Pyrosequencing reveals transient cystic fibrosis lung microbiome changes with intravenous antibiotics

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ABSTRACT Chronic airway infection in adults with cystic fibrosis (CF) is polymicrobial and the impact of intravenous antibiotics on the bacterial community composition is poorly understood. We employed culture-independent molecular techniques to explore the early effects of *i.v.* antibiotics on the CF airway microbiome.

DNA was extracted from sputum samples collected from adult subjects with CF at three time-points (before starting treatment, and at day 3 and day 8–10 of *i.v.* antibiotics) during treatment of an infective pulmonary exacerbation. Microbial community profiles were derived through analysis of bacterial-derived 16S ribosomal RNA by pyrosequencing and changes over time were compared.

59 sputum samples were collected during 24 pulmonary exacerbations from 23 subjects. Between treatment onset and day 3 there was a significant reduction in the relative abundance of *Pseudomonas* and increased microbial diversity. By day 8–10, bacterial community composition was similar to pre-treatment. Changes in community composition did not predict improvements in lung function.

The relative abundance of *Pseudomonas* falls rapidly in subjects with CF receiving *i.v.* antibiotic treatment for a pulmonary exacerbation and is accompanied by an increase in overall microbial diversity. However, this effect is not maintained beyond the first week of treatment.

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This article has supplementary material available from erj.ersjournals.com

Received: Nov 19 2013 | Accepted after revision: April 23 2014 | First published online: July 17 2014

Support statement: This work was supported by the Royal Hobart Hospital Research Foundation (Hobart, Australia) and the National Health and Medical Research Council of Australia (Canberra, Australia). D.J. Smith is the recipient of a Postgraduate scholarship from the National Health and Medical Research Council of Australia (APP1039309). G.J. Anderson is the recipient of a Senior Research Fellowship from the National Health and Medical Research Council of Australia (APP1024230). D.W. Reid is the recipient of a Practitioner Fellowship from the National Health and Medical Research Council of Australia. S.C. Bell and D.W. Reid are recipients of Queensland Health Medical Research Fellowships (QCOS013795 and QCOS013794).

Conflict of interest: Disclosures can be found alongside the online version of this article at erj.ersjournals.com

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Introduction

Despite advances in the management of cystic fibrosis (CF), chronic pulmonary infection remains responsible for most patient morbidity and mortality [1]. Culture-dependent analysis of CF airway infection reveals that *Pseudomonas aeruginosa* is the dominant bacterial pathogen in most adults with CF [2]. However, the recent application of culture-independent molecular techniques, based on the sequencing of the gene encoding bacterial 16S rRNA, have revealed a complex microbiome in the CF airway [3–9].

Chronic infection with *P. aeruginosa* is associated with an increased rate of lung function decline, increased frequency of pulmonary exacerbations, impaired quality of life and increased mortality [10, 11]. Guidelines recommend that pulmonary exacerbations are treated aggressively with a combination of at least two intravenous anti-pseudomonal antibiotics for 14 days [12, 13]. However, this practice is based on limited evidence [13].

Two studies, one prospective and one a large registry-based retrospective, have suggested that the maximum clinical and metabolic response to *i.v.* antibiotics occurs in the first week of treatment, with little additional benefit being achieved by extending treatment beyond this point [14, 15]. Unfortunately, these studies did not assess the relationship between clinical improvement and microbiological response and their findings have not been considered sufficiently robust to change clinical practice.

In this study, we utilised culture-independent molecular techniques to explore the effect on the CF airway microbiome of *i.v.* antibiotic therapy administered for the treatment of an acute pulmonary exacerbation. The focus was primarily on the impact of antibiotic therapy on microbial community composition during the first week of treatment.

Methods

Participants

23 adult subjects (aged 18–54 years) with CF, admitted to hospital for *i.v.* antibiotic treatment of an acute, infective pulmonary exacerbation were recruited from The Prince Charles Hospital (Brisbane, Australia) (n=14) and the Royal Hobart Hospital (Hobart, Australia) (n=9). Institutional human research and ethics committee approval was obtained at both sites (HREC2008:2885 and H0009813, respectively). Individuals who had undergone lung transplantation or were using systemic immunosuppression were excluded. Based on standard microbiological cultures, all patients were infected with *Pseudomonas aeruginosa* (table S1).

