1 TEMPORAL PROFILING OF THE BACTERIAL AND FUNGAL COMMUNITIES IN

2 ΔF508 ADULT CYSTIC FIBROSIS SPUTUM

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ABSTRACT

Aims: The purpose of this study was to analyse the bacterial and fungal turnover in the lungs of cystic fibrosis patients who were ΔF508 homo- and hetero-zygotes. Further to this we wanted to identify the effects that Intravenous (IV) antibiotic perturbations had on the community and most importantly, whether exacerbations in these patients could be attributed to microbial species or communities.

Methods: A total of 149 samples were collected from 18 adult CF patients attending a clinic at the RVI hospital, Newcastle upon Tyne. The samples were subject to DNA extraction followed by bacterial and fungal community DGGE analysis as well as qPCR analysis of the bacterial load.

Results: We have found that bacterial and fungal communities present in the CF lung are not different when patients are suffering an exacerbation. Further to this, we have found that bacterial communities in the CF lung are disturbed by IV antibiotic administration and cause increased species turnover. We have shown that fungal taxa are capable of chronically colonising the CF lung.

Conclusions: Our study adds further evidence to the assertion that changes in bacterial communities are not the cause of CF exacerbations. However, we were able to demonstrate that acquisition of new bacterial taxa was strongly associated with exacerbations in one patient. This study is the first to illustrate that fungi can persist in the CF lung but are not associated with clinical status.

INTRODUCTION

Bacterial succession of the major CF pathogens has been well characterised by the CF foundation which shows that children are most likely to be colonised by *S. aureus* and *H. influenzae* and during adolescence these organisms will largely be replaced by *P. aeruginosa* (Anon, 2009). Rogers et al. (2004) have found that the bacterial communities present in the CF lung are unique to each patient and this has been confirmed in other studies. Further to this, the functionality of the bacterial communities in the CF lung has also been shown to be unique to individual patients and vary through time (Lim et al., 2014). Cox et al. (2010) used an age stratified study of CF patients and demonstrated that bacterial diversity, richness, and evenness decreased with increasing age and attributable to an increase in members of the family Pseudomonaceae. The underlying mechanism that drives bacterial community composition and evolution in CF lung infection is beginning to be explored (Nelson et al., 2012). A core microbiome for CF lung samples but this may differ based upon geographical location (Stressmann et al., 2011a, van der Gast et al., 2011).

Many fungal species are frequently isolated from the sputum of CF patients with *Candida* sp. *and Aspergillus* sp. being most common (Bouchara et al., 2009). Duan et al. (2003) have shown that recurrent *C. albicans* infection occurs over a ten year period, suggesting that fungi may be capable of chronically colonising the lung. Previously, *C. dubliniensis* has been isolated from CF sputum at a prevalence of 11.1% in a study utilising microbial culture techniques (Peltroche-Llacsahuanga et al., 2002). However, previous studies suggest that colonisation by *C. dubliniensis* does not lead to invasive infection and its role as a pathogen in CF is yet to be defined (Peltroche-Llacsahuanga et al., 2002). Amin et al. (2010) found that patients colonised with *A. fumigatus* had significantly reduced lung function compared to controls and had increased risk of hospitalisation. Futhermore, patients who are receiving

69   antibiotics are at increased risk of *A .fumigatus* infection compared to controls (Bargon et al.,

70   1999). Our previous work has identified that ΔF508 homozygotes have more diverse fungal

71   communities than ΔF508 heterozygotes (Nelson et al., 2013). It has also been shown that a

72   reduced fungal diversity is associated with a reduction in clinical measurements such as BMI

73   and lung function scores (Delhaes et al., 2012).

74   Lung function decline in CF patients has largely been attributed to exacerbations, but the

75   cause remains unclear. A pulmonary exacerbation in CF can be defined as changes in many

76   factors such as patient wellbeing, cough, dyspnoea, loss of appetite and/or weight and

77   changes in spirometric tests (Goss and Burns, 2007). It is this vague definition of pulmonary

78   exacerbations in CF that makes concordance between studies exploring aetiology and

79   treatment difficult. Pulmonary exacerbations in CF are attributed to a number of phenomenon

80   including increase in bacterial load, decrease in susceptibility to antimicrobials, and

81   acquisition of a new organism. For example, the organisms that make up the *Streptococcus*

