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Food web and metabolic interactions of the lung inhabitants *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*

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Abstract

Bacteria that successfully adapt to different substrates and environmental niches within the lung and overcome the immune defence can cause serious lung infections. Such infections are generally complex, and recognized as polymicrobial in nature. Both *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* can cause chronic lung infections and were both detected in cystic fibrosis (CF) lung at different stages. In this study, single and dual species cultures of *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* were studied under well-controlled planktonic growth conditions. Under pH-controlled conditions, both species apparently benefited from the presence of the other. In co-culture with *P. aeruginosa*, *S. pneumoniae* grew efficiently under aerobic conditions, whereas in pure *S. pneumoniae* culture, growth inhibition occurred in bioreactors with dissolved oxygen concentrations above the microaerobic range. Lactic acid and acetoin that are produced by *S. pneumoniae* were efficiently utilized by *P. aeruginosa*. In pH-uncontrolled co-cultures, the low pH triggered by *S. pneumoniae* assimilation of glucose and lactic acid production negatively affected the growth of both strains. Nevertheless, ammonia production improved significantly, and *P. aeruginosa* growth dominated at later growth stages. This study revealed unreported metabolic interactions of two important pathogenic microorganisms and shed new lights into pathophysiology of bacterial lung infection.

INTRODUCTION

The lung is a stable, warm, humid, organic compound-rich environment and provides favourable conditions for the proliferation of many organisms. In a healthy lung environment, energy is produced from the oxidation of sugar, fatty acids and branched-chain amino acids. The presence of mixed substrates in the lung is the rule rather than an exception, especially during lung infection. Palmer et al. (2007) previously determined average concentrations of ions, free amino acids, glucose and lactate in different cystic fibrosis (CF) sputum samples. In their analysis, both glucose and lactate were found at different concentrations. The C-sources availability varied also with the type of infection and the microbial community involved.

In all sites of infection, different microbial cells are exposed to a wide spectrum of potential substrates. This leads to a competitive advantage of cell population capable of such a mixed substrates growth. Based on the C-source availability (Conrad et al., 2013) and using ecological and functional networking approach, Quinn et al. (2016) developed the 'Climax and Attack model' to describe the CF lung microbiota. The authors differentiated between two communities: An 'Attack Community' including species of *Streptococcus*, *Rothia*,

Lactobacillus and *Veillonella* that ferment sugars and produce acids and lower the pH; and the 'Climax Community' with *P. aeruginosa* and *Staphylococcus aureus* as representatives, which utilize amino acids and produce ammonia. Principally by changing the pH, the authors explained the community stability during Climax community growth and instability during growth of the Attack community at exacerbation. The causes of phase shift between these communities are still unknown but hypothesized to be due to changes in microbiome physiological processes and/or antibiotic treatment (Quinn et al., 2016). Here, the microbial interactions are crucial for a successful establishment and maintenance of a microbial population. Different forms of microbial interactions and mechanisms are previously reported (Sabra et al., 2010; Scott et al., 2019). Among others, competition between species is a well-recognized ecological force to drive microbial metabolism and diversity. *Streptococcus pneumoniae* is known to produce H_2O_2 to inhibit the growth of other lung inhabitants (Pericone et al., 2000). On the other hand, alginate synthesized by *P. aeruginosa* is required for the promotion of *S. parasanguinis* biofilm formation, and a commensal relationship between the two strains was proposed (Scoffield et al., 2017). Scott et al. (2019) have recently reported that *P. aeruginosa* enhances the growth of *S. sanguinis* in an in vitro biofilm culture.

In the United Kingdom, pneumonia caused by *P. aeruginosa* and *S. pneumoniae* remains a common cause of morbidity and mortality in HIV-1-infected individuals (Allen et al., 2003; McConnell et al., 2010). Moreover, *P. aeruginosa* and several species of *Streptococci* were isolated from the lungs of different CF patients (Filkins et al., 2012; Quinn et al., 2016; Whiteson et al., 2014). *Streptococcus milleri* can cause exacerbations and lung damage in synergy with *P. aeruginosa* (Sibley et al., 2006). In mouse models, it has been shown that the infection with *S. mitis* together with *P. aeruginosa* could alleviate lung inflammation in acute lung infection (Song et al., 2017). The relative abundance of streptococci in the CF lung, including members of the oral microflora, was shown from different microbiome studies (Scott & O'Toole, 2019). *Streptococcus pneumoniae* has been considered to predispose patients to acute and chronic airway infections for other CF microorganisms, including mucoid *P. aeruginosa* and *Staphylococcus aureus* (del Campo et al., 2005; Konstan & Berger, 1993). In fact, possible interactions between different fermenter strains and *P. aeruginosa* in the lung environment were proposed (Venkataraman et al., 2014). Such an interaction might cause a significant increase in the virulence factor production, antimicrobial activity and biofilm formation by *P. aeruginosa*. Although the understanding of interactions between cohabiting microbes can be important for pathogenesis, the nature of polymicrobial infection is rather complex and is not well understood. The current work investigates the single and dual species cultures of two common pathogens associated with lung infection, namely, *P. aeruginosa* and *S. pneumoniae* in controlled bioreactors. Studies of these two organisms in co-culture, to date, have not been performed. Here we show that the two microbes exchange nutrients in a complex food web, and lactate and acetoin produced by *S. pneumoniae* under different growth conditions are efficiently metabolized by *P. aeruginosa* strain. Interestingly, the aerobic growth of *S. pneumoniae* was facilitated by the presence of *P. aeruginosa* culture, and H_2O_2 which normally inhibits *S. pneumoniae* growth in pure culture at aerated condition, was never detected in co-culture with *P. aeruginosa*. We show here for the first time that ammonium production and pH elevation are correlated with the presence of lactate in the medium or acid challenge. Our findings suggest the importance of lactate as an inter-bacterial metabolite in the CF polymicrobial infections and could have a considerable impact on the lung microbiota.

EXPERIMENTAL PROCEDURES

Bacteria strain, culture medium and growth conditions

Cryocultures of *S. pneumoniae* TIGR4 serotype 4 (ATCC BAA-334) were first platted on Columbia blood agar plates containing 5% sheep blood (BD Biosciences) and incubated overnight at 37°C under 5% CO_2 atmosphere. Cells were then scraped from plate and used to inoculate Todd-Hewitt-Broth + 1% (wt./vol.) yeast extract (THBY, Sigma) liquid medium in filled anaerobic serum bottle. Bottles were incubated at 37°C without shaking under 5% CO_2 for 10–12 h. The resulting culture was used to inoculate (1% vol./vol.) prewarmed bottles containing THBY, which are then incubated till an OD (OD600nm) of 0.7 ± 0.1. The last precultures were then used to inoculate bioreactor or flask experiments (5% vol./vol. inoculum). Carbon sources (glucose, lactate) were autoclaved separately and added before inoculation.

Cryocultures of *P. aeruginosa* PAO1 strain were first revived on the first preculture with LB liquid medium for 18 h at 37°C under shacking. Second precultures were prepared by transferring 1% (vol./vol.) from the former flask to a second one containing THBY medium and incubated at 37°C on a shaker. At an OD of 5 ± 0.5, cells were taken for inoculation to bioreactors (1% vol./vol. inoculum).

OD600 determination

For the estimation of bacterial growth in mono or co-culture, the OD was measured at 600 nm. For the

determination of the OD_{600} within the linear range of the spectrophotometer (0.01–0.6), culture samples were diluted with sterile growth media and measured in triplicates.

Bioreactor experiments

Batch cultivations were performed in a 1.5 L well-equipped parallel bioreactor system (DASGIP Parallel Bioreactor System, Jülich, Germany) with 1.0 L initial working volume. Cultivations were started by inoculating 30 ml (3%) seed culture. pH was either maintained at 7.0 during all the cultivations using 5 N NaOH and 2 M HCL or kept constant only during the first phase of growth by the addition of 5 N NaOH or left uncontrolled depending on the experiment. Dissolved oxygen tension was controlled at different values by mixing air, N_2 and pure O_2 in the inlet gas stream.

