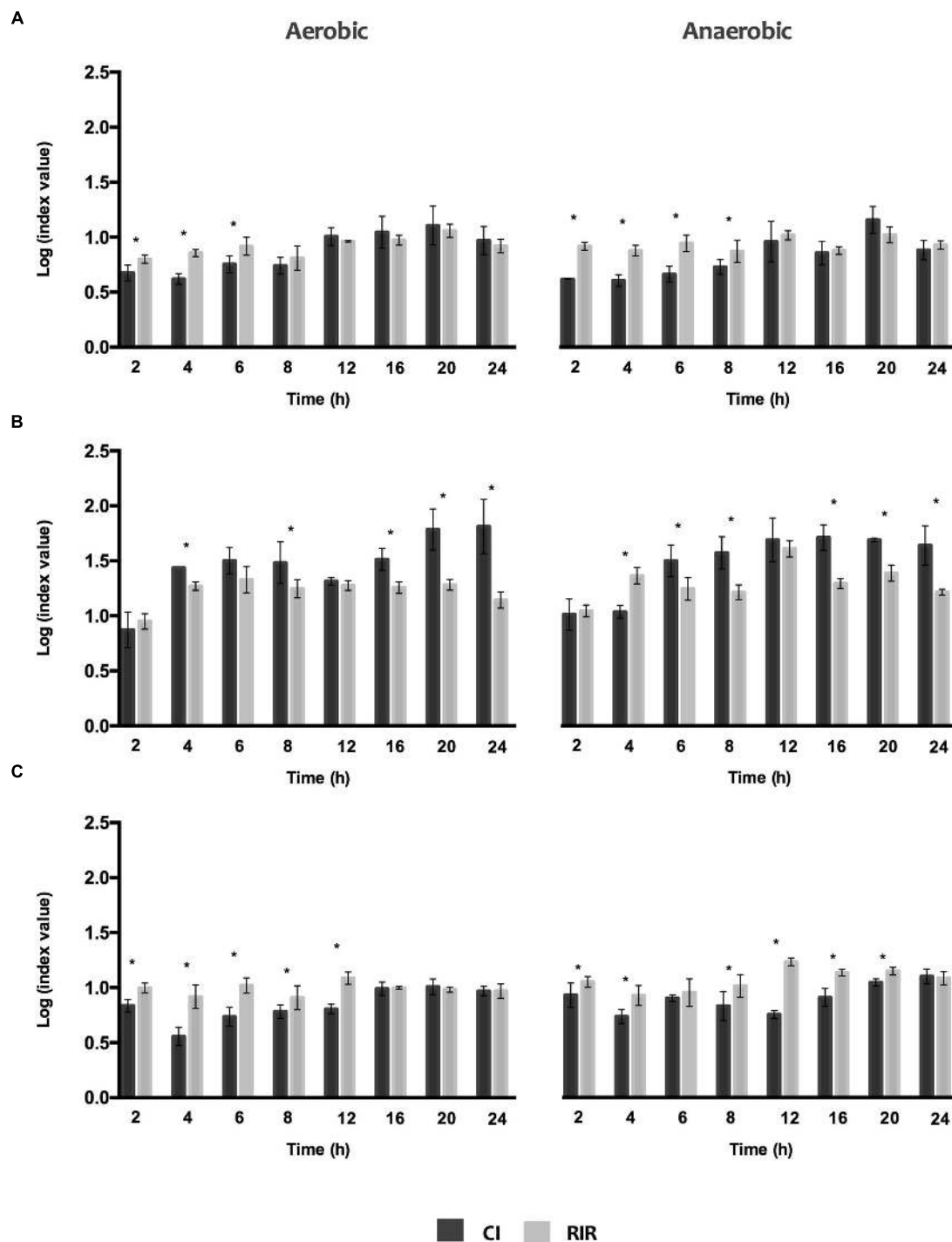
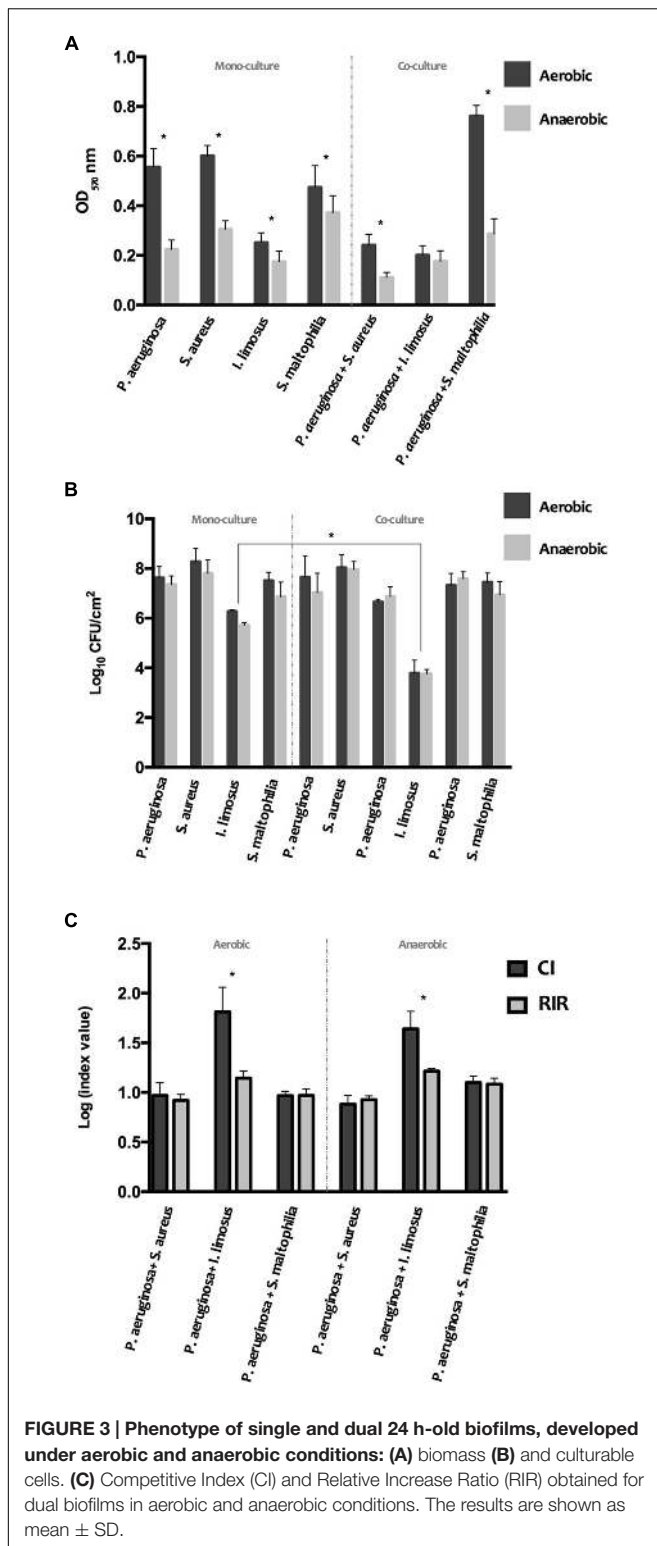