82   *milleri* group (SMG) have been found in CF patients only when presenting with an

83   exacerbation and that treatment of these organisms can alleviate symptoms (Sibley et al.,

84   2008). Tunney et al. (2011) examined the relationship between bacterial richness and load

85   during times of pulmonary exacerbation and showed that IV antibiotics reduced the numbers

86   of viable aerobic and anaerobic bacteria present in the lung. However, very little change in

87   the overall community richness was observed between exacerbation, post-IV antibiotics and

88   stable bacterial communities suggesting that the bacterial communities present in the CF lung

89   are resistant to perturbation by antibiotics which is supportive of earlier data (Mulcahy et al.,

90   2010). Further studies have also investigated the role of the bacterial community in

91   exacerbations and the effects of antibiotic treatment also showing that antibiotics only have a

92   minimal effect on the bacterial communities and no characteristic community assembles that

93   could be used to define an exacerbation (Fodor et al., 2012, Stressmann et al., 2012, Zhao et

94   al., 2012).

95   Here, we aimed to assess the diversity and stability of both bacterial and fungal communities

96   in a longitudinal study. We tracked changes in eighteen cystic fibrosis ΔF508 homo and

97   heterozygotes over a two year period to determine whether in bacterial and fungal community

98   structure or diversity have demonstrable links to pulmonary exacerbations in CF and whether

99   fungal species are chronic colonisers of the CF lung.

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MATERIALS AND METHODS

Ethics statement

Consultation with County Durham and Tees Valley research ethics committee review board was undertaken. The committee advised that because these samples were collected for routine clinical purposes they could have additional investigational diagnostic tests applied without formal written consent. No additional samples were requested specifically for the study.

Sample collection

A total of eighteen patients were enrolled in the study contributing a total of 149 (Mean 8.3 sample/patient; Range 4 – 16) spontaneously expectorated sputum samples across a 20 month time period from October 2008 to July 2010 (Table 1). After expectoration the samples were stored at -20˚C until processed. Information regarding the patients' age, sex, CFTR genotype, clinical status, FEV_1 % predicted, oral and IV antibiotic therapy and results from microbial culture were collected. The patients' clinical status was classified as stable, exacerbation and routine IV. An exacerbation was defined by the supervising clinician (SJB) in the presence of deterioration in their clinical status with an increase in cough, sputum or chest symptoms, and/ or a fall in FEV1 and was subsequently treated with IV antibiotics, except in one instance where the patient received oral antibiotics. A total of 49 exacerbation events were recorded across all patients throughout sampling period and 62 cases of IV antibiotic administration of which 13 were administered as "routine IV". The average number of days a patient remained stable before exacerbation was 68.2 (range: 6-246) with the average length in days that the next available stable sample was received was 57.7 (range: 12-156). An average of 2.72 (range 0-8) exacerbations per patient was recorded across the cohort (range: 0-8). A total of 12 combinations of IV antibiotics administered, of which Ceftazidime and Tobramycin in combination were the most frequently administered (n=25).

DNA extraction and PCR amplification

DNA was extracted from the sputum samples using the MoBio Ultraclean Microbial DNA isolation kit with a filtration step added to remove any bacterial cells that remained intact after the cell lysis stage. PCR amplification of the V3 region of the bacterial 16S rRNA gene was performed with primers targeting the V3 region of the 16S rRNA gene as described by Muyzer and colleagues (Muyzer et al., 1993) using the reaction and cycling conditions as previously (Nelson et al., 2010). Briefly, the reaction was performed with 0.5mM each primer, 1x Ex Taq buffer, 0.3mM each dNTP, 1mM MgCl2, 500mg BSA, 1.25U Ex-Taq and 1μl of gDNA in a total volume of 50μl. The cycling conditions used were 95°C 5 min, followed by 20 cycles of 95°C 1 min, 65°C (-0.5°C per cycle) for 1 min and 72°C for 30 s followed by 15 cycles of 95°C 1 min, 55°C for 1 min and 72°C for 3 min with a final extension step of 30 min to prevent spurious band formation.PCR amplification of the fungal community of sputum was amplified using PCR primers specific for the 28S rRNA region of the fungal genome. The primers used were U1 and U2-GC (Sandhu et al., 1995) with conditions as described by our group (Nelson et al., 2010). Denaturing Gradient Gel Electrophoresis (DGGE) analysis of the bacterial and fungal communities was performed using the D-Code DGGE system (Bio-Rad) as previously described (Nelson et al., 2010).