Viable cell count measurements

For determination of CFUs, 10-fold serial dilutions of cultures were prepared in 0.80% (wt./vol.) NaCl solution (pre-warmed to 37°C). A total volume of 100 μl from each dilution was then plated onto pre-warmed (37°C) Columbia blood agar plates (BD Biosciences), which were incubated at 37°C and 5% CO_2 for 24 h. The two strains can be differentiated on Columbia agar plates due to the different haemolytic activities of both.

Supernatant cytotoxicity

The culture supernatants were collected, filtered using 0.2 μm sterile filters and stored at 4°C prior to use. The mouse fibroblast cell line L929 acquired from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) was used to perform the Neutral Red uptake test according to ISO 10993-5. Cells were grown in culture flasks in RPMI1640 medium (Lonza) supplemented with 10% FCS (FCS Superior, Biochrom) and antibiotic mixture (100 U/ml penicillin and 100 μg/ml streptomycin, Lonza) in a humidified cell culture incubator at 5% CO_2. The cells were harvested during log phase and seeded into a 96 well plate, five wells per condition, 2×10^4 cells per well in 200 μl culture medium. The cells were allowed to attach to the culture dishes for 24 h. Then, the culture media were aspirated and replaced by the culture supernatants, negative control (culture medium), positive control (1% Triton X-100 in culture medium), respectively. Finally, the supernatants and control media were added to the culture plate and incubated for 24 h. The supernatants were then discarded and replaced by neutral red solution (Sigma Aldrich, 4 mg/ml in culture medium). Then the cell cultures were washed with phosphate buffer saline (pH 7.4) and the Neutral Red was extracted from the viable cells using ethanol/water/glacial acetic acid [50/49/1 (vol./vol./vol.)]. The OD was measured at 530 nm using a plate reader (Victor multilabel counter, Perkin Elmer).

Analytical methods

Cell concentration was measured optically, at 600 nm and correlated with cell dry weight determined directly. The concentrations of glucose, acetoin, organic acids in supernatant were determined by HPLC using an Aminex HPX-87H column (300 × 7.8 mm) and the detection was assessed by refractive index and ultraviolet detectors. The operating conditions were as follows: mobile phase, H_2SO_4 0.005 M; flow rate, 0.6 ml/min; temperature, 60°C. Pyocyanin measurement was performed as reported by Saha et al. (2008). Cyanide and ammonium in the supernatants were measured according to the German Institute of Standardization (Deutsche Institut für Normung, DIN) using DIN 38405-13 for cyanide and DIN ISO 15923-1:2014 for ammonium.

Hydrogen peroxide was determined in supernatants of *S. pneumoniae* cultures grown under semi-aerobic and aerobic conditions or in co-cultures with *P. aeruginosa*. The Amplex® Red Hydrogen Peroxide/Peroxidase assay kit (Invitrogen) was used to quantify H_2O_2 contents below 10 μM in the cell-free supernatants. Determinations of H_2O_2 up to 300 μM were performed as described by Pericone et al. (2003).

Statistical methods

Student's *t*-test (paired or unpaired) was used to compare the data set means in our experiments, and the probability values (*p*) were recorded. Probability value (*p*) less than 5% was taken to be significant. All calculations were done in Excel 2007.

RESULTS

Microaerobic condition supports the growth of *S. pneumoniae* TIGR4 in controlled bioreactors

The physiological oxygen concentrations or the amount of oxygen available for cellular respiration in the lung differs by the growth mode of the microorganism whether in biofilm or in planktonic mode. Here, we performed cultivations of *S. pneumoniae* TIGR4 in controlled bioreactors, where pH and dissolved oxygen tension (pO_2) are kept constant. Besides the growth under anaerobic conditions by continuous sparging of

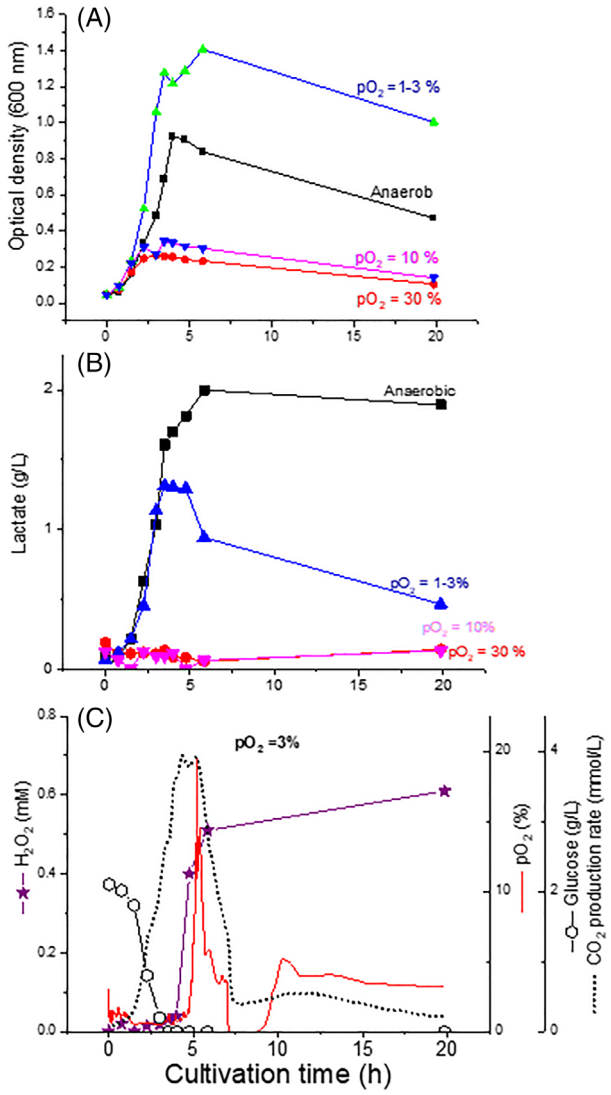

FIGURE 1 (A) Growth of *S. pneumonia* at different pO_2 values in pH-controlled bioreactors. (B) Lactate formation at the different pO_2 values; (C) CO_2 production rate, glucose consumption and H_2O_2 formation in the microaerobic culture (pO_2 = 1%–3% air saturation). All bioreactor experiments were inoculated with 5% (vol./vol.) using a 12 h old *S. pneumoniae* culture in THBY and a final OD of 0.75 (±0.04). The results obtained were reproduced in three independent trials

N_2 through the reactor, the pO_2 value was varied and controlled at microaerobic condition (pO_2 of 1%–3%), or aerobic conditions at either 10% or 30% of air saturation. To exclude the effect of the dissolved CO_2 on the growth of *S. pneumoniae* (Burghout et al., 2013), all bioreactor experiments were performed with a constant aeration rate of 0.1 vvm.

As shown in Figure 1(A), *S. pneumoniae* grew most efficiently under microaerobic conditions. In the aerobic cultures (pO_2 of 10% and 30%), a growth cessation occurred earlier, even in the presence of an excess amount of glucose. In contrast, in both anaerobic and microaerobic cultures, cells first entered the stationary phase after glucose limitation [~4 h, Figure 1(A)]. Moreover, the microaerobic growth condition (pO_2 = 1%–3%) sustained the highest biomass formation of *S. pneumoniae* in the THBY medium. Unlike the anaerobiosis, in which no H_2O_2 formation was detected, H_2O_2 production was detected in the microaerobic culture, though its concentration remained relatively low (<30 μM) during the glucose unlimited growth phase. After glucose limitation, and in synchronization with lactate re-assimilation in the microaerobic culture [Figure 1(B)], the H_2O_2 production increased significantly to a maximum concentration of about 0.53 ± 0.04 mM [Figure 1(C), $p = 0.003$ paired *t*-test analysis]. Consequently, *S. pneumoniae* growth ceased as evidenced by the decrease in the optical density (OD), the sharp decrease in the CO_2 production rate and the abrupt increase in the pO_2 value [Figure 1(C)].