FIGURE 2 | Competitive index (CI) and Relative Increase Ratio (RIR) obtained for single and dual biofilm of (A) *P. aeruginosa* and *S. aureus*, (B) *P. aeruginosa* and *I. limosus* and (C) *P. aeruginosa* and *S. maltophilia*. Data are shown as mean \pm SD.

species in dual cultures. The dominant inhibitory effect of *P. aeruginosa* can be noticed in the lag and exponential phases (between 2 and 8 h), for *P. aeruginosa*+*S. aureus* dual cultures (Figure 2A) or even all over 24 h for *P. aeruginosa*+*I. limosus* and *P. aeruginosa*+*S. maltophilia* (Figures 2B,C). A strong inhibitory

effect is observed for *P. aeruginosa*+*I. limosus* consortia, with statistical significances ($P < 0.05$) obtained for CI versus RIR for the 24 h of growth under both aerobic and anaerobic conditions.

In order to assess whether species interactions could interfere with the biofilm-producing ability, the biomass and number



of culturable cells of the 24 h-old dual biofilms formed under aerobic and anaerobic conditions were determined (Figure 3). Interactions among the species in the dual populations were evaluated by CI and RIR indexes. Data from single and dual

cultures revealed that the amount of biofilm mass declined markedly under low oxygen environments ($P < 0.05$) for most cases (Figure 3A), however with no significant changes in terms of cell cultivability (Figure 3B). These results led to put the hypothesis that the species within biofilms are more prone to produce extracellular polymeric substances (EPS) when grown under aerobic atmospheres.