Quantitative PCR was used to analyse the bacterial load in the sputum samples using the primer set EUB338 and EUB518 (Lane, 1991) as previously described (Nelson et al., 2013). Briefly, a plasmid standard was constructed containing the target region for each primer set using DNA extracted from the appropriate control strain. Standard curves were prepared using triplicate 10-fold dilutions of the plasmid standard to contain the target sequence at 300,000 – 30 copies/ml. The cycling conditions used were an initial enzyme activation step at 95 °C for 15 min, then 50 cycles of 95 °C for 10s, 65°C for 15s and extension at 72 °C for 20s on RotorGene 3000 instrumentation (Corbett life sciences). Target copy numbers for

150   each reaction, performed in triplicate, were calculated from the standard curve and were used

151   to ascertain the number of copies per ml of sputum.

152   **Data analysis**

153   A ladder of known organisms was loaded on each DGGE gel in the experiment so that

154   successful gel alignment could be achieved. The bands from the DGGE ladder were assigned

155   standard Rf values. A minimum of five bands per standard has been suggested for accurate

156   interpolation of multiple gels which was maintained or exceeded throughout our analysis

157   (Tourlomousis et al., 2010). Replicate DGGE gels were digitized and analysed using Phoretix

158   1D Pro (TotalLab, Newcastle Upon Tyne). Banding profiles were compared using band

159   matching algorithms to generate a band matrix of relative taxon abundance (band intensity)

160   for each community (lanes). Each lane was normalised by dividing the sum intensity of each

161   band class by the total intensity for each lane individually.

162   *Role of microbial communities during exacerbations*

163   A cluster analysis using the Dice similarity index for the bacterial and fungal binary

164   (presence/absence) band matrices for the entire cohort were compared to see if samples

165   collected when the patients presented at the clinic with an exacerbation clustered separately

166   from the stable samples. An RDA analysis was performed with clinical status as the only

167   factor to assess whether there was a significant difference between stable and exacerbation

168   samples. This analysis was repeated for individual patients who had suffered greater than one

169   exacerbation during the study period to account for inter patient variability. The biplots

170   created from the whole cohort and individual patients were further examined to determine if

171   particular taxa were being acquired that were the causative agent of exacerbations.

172   A one-way ANOVA analysis was used to compare Shannon diversity, bacterial richness,

173   bacterial evenness, bacterial load, fungal presence and fungal abundance with clinical status

174   to see if any of these factors could explain the cause of exacerbations in CF.

175   *Temporal dynamics of microbial communities*

176   The mean Dice (Sorensen) coefficient was calculated individually for each patient over the

177   course of the study period (Hammer, 2001). The inter-patient relationship between the Dice

178   similarity and number of antibiotic interventions was analysed using Pearson product

179   moment correlation (Minitab). Species richness (the number of taxa present), evenness (how

180   equal the different taxa are numerically), and Shannon diversity (a diversity indices giving a

181   measure of both species numbers and the evenness of their abundance), were calculated using

182   the DGGE band matrix. The band matrix for both bacterial and fungal community analysis

183   was further analysed using Canoco (v4.5.1). Mathematical testing using Pearson product

184   moment correlation was used to analyse the relationship between age, FEV1 (% Predicted),

185   Bacterial load, Shannon diversity, bacterial richness, bacterial evenness and fungal richness.

186   Pearson product moment correlation was also used to analyse the relationship between use of

187   IV antibiotics and bacterial community similarity.

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RESULTS AND DISCUSSION

Role of microbial communities during exacerbations

The progression of CF lung disease is known to be a constant decline in lung function towards respiratory failure due to inflammation and infection. Recent evidence has found that exacerbations in CF contribute to lung function decline (Amadori et al., 2009, Corey et al., 1997). A great deal of research has been aimed at finding the cause of exacerbation, with bacterial and viral agents being implicated. However, a definitive conclusion has yet to be reached. Shannon diversity index, bacterial species richness and bacterial community evenness were calculated for the abundance profiles and analysed against clinical status using one-way ANOVA. None of these factors were significantly different between samples obtained when the patients were stable or having an exacerbation ($P = 0.774$, 0.285 and 0.675 respectively). This confirms the findings of (Tunney et al., 2011) who examined the relationship between Bacterial richness and load during times of pulmonary exacerbation. They found very little change in diversity was observed between exacerbation, post-IV antibiotics and stable bacterial communities using T-RFLP.