It is well known that cells of *S. pneumoniae* produce autolysin A as virulence factor. The autolytic action of this enzyme leads to the cell lysis that is typical of pneumococcal cells growing in batch culture, especially at the stationary phase (Alonso DeVelasco et al., 1995; Kadioglu et al., 2008). Hence, cell viabilities in different cultures at late logarithmic phases were compared (~4 h). The viable cell counts decreased significantly from the microaerobic culture (1.01×10^9 CFU/ml), compared to the anaerobic growth condition (5.8×10^7 CFU/ml, $p = 0.0006$ using unpaired *t*-test). The diminished cell viability was even pronounced in the aerobic culture (between 9.3×10^6 and 9.5×10^6 CFU/ml; $p = 0.0005$). At the late stationary phase (~20 h), however, the cell viabilities decreased for all cultures but recorded a significant decrease at the aerobic ($p = 0.002$) and microaerobic range ($p = 0.0003$) compared to the anaerobic range (at 20 h, the CFU/ml was less than 3.4×10^3 at both microaerobic and aerobic ranges compared to an average value of 2.1×10^7 at the anaerobic growth condition).

In addition to organic acids and H_2O_2 production, analysis of *S. pneumoniae* TIGR4 culture supernatants revealed the production of acetoin. Acetoin is produced as a fermentation end product or as a metabolic intermediate for 2,3-butanediol production by many bacteria at low pH values (Sabra et al., 2016a; Xiao & Xu, 2007; Zeng & Sabra, 2011). In pH and pO_2 control cultures, the accumulated maximum amount of acetoin varied from 0.051 ± 0.012 $g/g_{biomass}$ in anaerobic cultures to 0.031 ± 0.008 $g/g_{biomass}$ in microaerobic cultures and to less than 0.01 $g/g_{biomass}$ in aerobic cultures (Figure 2). Moreover, compared to the anaerobic pH-controlled conditions, the acetoin production increased significantly in pH-uncontrolled anaerobic cultivations to a value of 0.35 $g/g_{biomass}$ ± 0.082 ($p = 0.006$ using unpaired *t*-test). Acetoin concentrations remained constant even after glucose limitation, indicating that no further metabolism of this compound by *S. pneumoniae* occurred.

Growth of *Pseudomonas aeruginosa* PAO1 in controlled bioreactors

Previous studies in our laboratories showed the importance of dissolved oxygen concentrations for the growth and virulence factors released by *P. aeruginosa* PAO1 (Kim et al., 2003; Sabra et al., 2002; Sabra et al., 2003; Sabra et al., 2014). Unlike the growth behaviour of *S. pneumoniae*, *P. aeruginosa* was shown to grow at a wide range of pO_2 values. However, compared to aerobic/anaerobic growth conditions, and similar to the growth behaviour of *S. pneumoniae*, the microaerobic conditions were shown to be favourable for the growth and virulence factors production by *P. aeruginosa* PAO1 (Sabra et al., 2002). As described above, acetoin and lactate were the two main metabolites produced and excreted by *S. pneumoniae*. We have therefore investigated the effect of these metabolites on the growth of *P. aeruginosa* in controlled bioreactors.

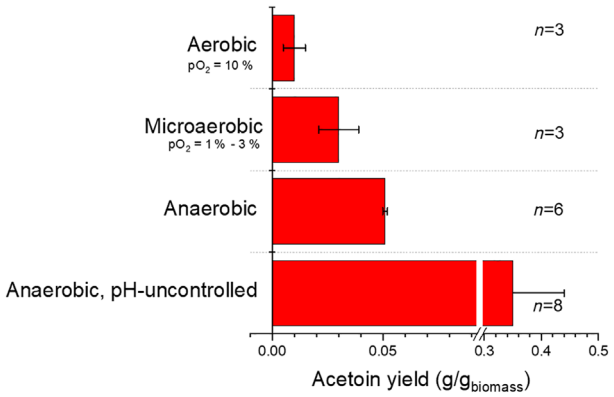

FIGURE 2 Acetoin production by *S. pneumoniae* TIGR4 grown at different conditions in pH and pO_2 controlled bioreactors compared to anaerobic serum bottles with neither pH nor pO_2 control. Acetoin was measured at the late stationary phase in all cultures and was correlated to the biomass produced (g/g biomass). THBY medium was used for the cultivation of *S. pneumoniae* and the initial pH was adjusted to 7.2 in the anaerobic bottles

Acetoin is metabolized efficiently by *P. aeruginosa* PAO1 without significant influence on pyocyanin production

Previous work has shown that the addition of either diacetyl or 2,3 butanediol to cultures of *P. aeruginosa* evokes the production of pyocyanin and hence enhances its virulence (Venkataraman et al., 2014). Acetoin is an intermediate compound that can be either oxidized to produce diacetyl or reduced to form 2,3-butanediol. It is of interest to know whether acetoin would have similar effects on *P. aeruginosa* as its oxidized and reduced derivatives. The effect of acetoin on the growth of *P. aeruginosa* was first tested by supplementing the THBY medium with acetoin in the concentration range of 0.1–2.5 g/L. While higher concentrations of acetoin inhibited the growth of PAO1 [Figure 3(A)], a concentration of 1 g/L of acetoin was found to slightly stimulate the growth in shake flask cultures with glucose alone and on the co-substrate comprising glucose and lactate [Figure 3(B)]. Pyocyanin production was not significantly enhanced in all

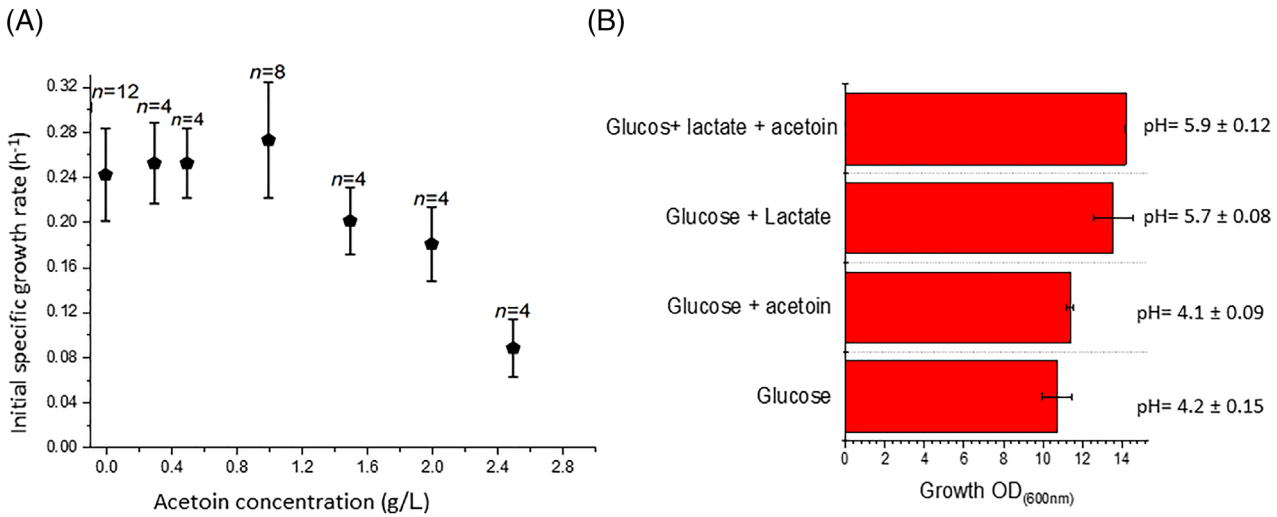

FIGURE 3 (A) Growth of *P. aeruginosa* in THBY medium supplemented with different concentrations of acetoin. Flasks were inoculated with 1 vol./vol. using a 24 h old *P. aeruginosa* grown in THBY medium. After inoculation, the flasks were incubated aerobically on a shaker and the OD was measured periodically. The initial specific growth rates were calculated at the first 6 h using the following formula [$\mu = \frac{\ln X_2 - \ln X_1}{6}$], where X_1 and X_2 are the OD_{600} after inoculation and 6 h, respectively. (B) The effect of acetoin on the growth of *P. aeruginosa* in uncontrolled shake flask cultures agitated at 150 rpm. An overnight culture of *P. aeruginosa* in THBY medium (200 µl, OD_{600} of 6.5) was used to inoculate 50 ml THBY medium in 100 ml baffled flasks supplemented with either glucose or a blend of glucose and lactate. In all flasks the initial pH was adjusted to 7.2, and the results show the mean of three independent experiments