In the co-infection scenario, the number of viable cells of *P. aeruginosa* and *S. aureus* that adhered to polystyrene were not affected comparing with the respective single biofilms (Figure 3B), as confirmed by comparable CI and RIR values (Figure 3C). However, the biofilm mass level was significantly lower than that for both single populations ($P < 0.05$). Therefore, it can be speculated that the reciprocal interference observed in *P. aeruginosa* and *S. aureus* dual-biofilms may have led to a decrease on the EPS production by the overall consortium. Similarly to *P. aeruginosa*+*S. aureus* consortium, the cultivability of *P. aeruginosa*+*S. maltophilia* biofilms was not significantly disturbed, compared with single biofilms (Figure 3B), with CI versus RIR not showing significant differences among both bacterial species within the biofilm (Figure 3C). However, this reciprocal interference triggered an increase in the biomass of the overall consortium, in particular when developed aerobically (Figure 3A). Regarding *P. aeruginosa* and *I. limosus* consortia, dual populations produced lower biomass compared to *P. aeruginosa* single populations ($P < 0.05$), under aerobic environments (Figure 3A). These data can suggest a potential inhibition of EPS production by *P. aeruginosa* under this condition in the presence of *I. limosus*. Nonetheless, a significant decrease in *I. limosus* cultivability (Figure 3B) was observed ($P < 0.05$), under both conditions, with no significant disturbances for *P. aeruginosa* cultivability in dual populations (Figure 3B). The significant difference between CI and RIR indexes obtained for these dual cultures of *P. aeruginosa* versus *I. limosus* ($P < 0.05$) (Figure 3C), under both oxygen atmospheres, led to conclude that the loss of cultivability in *I. limosus* resulted from the interaction with *P. aeruginosa*, which outcompetes *I. limosus* and affects its growth in dual biofilms.

Antimicrobial Activity of Ciprofloxacin on Single and Dual-Species Biofilm Formation

The effect of ciprofloxacin at $1/4 \times \text{MIC}$, MIC and $4 \times \text{MIC}$ (MIC = 0.125 mg/L, previously achieved for *P. aeruginosa*) on the development of biofilms under variable oxygen atmospheres was assessed by viable biofilm-cell count (Figure 4). Overall, increasing concentrations of antibiotic led to gradual reductions (maximum of $\sim \text{Log } 3.3$ achieved) of biofilm-cell viability in single and dual cultures, both under aerobic and anaerobic conditions, when compared to un-treated biofilms (CTRL). In turn, viability reduction was not so evident when ciprofloxacin was used against polymicrobial biofilms. When *P. aeruginosa* grown in dual biofilms with *S. aureus* or *S. maltophilia*, ciprofloxacin was only effective on *P. aeruginosa* (Figures 4A,C), with increasing antibiotic doses leading to drastic inhibition of *P. aeruginosa* cultivability. Regarding *P. aeruginosa*+*I. limosus*