Spontaneously expectorated sputum samples were collected at day 1, before *i.v.* antibiotics (time-point (TP)-1), and at day 3–4 (TP-2) and day 8–10 (TP-3) following commencement of *i.v.* antibiotics.

A pulmonary exacerbation was defined clinically, based on the attending physician's assessment that the subject required *i.v.* antibiotics to treat an increase in respiratory symptoms or a decline in lung function.

Participant demographics, lung function and antibiotic treatment are outlined in table 1. Disease severity was determined based on the participants best forced expiratory volume in 1 s (FEV₁) in the 12 months prior to recruitment (FEV₁ >70% mild, 40–70% moderate and <40% severe).

Sputum collection

Sputum was expectorated directly into 10 mL of RNAlater (Life Technologies, Mulgrave, VIC, Australia) and stored for at least 24 h at 4°C to allow full penetration into the sputum sample. Sputum samples were stored at -80°C for later batch DNA extraction.

DNA extraction

A saliva-free aliquot of each frozen sputum sample was selected and manually homogenised with 500 µL lysis buffer (50 mM Tris-HCl (Sigma-Aldrich, St Louis, MO, USA), pH 6.8, 50 mM ethylenediamine-tetraacetic acid (AnalaR, PA, USA), 50 mM sucrose (AnalaR), 100 mM sodium chloride (Univar, Ingleburn, NSW, Australia) and 1% SDS (Amresco, Solon, OH, USA). The samples were incubated at 37°C for 1 h with 100 µL chicken egg lysozyme (100 mg·mL⁻¹; Sigma-Aldrich), then at 56°C with shaking overnight with 100 µL of proteinase K (20 mg·mL⁻¹; Promega, Alexandria, NSW, Australia) and 75 µL of 10% SDS. If samples were not completely digested, overnight incubation with additional proteinase was repeated until complete digestion was achieved. DNA was extracted and purified using PureLink Genomic DNA Mini Kit (Life Technologies) and eluted in 50 µL kit elution buffer. Purified genomic DNA was analysed for quality (A₂₆₀/A₂₈₀ ratio of 1.8–2.0) and concentration using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Denver, CO, USA) and samples were diluted to 20 ng·µL⁻¹ prior to DNA amplification and sequencing.

TABLE 1 Participant demographics, cystic fibrosis genotypes and antibiotic treatment

Sex	
Male	17
Female	6
Age years	27 (18–54)
BMI kg·m⁻²	20 (17–26)
FEV₁ L	1.7 (0.9–4.2)
FEV₁ % predicted	50 (21–97)
Absolute improvement in FEV₁ L	0.2 (0–1.2)
Relative improvement in FEV₁ %	16 (0–76)
Genotype	
p.F508del homozygote	12
p.F508del heterozygote	8
G551D heterozygote	1
Other/other	2
Intravenous antibiotic treatment[#]	
β-lactam + aminoglycoside [†]	18
Dual β-lactams	2
Dual β-lactams + aminoglycoside	1
Dual β-lactams + colistin	1
β-lactam + monobactam + aminoglycoside	1
Azithromycin^{#+}	18

Data are presented as n or median [range]. Individual participant data are available in tables S1 and S2. BMI: body mass index; FEV₁: forced expiratory volume in 1 s. [#]: maintenance usage of oral azithromycin, dosage either 250 mg daily or 500 mg on alternate days; [†]: nebulised aminoglycoside was used in one subject; ⁺: data were unavailable for one subject.

DNA amplification, sequencing and statistical analysis

Sequencing was performed by Research and Testing Laboratories (Lubbock, TX, USA) applying Molecular Research DNA protocols (Shallowater, TX, USA). The 16S rRNA gene was amplified using primers 939F (5'-TTGACGGGGGCCCGCAC-3') and 1492R (5'-TACCTTGTTACGACTT-3') and products were sequenced using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) as described previously [16]. 16S rDNA amplicon sequences were processed with QIIME 1.5 [17]. Sequences with ambiguous base calls ("quality score" <25 and ≥6 homopolymers) were discarded. Forward and reverse primer sequences were removed allowing one mismatch. Chimeras were removed with ChimeraSlayer [18] using default parameters. Samples with <500 sequence reads were excluded. 16S rDNA sequences were clustered into operational taxonomic units (OTUs) using UCLUST v5.2.32 [19], employing an identity threshold of 97%. RDP Classifier v2.2 was retrained on the database Greengenes version 13.8 and used for the taxonomic assignment of representative sequences of each OTU with a confidence threshold of 0.6 [20]. Genera were categorised as aerobic or anaerobic, as described previously [21]. Data-mining, statistical analysis and data visualisation were carried out using the Calypso software (bioinfo.qimr.edu.au/calypso) and Krona [22].