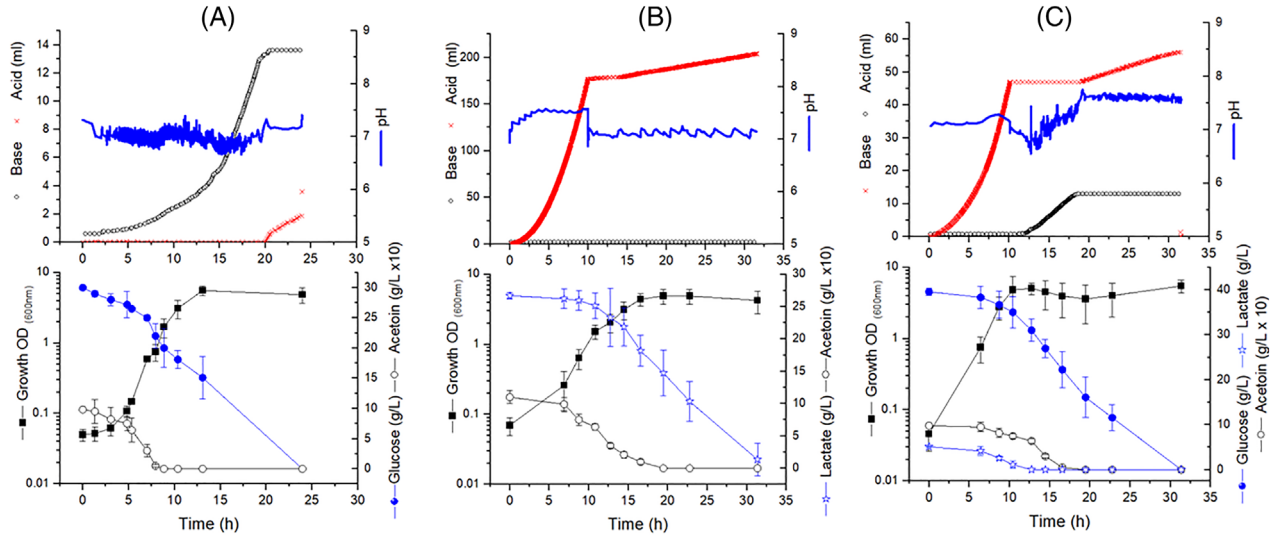

FIGURE 4 pH-controlled microaerobic growth of *P. aeruginosa* on glucose (A), lactate (B) or blend of both (C). An overnight culture of *P. aeruginosa* (2500 µl, OD_{600} of 5.9) was used to inoculate bioreactors with 700 ml medium supplemented with 1 g/L acetoin and glucose, lactate or a blend of glucose and lactate. All values are averages of two independent cultivations in controlled bioreactors

cultures, irrespective of presence or absence of acetoin (*t*-test unpaired analysis, $p = 0.66$). Pyocyanin concentrations were determined to be around 0.7 (±0.18) µl/ml.

Pseudomonas aeruginosa cultures grown in medium containing lactate recorded higher growth OD after 24 h compared to those without lactate, irrespective of the presence of acetoin [Figure 3(B)]. The final pH recorded in flasks cultures with lactate was also significantly higher than those without lactate ($p = 4.6 \times 10^{-9}$ using unpaired *t*-test analysis), and might explain the better bacterial growth.

pH control in bioreactors and carbon source utilization by *P. aeruginosa*

The effects of lactate and acetoin on *P.* aeruginosa were examined in controlled bioreactors on dual-substrate, with either glucose or lactate, and in mixed substrate fermentation. Growth was monitored at microaerobic conditions (pO_2 adjusted to ≈3% air saturation). For the automatic control of pH, either the acid (5 N HCl) or alkali (5 N NaOH) is being pumped into the medium, if the pH deviates from the set point (7). The growth rates of *P. aeruginosa* differ among the various C-sources (Figure 4). *Pseudomonas aeruginosa* grew at a moderate rate of 0.29 ± 0.02 (h^{-1}) on lactate [Figure 4(B)] but increased to 0.58 ± 0.05 (h^{-1}) using glucose as the main C-source [Figure 4(A)]. With mixed substrate [Figure 4(C)], an intermediate value of the specific growth rate (0.47 ± 0.03) was recorded. The growth of *P. aeruginosa* PAO1 did not show any preference towards the given carbon sources and all C-sources were utilized simultaneously (Figure 4).

Although the bacterium can grow efficiently with either glucose or lactate alone, lactate limitation in the mixed substrate fermentation leads to growth cessation. Similar behaviour was reported previously in *Clostridium pasteurianum* grown on mixed substrate. The imbalance in anaplertic synthesis of some key metabolites was reported to trigger such phenomenon in *C. pasteurianum* (Sabra et al., 2016b).

Similar to the results obtained in flask cultures and independent of the used C-sources, the presence of acetoin did not trigger pyocyanin production in all bioreactors and concentrations of 0.6 (±0.27) µl/ml were measured in the different bioreactors. Interestingly, for pH control in these cultures, base was automatically pumped into the bioreactors with *P. aeruginosa* growing on glucose, whereas only acid was pumped in cultures growing on lactate. In both cultures acetate was produced and maximum concentrations of 0.6 ± 0.2 g/L were detected. In mixed substrate fermentation, the different C-sources were assimilated simultaneously [Figure 4(C)], and the presence of lactate, even in a relatively small concentration (5 g/L), resulted in a similar behaviour, and only acid was pumped into the culture in presence of lactate followed by the addition of base in the lactate limited growth phase. Only at later growth stage in the presence of glucose as the sole C-source, acid was pumped again to control the pH.

Co-occurrence of *P. aeruginosa* and *S. pneumoniae* is dependent on the growth conditions

The metabolic coexistence between *S. pneumoniae* and *P. aeruginosa* PAO1 was tested in THBY medium

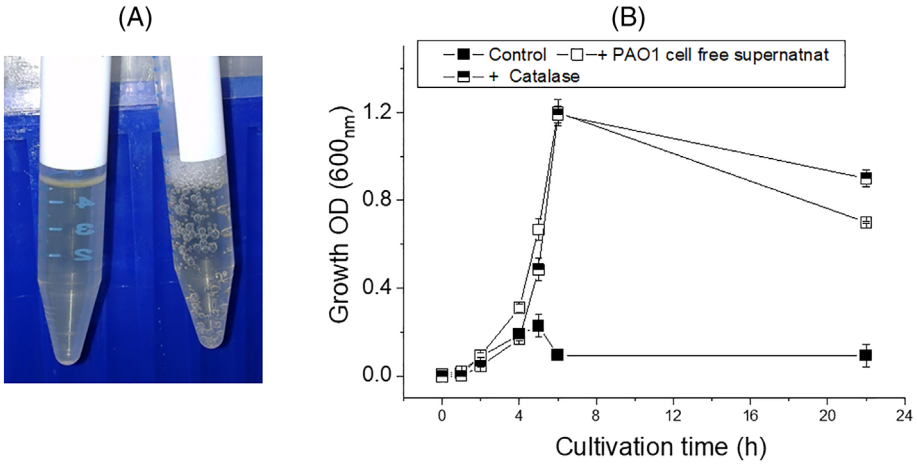

FIGURE 5 (A) Reaction of 30% H_2O_2 on either fresh un-inoculated THBY medium (left) or cell-free supernatant of *P. aeruginosa* PAO1 culture grown for 24 h on THBY medium at 200 rpm (right). In both cases, 100 µl of H_2O_2 was added to 5 ml of culture media. (B) The effect of *P. aeruginosa* PAO1 cell-free supernatant on the aerobic growth of *S. pneumoniae* in shaking flask cultures. All flasks were incubated at 37°C and agitated at 150 rpm. Negative and positive controls were performed with aerobic growing *S. pneumoniae* cultures without the addition of *P. aeruginosa* cell-free supernatants, or with the addition of pure catalase enzyme (100 units/50 ml THBY medium), respectively. The third set of aerobic growing *S. pneumoniae* cultures was treated with *P. aeruginosa* cell-free supernatants. Cell-free supernatant of *P. aeruginosa* was taken from culture grown in THBY media for 1 day at 200 rpm. After cell centrifugation and sterile filtration, the supernatant was added to freshly inoculated *S. pneumoniae* cultures. The results show the mean of three independent experiments