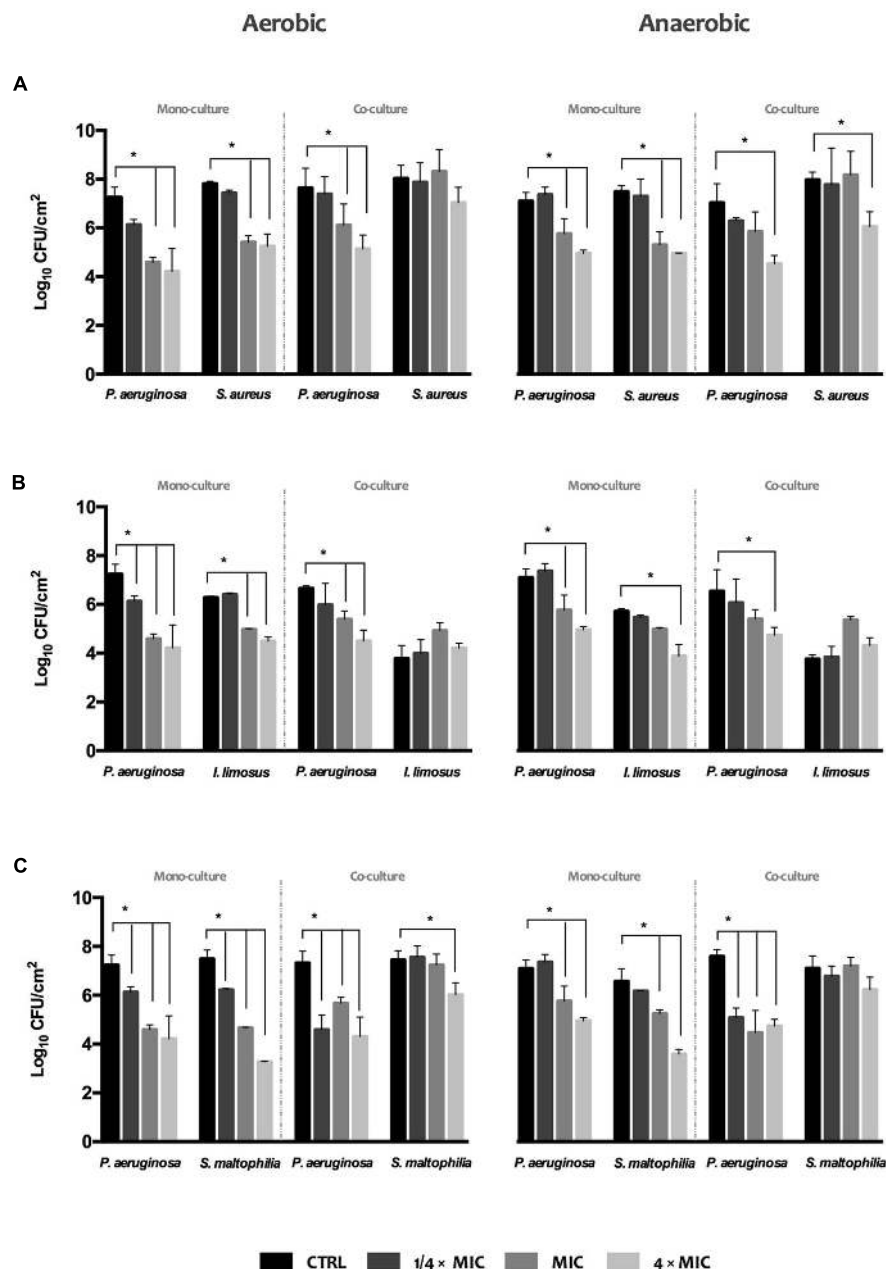


FIGURE 4 | Activity of ciprofloxacin against single and dual 24 h-old biofilms of (A) *P. aeruginosa* and *S. aureus*, (B) *P. aeruginosa* and *I. limosus*, and (C) *P. aeruginosa* and *S. maltophilia*. The results are shown as mean \pm SD.

consortium, the viability of *I. limosus* remains the same or increases relatively to the un-treated consortium and its single biofilm, highlighting loss of susceptibility toward ciprofloxacin (Figure 4B).

Together, these findings revealed that *S. aureus*, *I. limosus*, and *S. maltophilia*, when grown in consortia with *P. aeruginosa*, seem to be overprotected by it against ciprofloxacin action. In fact, these species showed increasing tolerance to ciprofloxacin in comparison with the corresponding single species biofilms and with un-treated biofilms.

DISCUSSION

The complex microbial communities of the CF respiratory tract constitute a challenging niche in which a number of microbial species can interact, contributing to disease progression and clinical outcome. The significance of microbe-microbe interactions in CF infections and the consequences of such interplay remain poorly understood, thus studies helping to comprehend the role that species interactions may play in infection prevalence and disease progression are welcome.

Thus, in this study, it was aimed to gain insights into the role of bacterial interactions, in particular among *P. aeruginosa* with other CF-associated species (*S. aureus*, *I. limosus*, and *S. maltophilia*), in biofilm formation and phenotype and in driving a response to antibiotherapy, often used for CF lung infections treatment, under variable-oxygen atmospheres.

Unlike *P. aeruginosa*, which has been extensively studied under similar CF environments (Field et al., 2005; Hogardt and Heesemann, 2010; King et al., 2010; Schertzer et al., 2010; Schobert and Jahn, 2010), the behavior of other bacterial species under the oxygen conditions found in *in vivo* CF airways are still poorly understood, often failing to consider the role of biofilm development, oxygen availability, and the interplay among microorganisms within polymicrobial infections in CF context.