Groups were compared using paired t-tests: TP-1 *versus* TP-2; TP-1 *versus* TP-3; and TP2 *versus* TP-3. Analysis was limited to subjects for whom samples at both compared time-points were available. P-values were adjusted for multiple testing using the False Discovery Rate (FDR). Microbial community diversity was assessed by Shannon index (OTU level). Canonical Correlation Analysis (CCA) and ANOSIM were performed on the sputum microbiome at TP-1 using the relative genera abundance (number of 16S sequences assigned to each genus divided by the total number of sequences obtained for each sample). Associations between clinical variables (age and FEV₁) and the relative abundance of each genus at TP-1 or TP-3 were determined by the Pearson product-moment correlation coefficient. Analogously, Pearson correlation was utilised to infer associations between the relative abundance of genera, age and relative improvement in FEV₁ between early and late treatment. For the purpose of determining the differences between the relative abundances of bacterial groups at the genus level, Shannon diversity and FEV₁ at TP-1 and TP-3 were calculated, and then evaluated for relationships by means of Pearson correlation.

Real-time PCR quantification of *P. aeruginosa*

In 18 sputum samples from nine subjects, *P. aeruginosa* concentration was determined by real-time quantitative PCR using previously established methodologies [23]. Pearson's correlation was used to explore the relationship between *P. aeruginosa* load and relative abundance of *Pseudomonas* at the genus level.

Results

Sample quality

59 sputum samples were collected during 24 pulmonary exacerbations from 23 subjects. Sequencing yielded 420 145 reads with an average read length of 457 base-pairs. After quality control and chimera detection, 57 samples (18 for TP-1, 18 for TP-2 and 21 for TP-3) and 336 973 high-quality sequencing reads remained, with a median of 5478 sequences per sample and a range from 1468 to 14 164.

Sputum microbiota before antibiotic treatment: TP-1

18 sputum specimens were analysed at TP-1 and showed a complex microbiota, with a median (range) of 113 (23–210) OTUs per sample. A total of 100 different genera were observed (median (range) 13 (2–39) per sample) (fig. 1). *Pseudomonas* was the dominant genus in 94% (17 out of 18) of samples, with a mean (range) relative abundance of 78.5% (41.9–99.6%). In one subject, *Fusobacterium* was the dominant genus (49% of 16S sequences), followed by *Pseudomonas* (41.9% of sequences). *Streptococcus* was the second most abundant genus in 13 sputum samples, with a mean relative abundance of 7.3%. The most prevalent anaerobic genera were *Prevotella* (mean (range) relative abundance 2.7%, (0–13.8%)) and *Veillonella* (mean (range) relative abundance 1.2% (0–5.9%)), which were present in 72% and 78% of the subjects, respectively. Other pathogenic genera were recovered infrequently, including *Actinomyces* (44% of samples), *Staphylococcus* (17% of samples) and *Haemophilus* (11% of samples). The mean percentage of sequences that could not be assigned to any known genus was 3% (table S3). Statistical analysis of the taxonomic profiles at TP-1 did not identify a relationship between global microbial community composition (rank genus) and age (CCA: $p=0.55$; ANOSIM: $p=0.59$) or FEV₁ (CCA: $p=0.99$; ANOSIM: $p=0.7$).

The relative abundance of *Pseudomonas* showed a strong negative correlation with microbial diversity (Shannon index; $r=-0.81$, $p<0.01$, Pearson correlation). Conversely, the relative abundance of *Streptococcus* ($r=0.63$, $p<0.01$) and *Prevotella* ($r=0.63$, $p<0.01$) were positively correlated with community diversity (fig. S1). We did not observe an association between microbial diversity and subject age ($p=0.29$) or lung function (FEV₁; $p=0.3$).