with glucose as the sole carbon source. Co-culture cultivations were either done under pH uncontrolled conditions or with the automatic addition of alkali to avoid fast pH decrease at the beginning. Co-culture experiments were tested under either aerobic (pO_2 of 10% air saturation), microaerobic (pO_2 at 1%–3%) or anaerobic conditions. Viable cell counts of both strains were determined and ratios between *S. pneumoniae* and *P. aeruginosa* (*S*/*P*) were shown.

Extracellular metabolic products of *P. aeruginosa* enhance the aerobic growth of *S. pneumoniae* in controlled bioreactors

First result showed that the cell-free supernatant of *P. aeruginosa* PAO1 (grown for 24 h in THBY with vigorous aeration in flask cultures) had a catalase activity as shown by the reaction with pure H_2O_2 [Figure 5(A)]. To test the hypothesis, if the catalase enzyme produced by *P. aeruginosa* had a positive effect on the aerobic growth of *S. pneumoniae*, cell-free supernatants of *P. aeruginosa* cultures were added together with *S. pneumoniae* inocula and incubated under agitation. The results obtained by the addition of 7% (vol./vol.) of *P. aeruginosa* PAO1 cell-free supernatant to *S. pneumoniae* shacked cultures are shown in Figure 5. Compared to the growth restriction of *S. pneumoniae* at agitated aerobic condition in THBY medium, the addition of either pure catalase enzyme or cell-free supernatant of overnight grown *P. aeruginosa* enhanced the growth of *S. pneumoniae* significantly at agitated aerobic condition (using unpaired *t*-test analysis, *p*-values of 4.6×10^{-3} and 1.02×10^{-3} were obtained, respectively) [Figure 5(B)].

The metabolic interaction of both strains was further investigated in controlled bioreactor. Seed cultures of *P. aeruginosa* and *S. pneumonia* were grown on THBY medium with glucose as the sole carbon source. Anaerobic and agitated aerobic conditions were used for the seed cultures preparation of *S. pneumoniae* and *P. aeruginosa* and incubated for 12 and 24 h, respectively. The seed cultures were then mixed and used as inocula for the growth of the co-culture in controlled bioreactors. Different ratios of *S. pneumoniae*/*P. aeruginosa* (SP/PA) between 1.5 and 0.5 were also tested (Supplementary Figures 6–8).

Under controlled aerobic growth conditions (pO_2 = 10% air saturation) and compared to *S. pneumoniae* growth in pure culture [Figure 1(A)], the presence of *P. aeruginosa PAO1* enhanced the growth of *S. pneumoniae* significantly as manifested by the viable cell counts (Figure 6, $p = 2.7 \times 10^{-5}$). Different from *S. pneumoniae* pure culture grown under similar conditions, H_2O_2 was never detected in the co-cultures. In the presence of glucose, the *S. pneumoniae* colony-forming units (CFUs) increased by more than two orders (log) compared to the monoculture grown at the same aerobic pO_2 values. In general, the overall course of the co-culture in controlled bioreactors was all the time reproducible, with *S. pneumoniae* being dominating at the beginning in the first phase ($p = 0.003$, unpaired *t*-test of six different trials) with a significant increase in lactate production. A second phase was characterized by lactate re-consumption and a continuous acetate production parallel to an elevation of the

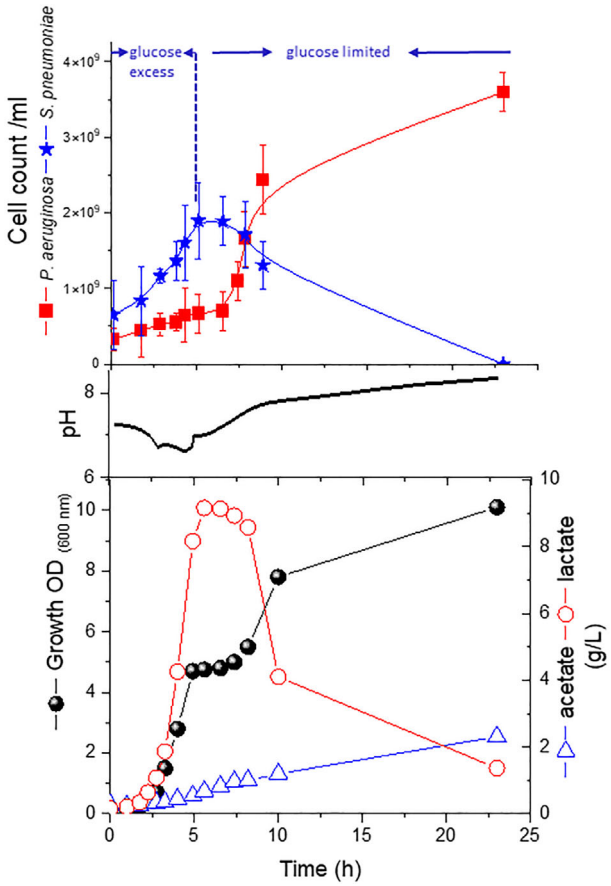

FIGURE 6 Growth of *S. pneumoniae* and *P. aeruginosa* co-culture in bioreactor with the control of dissolved oxygen tension (pO_2) at 10% of air saturation. To avoid initial acidification, only the alkali pump was activated for the automatic pH-control in bioreactor. The seed culture of *S. pneumoniae* was prepared in THBY medium and incubated anaerobically for 12–14 h, while for *P. aeruginosa*, an overnight aerobically grown cells in THBY medium with glucose were taken. Seed cultures were mixed directly before inoculation to the bioreactor

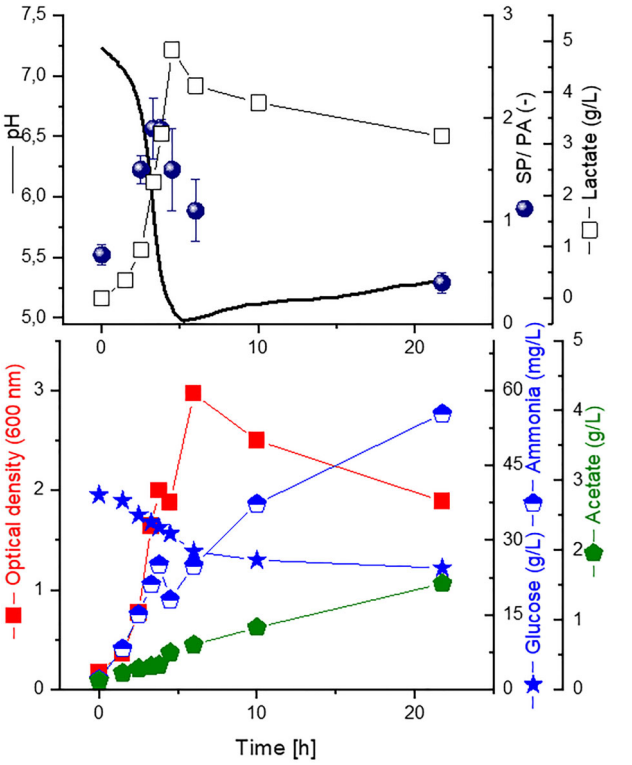

FIGURE 7 Growth of co-culture in controlled bioreactor at a microaerobic range (pO_2 of 3%) without pH control. The seed cultures of both strains were mixed and directly added to the pre-wormed bioreactor medium containing glucose as the sole C source. Seed cultures preparation is as indicated in Figure 5

pH. Coinciding with such pH elevation a drastic decrease in the *SP/PA* ratio (*p*-value of 0.054) and the CFU of *S. pneumoniae* dropped below the detection limit (Figure 6).