The biofilm formation kinetics, under aerobic and anaerobic conditions, and the significant differences between the CI and RIR indexes indicated a competitive advantage of *P. aeruginosa* over the remaining species, when in dual biofilms. This competition was observed during the first stages of biofilm growth (lag and exponential growth phases) or even for all over 24 h, resulting in higher numbers of *P. aeruginosa* compared to *S. aureus*, *I. limosus*, and *S. maltophilia* cells. In fact, antagonism between microorganisms within a community can be a result of bacterial competition for both nutrients and space, or to direct antagonistic effects (Harrison, 2007; Hibbing et al., 2010). There is evidence supporting antagonism between *P. aeruginosa* and *S. aureus* as it has been reported that *S. aureus* is susceptible to *P. aeruginosa* exoproducts, such as pyocyanin, hydrogen cyanide or alkyl-hydroxyquinoline *N*-oxides (HQNO), which are able to suppress its aerobic metabolism and growth (Hoffman et al., 2006; Biswas et al., 2009). Recently, Filkins et al. (2015) reinforced that HQNO and siderophore produced by *P. aeruginosa* additively induce a transition of *S. aureus* metabolism from aerobic respiration to fermentation and eventually lead to loss of *S. aureus* viability.

Interestingly, when simultaneously cultured in dual biofilms, *I. limosus* and *S. maltophilia* cannot coexist with *P. aeruginosa* in a dynamic equilibrium. The results from CI versus RIR suggested that *I. limosus* and *S. maltophilia* are, in a general point of view, outcompeted. These findings are in agreement with Pompilio et al. (2015) that found that *P. aeruginosa*, in dual biofilms, significantly affect *S. maltophilia* growth. They also shown that, when grown with *S. maltophilia* in dual biofilms, some *P. aeruginosa* virulence factors, as alkaline protease and alginate, were up-regulated.

An important feature of *P. aeruginosa* infection is its ability to form biofilms, which is one of the contributing factors to reduce antibiotic efficacy and provide tolerance to the host inflammatory defense mechanism (Høiby et al., 2011). Data obtained from single and dual cultures showed that the biofilm-producing ability was markedly higher under aerobic atmospheres. Kadouri and Tran (2013) measured biofilm formation by a variety of opportunistic pathogens, at different oxygen concentrations, and also established a positive correlation between oxygen levels and biofilm formation ability. Data also suggested that the changes in the overall biofilm

mass and culturable cells of dual biofilms can be due to reciprocal interference between species in the consortia or even only triggered by *P. aeruginosa*. Dual biofilms encompassing *P. aeruginosa*+*S. aureus* or *P. aeruginosa*+*S. maltophilia* were not significantly affected in terms of biofilm-cell number, as the whole consortia had similar proportions of both species (~50% each), for aerobic and anaerobic atmospheres. Nonetheless, the reciprocal species interference (CI versus RIR without significant differences) led to marked changes in biofilm mass of the final consortia, suggesting that EPS production changes may occur. In chronic CF, the matrix of mucoid *P. aeruginosa* biofilms is majority constituted by alginate, a linear polyanionic EPS (Bjarnsholt et al., 2009), that has shown to significantly contribute to decreased susceptibility of biofilms to antibiotic treatment and to human antibacterial defense mechanisms (Pier et al., 2001). For instance, the production of biofilm biomass by *P. aeruginosa*+*S. aureus* was significantly reduced under both oxygen environments whereas an increase in EPS accumulation was observed for *P. aeruginosa*+*S. maltophilia* biofilms, in particular under aerobic conditions. In apparent contradiction with the overview of the results obtained from kinetic data, that revealed a clear dominant advantage for *P. aeruginosa*, biofilm-formation ability of dual cultures seems to result from a reciprocal interference between both species in *P. aeruginosa*+*S. aureus* and *P. aeruginosa*+*S. maltophilia* consortia. These findings could be explained considering the complexity of the microbial interactions in the CF lung, and the presence of many factors contributing to the biofilm formation.