Effects of antibiotic treatment on the airway microbiome

There was a significant reduction in the relative abundance of *Pseudomonas* during the first 72 h of antibiotic treatment ($p<0.005$; FDR=0.02, paired t-test TP-1 versus TP-2). The mean relative abundance of *Pseudomonas* decreased from 78.5% of 16S sequences before antibiotic treatment to 47% after 72 h of treatment. The reduction in *Pseudomonas* was accompanied by a significant increase in overall microbial diversity (Shannon index paired t-test $p=0.012$) and a trend towards an increase in the relative abundance

FIGURE 1 Taxonomic profile of all sputum samples segregated by time-point. Only genera which were present in at least one sample at a relative abundance of $\geq 10\%$ are presented.

FIGURE 2 Changes in cystic fibrosis sputum microbiota between time-point (TP)-1, TP-2 and TP-3. a) Community diversity assessed by Shannon index (OTU level) demonstrating changes in overall microbial diversity. b) Changes in the relative abundance of *Pseudomonas*. c–e) Circle plots demonstrating changes in global community structure at c) TP-1, d) TP-2 and e) TP-3. Each ring represents a taxonomic rank (phylum, class, order, family and genus from inner to outer circle, respectively).

of anaerobes, which was mainly driven by increases in the abundance of *Prevotella* (TP-1 2.7% of 16S sequences; TP-2 12.2%; $p=0.06$; FDR=0.13) and *Veillonella* (TP-1 1.2%; TP-2 6.8%; $p<0.02$; FDR=0.07) (fig. 2).

At TP-3, overall microbial diversity (Shannon index) and *Pseudomonas* relative abundance were similar to TP-1, suggesting a return to pre-treatment community composition (fig. 2). The change in overall microbial diversity correlated negatively with the change in abundance of *Pseudomonas* ($r = -0.7$, $p<0.01$) and positively with change in abundance of *Streptococcus* ($r=0.6$, $p<0.05$) (fig. S2).

A principal coordinates analysis demonstrated samples from TP-1 and TP-3 clustered together; however, TP-2 formed a distant separate cluster (fig. S3c). This clustering was significant; comparison of intra- and intergroup Jarrard distances of TP-1 versus TP-3 were not significant ($p=0.75$), but were significant between TP-1 versus TP-2 and TP-2 versus TP-3 ($p<0.01$).

We did not observe any significant associations between the changes in abundance of individual genera and improvement in FEV₁ between TP-1 and TP-3. A negative relationship between relative abundance of *Pseudomonas* and FEV₁ was observed at TP-3, but did not reach statistical significance ($r = -0.46$, $p=0.07$).

In the nine subjects for whom *P. aeruginosa* quantification was performed, there was a weak, positive correlation between *P. aeruginosa* load and the relative abundance of *Pseudomonas* at the genus level ($r^2=0.24$, $p=0.04$) (fig. S4).

Sub-group analysis based on the relative abundance of *P. aeruginosa*

Clustering of OTUs based on the relative abundance of *Pseudomonas* spp. revealed three distinct groups: low abundance of *Pseudomonas* spp. (<40%) with a complex community structure and presence of various other bacterial genera; medium abundance of *Pseudomonas* spp. (40 to <75%); and high abundance of *Pseudomonas* spp. ($\geq 75\%$) (fig. S3b).

Samples from subjects with severe disease tended to cluster in the high and medium categories, whereas samples from subjects with mild-to-moderate disease clustered in the low category (fig. S3c). Antibiotic treatment rarely resulted in a change in a subject's category at the end of treatment, and changes in the relative abundance of *Pseudomonas* in response to treatment were not associated with an improvement in lung function (fig. S2).

Non-*Pseudomonas* genera were categorised as obligate anaerobes or aerobes/facultative anaerobes (fig. 3). By TP-2 the median relative abundance of aerobic bacteria increased (TP-1: 4.5% of 16S sequences; TP-2: 11.7% of 16S sequences; $p=0.03$, FDR=0.049) and a similar trend was observed in the abundance of anaerobic bacteria (TP-1: 3.4%; TP-2: 22.6%; $p=0.1$). A reversal in these trends was seen between TP-2 and TP-3 (aerobes TP-3: median 9.5%, paired t-test between TP-2 and TP-3 $p=0.44$; anaerobes TP-3: median: 2.4%, $p=0.048$, FDR=0.1) and at TP-3, the sputum community composition once again resembled TP-1 (fig. 3).