To test whether the catalase contained in the inoculum of *P. aeruginosa* would affect the co-culture growth at aerobic conditions, the pre-culture of *P. aeruginosa* was first centrifuged, re-suspended in phosphate buffer and together with *S. pneumoniae* precultures were used to inoculate the bioreactor. Again, similar trend of both bacterial growth and pH profile was observed (Supplementary Figure 1).

Persistence of *P. aeruginosa* in microaerobic co-cultures even at lower pH

The relative abundance of both strains was also studied in bioreactors controlled at microaerobic condition, condition known to mimic the in vivo CF conditions (Sanderson et al., 2008), and favoured by both strains (Sabra et al., 2002). The growth behaviour of both strains in bioreactors with or without pH control was also compared. In pH-uncontrolled experiment, the culture profile can be divided into two phases. *Streptococcus pneumoniae* population increased in the first phase, coupled with a rapid decrease in the medium pH (≈5). The second phase has begun even in presence of excess glucose, and the SP/PA ratio decreased with a continuous assimilation of lactate. Despite the low growth rate of *P. aeruginosa* at such low pH, its persistence resulted in a gradual increase in the pH (Figure 7).

The growth of the co-culture at microaerobic range with glucose as the sole C-source was tested in bioreactors with pH control. As shown in Figure 8(A), more acids were produced by both strains compared to pH uncontrolled condition (Figure 7). Similar to the growth behaviour at pH uncontrolled condition, the SP/PA ratios slightly increased in the first phase, and lactic acid and acetic acid were the major products obtained. This was followed by a second phase with lactate re-consumption, continuous acetate production and an overwhelming growth of *P. aeruginosa*. The controlled

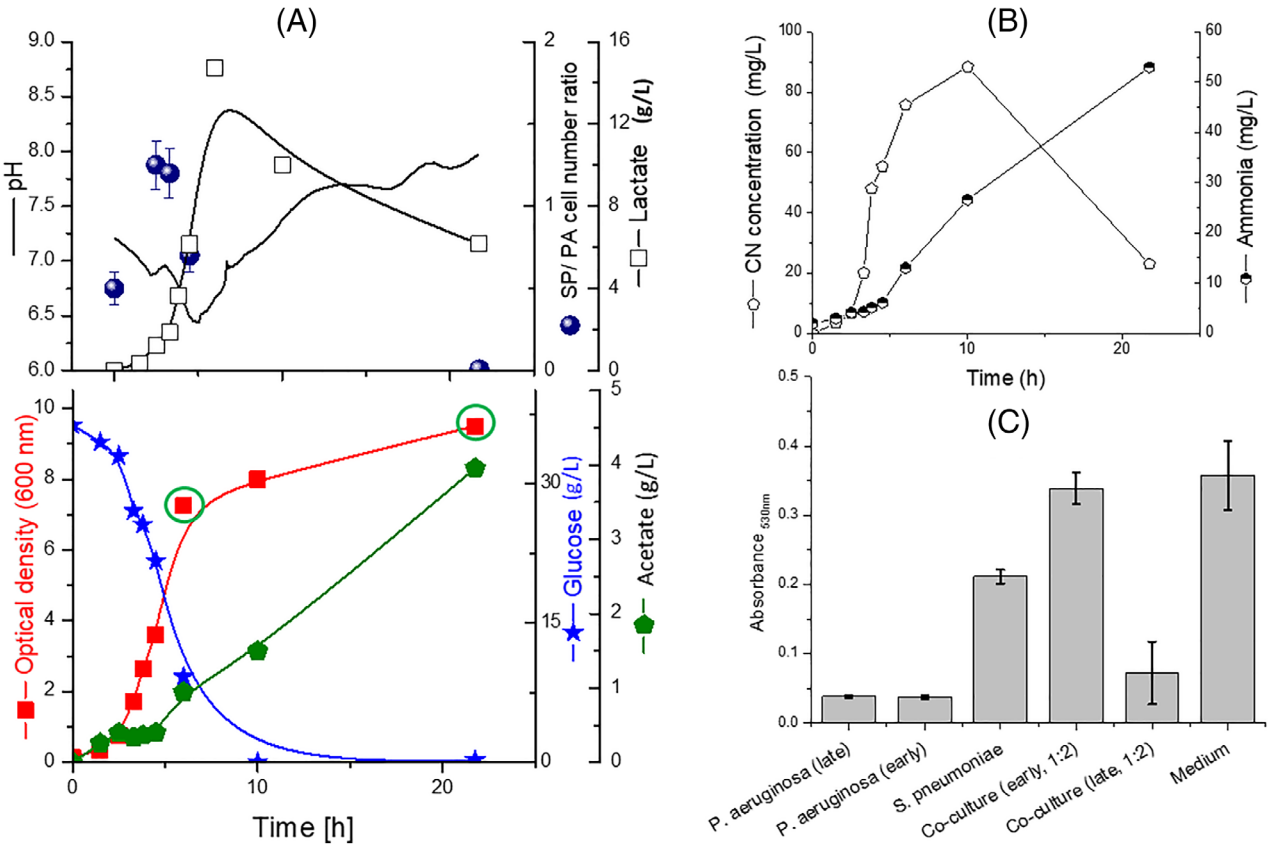

FIGURE 8 Co-culture growth of *P. aeruginosa* and *S. pneumoniae* in bioreactor with pO_2 control at microaerobic range and with an initial pH adjustment with base (A); cyanide (CN) and ammonia production during microaerobic growth of co-culture (B). Neutral red uptake test with mouse fibroblast cell line for the measurements of the total toxicity of cell-free supernatant (CFS) from mono- and co- bacterial cultures. The mammalian cell lines were treated with CFS of *P. aeruginosa* at either mid logarithmic or late stationary phases, with CFS of *S. pneumoniae* at late stationary phases in presence of glucose, with CFS of co-culture at an early stage (in presence of glucose) and a later phase (in absence of glucose), and with THBY medium as a positive control. Co-culture samples taken for toxicity test are marked with green circle in 'A'

pH conditions also promoted the *P. aeruginosa* growth on glucose even at the beginning of the co-culture in comparison to the uncontrolled pH conditions.

Overall toxicities of samples taken from mono- or co-cultures

Both *S. pneumoniae* and *P. aeruginosa* produce several virulence factors that are involved in the disease process. In this study, cyanide (CN) and ammonia were detected in the co-culture supernatant in the first phase when glucose was in excess, whereas ammonium increased at the second stage, when lactate was the sole C-source [Figure 8(B)]. Similar concentrations of CN were measured in pure *P. aeruginosa* culture grown on complex medium with glucose under microaerobic conditions, while CN was never detected in the supernatants of *S. pneumoniae*.