Concerning *P. aeruginosa*+*I. limosus* biofilms, results demonstrated that this biofilm was significantly disturbed in terms of overall biomass that could be justified by the predominance of *P. aeruginosa* in the consortium. A significant difference among CI versus RIR indexes under both oxygen conditions led to conclude that a strong competition between the species occurred, with *P. aeruginosa* predominating and outcompeting *I. limosus*. In a previous work, Lopes et al. (2012) has demonstrated that, in dual biofilms, *P. aeruginosa* biomass was markedly reduced by the presence of *I. limosus*.

Understanding the interspecies interactions in dual infections is crucial, not only because they can modulate the virulence and persistence of pathogens, but also because that knowledge can assist the design of tailored therapy regimens and the definition of new antimicrobial agents, new targets, and strategies for CF disease control. Ciprofloxacin is one of the most commonly used oral agent used to control pulmonary infections caused by *P. aeruginosa* in CF patients (Bittar and Rolain, 2010; Guss et al., 2011; Sriramulu, 2013). The exposure of biofilms to increasing concentrations of ciprofloxacin lead to gradual inhibition in biofilm cells, for both aerobic and anaerobic environments. Furiga et al. (2015), when assessing the efficacy of a new quorum sensing inhibitor in combination with ciprofloxacin, against *P. aeruginosa* biofilms, observed a synergistic anti-biofilm activity regardless the oxygen conditions. When applied to dual consortia, ciprofloxacin only reduced *P. aeruginosa* viability, being the viability of the other species of the consortia unchanged. Data suggest a potential protective effect of *P. aeruginosa* over the other species against ciprofloxacin. Similar hints

were pointed out by Pompilio et al. (2015) as they stated that *P. aeruginosa* might be responsible for the protection of *S. maltophilia* against tobramycin in dual biofilms due to alginate overproducing. Moreover, data also shown that, independently of the reduced biomass noticed in *P. aeruginosa*+*S. aureus* and *P. aeruginosa*+*I. limosus* dual biofilms, the high number of biofilm-encased cells was enough to imply an increased tolerance on those consortia. In fact, biofilm tolerance is thought to be multifactorial, resulting by (i) decreased growth rates, due to oxygen and nutrient microscale heterogeneities within the biofilm; (ii) the protective barrier provided by the EPS, retarding or inactivating the penetration of antibiotics into the biofilm; (iii) the number and spatial distribution of bacterial cells within biofilms; (iv) the expression of biofilm-specific resistance genes; (v) the presence of “persisters”, i.e., a subpopulation of microorganisms that differentiate into a dormant and protected state, like a spore-bacterial form (Hill et al., 2005; Hassett et al., 2010).

The majority of studies about interactions in the polymicrobial CF community focus on the traditional pathogen *P. aeruginosa*, due to its prevalence in CF lung, examining its ability to form biofilms, as this lifestyle protects the microorganism to the host responses and to numerous antibiotics, and its potential to develop chronic infections (Magalhães et al., 2015). However, several studies have highlighted the crucial role of interspecies interactions in influencing infection status, clinical outcomes, and response to therapy in CF infections, suggesting that the role of other microbial species needs to be considered (Harrison, 2007; Ryan et al., 2008; Sibley et al., 2008; Rogers et al., 2010). In the present work, *P. aeruginosa* exhibited a dominant inhibitory effect, when co-cultured in biofilms with *S. aureus*, *I. limosus*, and *S. maltophilia* under both aerobic and anaerobic conditions. On the other hand, biofilm formation ability results from the reciprocal interaction between *P. aeruginosa* and *S. aureus* or *S. maltophilia*. Furthermore, it was found that the exposure of dual biofilms to ciprofloxacin reduced the viability of *P. aeruginosa* but not of *S. aureus*, *I. limosus*, and *S. maltophilia*. These findings suggest that *P. aeruginosa* might be responsible for the protection of *S. aureus*, *I. limosus*, and *S. maltophilia*, in dual biofilms toward ciprofloxacin. In conclusion, this study underlines the importance of bacterial

interactions in lung infections and in particular the complexity of the interactions of different pathogens that coexist in the CF airways.

Nonetheless, further investigations are required for inspecting the role of the EPS matrix in the behavior the polymicrobial consortia studied and considering host response and the molecular mechanisms involved in the microbe-microbe interactions, using both *in vitro* and *in vivo* models of chronic infection that better mirror the progression of CF lung infections.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: SPL and MOP. Performed the experiments: APM and SPL. Analysed the data: APM, SPL, and MOP. Wrote the paper: APM, SPL, and MOP.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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