FIGURE 3 Relative abundance (median) of aerobic and anaerobic genera in cystic fibrosis sputum samples after exclusion of reads assigned to *Pseudomonas*. There was a significant increase in the relative abundance of aerobic bacteria between time-point (TP)-1 and TP-2, with a similar trend seen in anaerobic bacteria, followed by a significant decrease in anaerobic bacteria between TP-2 and TP-3. The p-values were calculated using a paired t-test. Changes in the relative abundance of aerobic and anaerobic genera in response to treatment in individual subjects are presented in figure S6.

Discussion

In this study, we demonstrate a significant perturbation of the airway microbiome over the first 72 h of *i.v.* antibiotic treatment for pulmonary exacerbations in adult subjects with CF. This perturbation was characterised by a reduction in the dominance of *Pseudomonas* and an accompanying increase in microbial diversity. These changes were, however, short-lived, with bacterial community composition resembling that of the initial profile after 1 week of antibiotic treatment. These novel findings suggest that disturbance of the bacterial composition of the CF airway in response to *i.v.* antibiotics is transient, and potentially challenge current antibiotic management strategies for CF pulmonary exacerbations.

Evidence in the literature to support the application of culture-independent techniques to examine the effect of antibiotic therapy on the CF airway microbiome remains limited. TUNNEY *et al.* [9] have demonstrated that despite total bacterial numbers being reduced in response to antibiotics (culture-based assessment), there was relative stability in overall community composition as assessed by terminal restriction fragment length polymorphisms analysis when sputum samples were collected from subjects with CF before and after treatment of a pulmonary exacerbation. More recently, DANIELS *et al.* [24] examined the impact of antibiotics on CF sputum microbial diversity in a group of adult CF subjects. Sputum samples collected following the initial 72 h of antibiotic therapy were compared with samples collected during a period of clinical stability prior to the exacerbation and after 10–14 days of treatment. In comparison to samples collected at 72 h, samples collected at 10–14 days demonstrated an increase in relative abundance of *Pseudomonas* species compared with non-pseudomonads accompanied by a reduction in community diversity [24]. The authors concluded that antibiotic therapy was exerting a significantly greater effect on bacterial species other than *Pseudomonas*, culminating in the dominance of *P. aeruginosa*.

By examining microbial diversity at the onset of the exacerbation, prior to the administration of antibiotics, we have advanced the findings of DANIELS *et al.* [24] and shown that bacterial communities appear most susceptible to *i.v.* antibiotics at the beginning of antibiotic therapy. In addition to performing sampling prior to the onset of antibiotics, a number of important differences between the current study and that of DANIELS *et al.* [24] should be highlighted. In the earlier study, subjects were treated with a range of oral, inhaled and *i.v.* antibiotics which may have affected the ability to pick up consistent changes in community composition, whereas the subjects reported here were consistently treated with conventional *i.v.* antibiotic combinations. Additionally, sample processing and sequencing techniques differed between the two studies, most notably the samples in the study DANIELS *et al.* [24] were treated with propidium monoazide (PMA) to cross-link DNA from non-viable bacteria, a technique we did not employ. Nevertheless, both studies confirm that community composition remains essentially unchanged after at least 1 week of *i.v.* antibiotic therapy, which, given the early alterations in the microbiome that we observed, suggests that current treatment practices may need to be revised [25, 26].

The reduction in relative abundance of *Pseudomonas* from day 1 to days 3–4 suggests initial preferential killing of *Pseudomonas* in response to *i.v.* antibiotics. Recrudescence of *P. aeruginosa* infection under antibiotic pressure after this early response may occur as a result of increased replication of an inherently antibiotic resistant sub-population, or through population-wide adaptive mechanisms involving the upregulation of antibiotic resistance genes, which may occur rapidly in the treatment course [27, 28]. Whilst alternative explanations for the clinical improvements (reduction in respiratory symptoms and subjective wellbeing) in subjects beyond the first few days of treatment should also be considered, including the impact of adjunctive therapies, such as inhaled mucolytics, airway clearance techniques, rehydration and nutritional support [14, 29, 30], our findings challenge the convention of prescribing the same *i.v.* antibiotic combination for more than a few days at a time. A potential new strategy may involve the rapid cycling of different antibiotic regimes during treatment, but this would require investigation in large-scale, adequately powered randomised and blinded clinical trials.