To test whether metabolic collaboration would increase the bacterial virulence of the co-cultures, toxicity measurements were performed with the Neutral Red uptake test using the mouse fibroblast cell line. For the assay, the experimental groups were treated with cell-free supernatants of *P. aeruginosa* grown in pure culture (at mid-logarithmic phase and early stationary phase), cell-free supernatants of *S. pneumoniae* grown in pure culture and cell-free supernatants of co-culture {before and after glucose limitation [-Figure 8(C)]. The control group contains only the growth medium used for bacterial cultivation. All samples for toxicity tests were taken from pH-controlled, microaerobic growing pure- or co-cultures. Compared to samples taken from *S. pneumoniae* monoculture, samples taken from *P. aeruginosa* cultures in early or late growth phases showed the most toxicities, as indicated by the decrease in uptake of the neutral red dye in the treated mouse fibroblast cells [Figure 8(C)]. The same trend was observed for samples from co-culture experiments. At the first phase of the co-culture growth, at which *S. pneumoniae* dominated in the presence of glucose (the highest SP/PA ratio), the culture supernatant caused relatively low toxicities [-Figure 8(A,C)], while the second phase under glucose-limited conditions with the overwhelming growth of *P. aeruginosa* was characterized by a relatively higher toxicity to the mouse fibroblast cell lines [-Figure 8(A,C)].

DISCUSSION

The nutritional environment of most infection sites is poorly defined and often inadequately modelled by laboratory growth media (Palmer et al., 2007). In CF patients, mimicking the mucous viscous layer as a complex substrate and its unique physical environmental conditions (osmotic pressure, pO_2 or pH) to investigate the high-density growth during chronic colonization remain a challenging problem. The problem becomes exacerbated by the multi-microbial nature of such infections, and the unknown consequences on the members of the microbial community if a specific pathogen is treated. Both *P. aeruginosa* and *Streptococci* are highly versatile organisms, which readily adapt to a wide variety of environments and stress factors. In fact, in the three interaction groups within the CF lung postulated Quinn et al. (2016), both *P. aeruginosa* and *S. aureus* antagonize the anaerobic fermenting group. In the current investigation, the metabolic interactions between *S. pneumoniae* and *P. aeruginosa*, representative members of two different microbial groups frequently isolated from CF lung, are investigated.

The decrease in pH triggered by lactic acid production, the main product of pneumococcal glucose fermentation, and the secretion of H_2O_2 are, among others, of significant importance for *S. pneumoniae* lung colonization (Chao et al., 2014; Kadioglu et al., 2008). Lactate is therefore a central metabolite in the CF lung environment (Bensel et al., 2011). Besides its bacterial origin, lung lactate production may become clinically evident in patients with acute lung injury or with acute respiratory distress syndrome (Iscra et al., 2002). Irrespective of the source of lactate, its efficient utilization was shown by both *S. pneumoniae* and *P. aeruginosa* at low concentration of oxygen. While aerobic condition inhibits the growth of *S. pneumoniae* in pure culture, both strains have been shown to grow favourably under microaerobic conditions in controlled bioreactors (Figure 1) (Sabra et al., 2002).

Indeed, fulfilling the metabolic demands of glucose-limited *S. pneumoniae* by the uptake of its own metabolite is an advantage. However, the H_2O_2 produced not only inhibits other competing lung microbes as reported previously (Pericone et al., 2000) but also exerts negative effect on its own metabolism as indicated by the decreased cell viabilities and the sharp decrease in CO_2 production rate at the lactate assimilation phase at aerated condition (Figure 1). Beside lactate and H_2O_2 production by *S. pneumoniae*, acetoin was shown to be produced under anaerobic or microaerobic conditions (Figure 2). Under anaerobic conditions, acetoin production by *S. pneumoniae* showed a sevenfold increase in pH uncontrolled conditions compared to pH-controlled anaerobic growth (Figure 2). This was not surprising, since its production and secretion are known to be a mechanism utilized by different microbes to maintain the internal pH during the accumulation of acidic fermentation products (Xiao & Xu, 2007). Acetoin and its derivatives are detected in CF lung environments (Camus et al., 2020) and in lungs infected with pneumonia (Filipiak et al., 2012), and hence play a significant role in pathogenesis. Here, the effect of the different metabolites secreted by *S. pneumoniae* on *P. aeruginosa* growth is first studied in pure culture in controlled bioreactors.

Previously, it was reported that either 2,3-butanediol (the reduced derivative of acetoin) or diacetyl (the oxidized derivative of acetoin) triggered phenazine production in several *P. aeruginosa* strains (Venkataraman et al., 2014). Recently the role of acetoin for the survival of bacteria, especially for *P. aeruginosa* and *S. aureus* in the CF lung was discussed (Camus et al., 2020). While acetoin was efficiently metabolized by *P. aeruginosa* PAO1, in the presence of either lactate or glucose (Figure 3), its utilization did not evoke pyocyanin production (Figure 3). Definitely, the presence of acetoin as an additional C-source might improve the survival of *P. aeruginosa* in the CF lung. The growth of *P. aeruginosa* PAO1 did not show any preference towards the given carbon sources, i.e. no diauxic (acetoin with lactate or glucose) or triauxic (acetoin with lactate and glucose) growth could be observed (Figure 4). The excellent switching between the different C-sources that allows *P. aeruginosa* to maximize the energetic yield was described recently (Dolan et al., 2020). Interestingly, the utilization of lactate in co-substrates (with acetoin) or mixed substrates (with acetoin and glucose) in pH uncontrolled conditions resulted in lower acidification of the growth media, and hence a better growth of *P. aeruginosa* (Figure 3). Similar behaviour was also seen if *P. aeruginosa* grew under controlled microaerobic conditions in bioreactors on lactate, glucose or mixture of both (Figure 4). Overall, the utilization of the major metabolites produced by *S. pneumoniae*, namely lactate and acetoin, in *P. aeruginosa* pure cultures might indicate a possible interaction between both strains and explain the co-occurrence of these species in mixed lung infection, in CF lung or in pneumoniae disease at different stages (Bradshaw et al., 2018; Conrad et al., 2013; Scott et al., 2019; Scott & O'Toole, 2019).

In our examination of the co-culture using controlled bioreactors, *S. pneumoniae* predominates when glucose is present probably because of its inefficient carbon usage and higher substrate turnover rate. In general, the cell ratio between both strains varies with the growth conditions. Only under pH-uncontrolled anaerobic conditions, *S. pneumoniae* dominated and *P. aeruginosa* was only observed under the microscope but not detectable on the plate. On the other hand, when oxygen is introduced into the bioreactor, and controlled at either microaerobic condition ($pO_2 \sim 3\%$) or aerobic condition

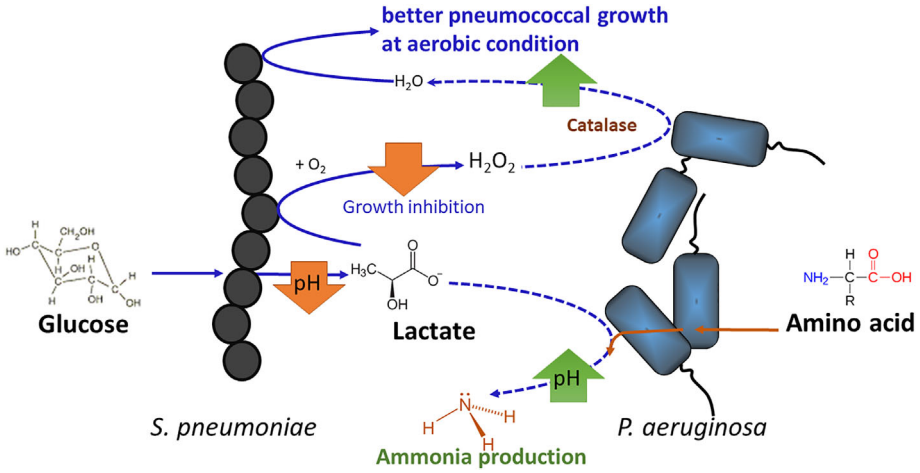

FIGURE 9 Possible interactions between *S. pneumoniae* and *P. aeruginosa*. *S. pneumoniae* produces lactate, acetoin and H_2O_2 as a result of its aerobic or microaerobic growth. *Pseudomonas aeruginosa* utilize the different metabolites produced by *S. pneumoniae* and produces ammonia, which prevent excessive acidification, and catalase enzyme, which protect both strain from the damaging effect of H_2O_2

($pO_2 = 10\%$), the growth dynamic was reproducible for both strains, with *S. pneumoniae* being dominating at the beginning with a significant increase in lactate production in the first phase, followed by a lactate re-consumption, growth of *P. aeruginosa* and elevation of the medium pH. In microaerobic and aerobic co-culture experiments (six experiments), the average growth rate of *S. pneumoniae* in the first phase with glucose was $0.24 \pm 0.06\ h^{-1}$ compared to $0.14 \pm 0.03\ h^{-1}$ with *P. aeruginosa*. Under glucose limitation, *S. pneumoniae* growth ceased, while the average rate of *P. aeruginosa* growth increased to $0.40 \pm 0.12\ h^{-1}$. Compared to growth in pure culture, *P. aeruginosa* recorded higher growth rate on glucose over lactate (Figure 4). These significant differences of *P. aeruginosa* behaviour in monoculture and co-culture with different substrates deserve more in-depth investigations. McGill et al. (2021) have shown that under certain conditions in mixed cultures, *P. aeruginosa* prefers non-fermentable substrates (such as lactate) and can coexist with bacteria that ferment glucose, thus show enhanced resource acquisition and increased virulence.

Indeed, co-culture of both *P. aeruginosa* and *S. pneumoniae* has proven some advantages for both strains. In contrast to the growth failure of *S. pneumoniae* under controlled aerobic condition, growth of *S. pneumoniae* was enhanced significantly in co-culture under the same pH controlled and pO_2-controlled conditions ($pO_2 = 10\%$). The production of catalase by *P. aeruginosa* is assumed to be the reason behind the aerobic growth of *S. pneumoniae* (Figure 5). H_2O_2 is never detected in co-culture fermentation despite the growth dominance of *S. pneumoniae* in the first phase (Figure 6). It should be stressed here that *P. aeruginosa* inoculum addition from the beginning was found to be crucial for the growth of *S. pneumoniae* in co-culture. On the other hand, the addition of *S. pneumoniae* even at the early logarithmic phase of *P. aeruginosa* culture resulted in the dominant growth of the first strain till the exhaustion of glucose. Thereafter, *P. aeruginosa* prevails and lactate is consumed efficiently. Previously, it was shown that staphylococcal species that secrete higher concentrations of catalase are resistant to *S. pneumoniae* (Regev-Yochay et al., 2006). In fact, *P. aeruginosa* and *S. aureus*, the two major groups of clinical significance in CF lung are both catalase positive and can produce catalase enzyme extracellularly (Elkins et al., 1999; Mandell, 1975), and its activity was shown to be important for their virulence (Mandell, 1975). While it was previously reported that H_2O_2 contributes to the mucoid conversion of *P. aeruginosa* and thus to the activation of virulence in the CF lung (Mathee et al., 1999), the environmental conditions and mechanisms that influence the production of alginate and the resulting mucoid phenotype are multifactorial and need further investigations. It is worth mentioning that neonates with CF have histologically normal lung and no mucoid variant of *P. aeruginosa* can be identified (Li et al., 2005), On the other hand, *S. pneumoniae* is frequently isolated from CF patients especially in early childhood (Pimentel de Araujo et al., 2014).

Accumulating evidence indicates that ammonia is an important metabolite and its elevated level has been observed in CF airway secretions (Newport et al., 2009). In our bioreactor experiments done under microaerobic conditions, ammonia production and pH elevation were observed during lactate assimilation phase in either pure culture of *P. aeruginosa* [Figure 4(B,C)] or in co-culture (Figure 7). In the pH-uncontrolled co-culture, conditions similar to that of in vivo, ammonia production was initiated by the acid challenge at lower pH even in the presence of excess glucose (Figures 7 and 8). In fact, *P. aeruginosa* was reported to preferentially use lactate compared with glucose in a synthetic CF medium (Palmer et al., 2007). This is also evident in co-culture experiment grown at pH uncontrolled conditions, where

lactate assimilation began in the presence of excess glucose (Figure 7). Overall, the increase in pH associated with lactate utilization and ammonia production is another benefit for both bacteria living in a co-culture as shown in Figures 7 and 9. Quinn et al. (2016) hypothesized that ammonia production contributes to the stabilization of lung mucus pH for the Climax Community.

Understanding the microbial interactions in lung microbiota is central for treating lung infection. Recent studies showed the presence of both *S. pneumoniae* and *P. aeruginosa* in infected CF lungs (Conrad et al., 2013; Quinn et al., 2016). In fact, Conrad et al. (2013) proposed in their 'climax-attack model for cystic fibrosis' a possible interaction between these two strains. Here, we investigated the interaction between these two strains at different environmental conditions in controlled bioreactors. At the first glance, these bacteria seem to have an antagonistic relationship as *S. pneumoniae* acidifies the medium very quickly and lowers the growth rate of *P. aeruginosa* and frequently outcompetes it in co-culture. This antagonistic behaviour is mainly shown in anaerobic co-culture during planktonic growth and under traditional cultural conditions, where no host factors or antibiotics are present. However, under microaerobic conditions, a condition known to be prevalent in the lungs, both bacteria can coexist well (Figures 7 and 8). It should be stressed here that the observed fast decrease in the pH as a result of the fermentative metabolism of *S. pneumoniae* is dependent on both the glucose concentration and the medium used for the growth. In the CF lung sputum, and beside the diverse set of ecological niches, much lower concentration of both glucose (≈0.58 g/L) and lactate (≈0.8 g/L) was measured (Palmer et al., 2007). At such lower concentration of glucose, the pH drop should be not that significant as shown in bioreactor without pH control (Figure 7). Moreover, the ammonium production triggered by lactate assimilation or excessive acidification was shown in our co-culture experiments (Figures 7 and 8), and is definitely playing a major role in pathogenicity in vivo (Figure 9). Aside from the role of pH variation on the growth dynamics of both strains, cross-feeding seems to benefit *P. aeruginosa* by precluding nutrient competition with the faster growing *Streptococci* at an early stage on glucose, allowing it to persist and cause infection later. In fact, lactate and acetoin assimilation by *P. aeruginosa* might explain the tight association between *P. aeruginosa* and fermenters (McGill et al., 2021).

It is generally known that both *S. pneumoniae* and *P. aeruginosa* produce large varieties of virulence factors (Alonso DeVelasco et al., 1995; Basavanna et al., 2013; Cabeen, 2014; Kim et al., 2003; Mitchell & Mitchell, 2010). In the current study, the supernatant toxicities of mono- and co-cultures on mouse fibroblast cells were compared. While a more toxic effect of the supernatants of *P. aeruginosa* in mono- or in co-culture on the mammalian cell is shown, it should be stressed here that, such a test has many limitations. Besides the test cell line, which might be not representative to the lung cells, the toxicity test using cell-free supernatants does not recognize many virulence factors, such as the type three secretion system, known to be present in *P. aeruginosa* (Horna & Ruiz, 2021), or the effect of EPS production and the adhesion ability by both strains and their biofilm growth mode. In the current study, ammonia, cyanide, H_2O_2, pyocyanin were measured. In fact, the CN (and pyocyanin by other *P. aeruginosa* strains) production observed in the presence of glucose in the first phase (Figure 8), while inhibiting other aerobes including *S. aureus* (Nair et al., 2014), will then indirectly enhance the growth of *S. pneumoniae*, a CN insensitive anaerobes. The interactions between those studied bacteria, whether cooperative or competitive, are rather complex and determined by the environmental conditions. Indeed, the metabolic interactions in the planktonic growth shown in this study may differ from that in biofilm cultures (Scott et al., 2019), with the extracellular matrix components of the latter likely being a key, and largely underexplored, factor. Despite the obvious limitations of in vitro experiments, these studies may contribute to understanding of bacterial metabolism in CF lung infection.

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DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are presented graphically in this published article (and the supplementary information file), and are available upon reasonable request from the corresponding author.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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