To explore the effect of antibiotics on bacterial species other than *P. aeruginosa*, we examined microbial community changes with *Pseudomonas* reads excluded. Contrary to the earlier study by DANIELS *et al.* [24] the relative abundance of both aerobes and anaerobes increased in the early stages of treatment, suggesting antibiotic therapy was having a lesser impact on the other bacterial species present compared to *Pseudomonas*. The effects of *i.v.* antibiotics in adults not infected with *P. aeruginosa* who are experiencing an acute exacerbation warrants further investigation, particularly as these individuals represent an increasing group of subjects transitioning from paediatric to adult care [31].

Greater sputum microbial diversity in our study subjects was positively associated with the abundance of *Streptococcus*, accompanied by a reduction in the relative abundance of *Pseudomonas*. These findings are consistent with a previous cross-sectional study in which microbial profiles of stable CF outpatients and inpatients were compared [32]. In this earlier report, three distinct sub-groups of CF subjects were identified by the relative abundance of *Streptococcus* and *Pseudomonas* in sputum. Importantly, outpatients

with high *Streptococcus* and low *Pseudomonas* abundance had greater lung function stability over time. In our study, sputum samples with a high relative abundance of *Pseudomonas* and low community diversity formed a cluster, predominantly in subjects with severe lung disease. However, in contrast to other studies, we were unable to confirm a reduction in microbial diversity with increasing age and severity of lung disease in the CF subjects we studied [25, 33].

One potential limitation of our study is that PMA was not used to exclude DNA from non-viable bacterial cells in the sputum samples prior to analysis [34]. The use of PMA has been advocated due to appropriate theoretical concerns that molecular-based techniques may identify both viable and non-viable bacteria and limit the ability to detect changes in bacterial number in antibiotic treatment responses studies [35]. However, the reduction in the relative abundance of *Pseudomonas* we observed at day 3 would not be consistent with the inclusion of non-viable organisms [34]. The use of PMA remains a topic of debate and the method carries its own potential drawbacks, including a limited ability to expediently penetrate purulent, non-homogenised sputum prior to the death of resident organisms deep within the sputum sample. Furthermore, the extended processing time involved may result in less hardy bacteria (e.g. anaerobes) preferentially dying *ex vivo*, while more robust bacteria proliferate and skew the true bacterial composition, even if samples are maintained at 4°C [9, 36].

Our conclusions are based on changes in relative abundance and not quantitative bacterial load. We have previously performed enumeration of *P. aeruginosa* number by real-time quantitative PCR and demonstrated heterogeneous changes in bacterial numbers in response to antibiotics [23]. The findings for some subjects in this earlier work were consistent with the current study, with an early reduction in *P. aeruginosa* numbers being followed by a recrudescence by the end of the first week of treatment [23]. Quantification of *P. aeruginosa* by real-time quantitative PCR in nine subjects from the current study revealed a positive correlation between *P. aeruginosa* load and the relative abundance of *Pseudomonas* at the genus level (fig. S4), and supports conjecture that changes in community composition in the CF lung reflect changes in *P. aeruginosa* concentration; however, this relationship was weak. Matching changes in community structure with enumeration of individual bacterial species is complex and changes in individual species may not be equivalent to changes at the level of the genus. Future advances in pyrosequencing technology may allow for identification and enumeration of bacteria at the species level, which will substantially advance understanding of the dynamics of the lung microbiome.

Approximately 3% of all obtained sequences in our study could not be assigned to a genus, which is consistent with the findings of other studies [37]. The success of read assignment is dependent on read length, primer pairs and the particular reference database applied to the analysis [38]. To date, there no universal method of analysis is agreed upon, which further confounds direct comparisons of the human microbiome between studies.

In summary, we have demonstrated for the first time that the relative abundance of *P. aeruginosa* falls rapidly in subjects with CF receiving *i.v.* antibiotics for pulmonary exacerbations, and that this is accompanied by an increase in microbial diversity. This effect was not maintained beyond the first week of treatment. These findings have implications for how *i.v.* antibiotic treatment should be employed for exacerbations and for how long. Future clinical trials should consider the impact of *i.v.* antibiotics on the whole lung microbiome in CF, how changes in microbial community composition relate to reductions in absolute bacterial counts and, in turn, how these parameters relate to the clinical response to treatment.

Acknowledgements

We would like to thank to Dana Willner (Australian Centre for Ecogenomics, University of Queensland, Brisbane, Australia and Diamantina Institute, University of Queensland, Brisbane, Australia) for help and advice with the initial optimisation of DNA extraction protocols.

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