A cluster analysis was performed to determine whether bacterial community composition when the patients presented with an exacerbation group separately from those obtained when the patients were stable. The cluster analysis shows that there is no split between stable and exacerbation derived samples. The relationship between bacterial communities and exacerbations was further explored using constrained ordination to maximise the separation by clinical status. An RDA analysis was performed with clinical status as the only environmental variable considered which found that bacterial communities present in the CF lung were not different when patients presented at the clinic with an exacerbation ($P= 0.192$). Biplots of species against clinical status were created to see if individual taxa (bands) in

either profile displayed a strong correlation with clinical status. The biplots show that there were no significant correlation present between individual taxa and clinical status when examining the entire cohort (Fig 1). The DGGE profiles for individual patients against clinical status were then analysed using RDA to see if the uniqueness of the microbiome between patients was masking any changes. Only patients who had greater than one exacerbation were included in this analysis to prevent casual trends being linked to exacerbations. The biplot from patient 16 shows that two taxa that were strongly correlated with an exacerbation (Fig 2). Further investigation found that bands 21 and 76 were present in all of the samples obtained when the patient presented with an exacerbation ($n = 3$) and none of the samples obtained when the patient was stable ($n = 4$). The acquisition of new bacterial taxa has been suggested as a possible cause for exacerbation in CF (Sibley et al., 2008). They found that members of the SMG were only isolated when patients presented with an exacerbation. Furthermore, they found that treatment directed at these organisms resolved the exacerbation. Although DGGE is capable of detecting the major changes in community structure, we were unable to confidently assign IDs to bands 21 and 76.

The presence and richness of fungal species was used to determine whether fungi were a cause of exacerbations in CF. The analysis found that fungal presence and richness were not the cause of exacerbation in CF ($P = 0.802$ and 0.795 respectively). Traditionally in CF, only *A. fumigatus* and *C. albicans* were identified as fungal pathogens. However, it is only recently that these species are being linked with CF exacerbations (Amin et al., 2010, Chotirmall et al., 2010). We have previously shown that our method is capable of detecting both *C. albicans* and *A. fumigatus* but we could not demonstrate a link between these species and exacerbations in our cohort (Nelson et al., 2010, Nelson et al., 2013). We have found that the presence of fungal species, richness and the relative abundance are not associated with exacerbations in our cohort.

239   Bacterial load as determined by qPCR was subjected to one way ANOVA using clinical

240   status as the discrete variable. Total bacterial load was not significantly different between

241   samples obtained when the patients were stable or having an exacerbation (P = 0.580). This

242   finding supports those of a previous study where increased bacterial load as measured by

243   qPCR was not indicative of an exacerbation (Stressmann et al., 2011b). This analysis was

244   repeated for each individual to examine whether changes in bacterial load were being masked

245   by the variation between patients. The bacterial load for individual patients was not

246   significantly different between samples obtained when the patients were stable or presenting

247   with an exacerbation (Table 1).

248   **Temporal dynamics of microbial communities**

249   Shannon diversity index, species richness and bacterial community evenness were calculated

250   to assess the relationship between patients. These data along with FEV_1 % predicted, age and

251   fungal species richness were analysed using Pearson product moment correlation to assess

252   which of the continuous variables affected the microbial community. Pearson product

253   moment correlation found that FEV_1 % predicted, Shannon diversity and bacterial species

254   richness had a significant negative correlation with age (P = <0.0001, 0.004 and <0.001

255   respectively). Furthermore, Shannon diversity, bacterial species richness and bacterial

256   community evenness displayed a positive correlation with FEV_1 % predicted (P = <0.0001,

257   0.003 and 0.008 respectively). Similarly, the decline of bacterial diversity and bacterial

258   community richness has also been identified previously (Cox et al., 2010). Our data suggests

259   that, as has been previously reported, DGGE analysis is capable of detecting major changes

260   in bacterial communities (0.1 – 1 %) which are normally due to the more prevalent taxa

261   (Muyzer et al., 1993).

262   Dice similarity was calculated to evaluate the percentage similarity of banding patterns over

263   time between consecutive sampling points for individual patients. The mean pair-wise

264   percentage similarity over the entire sampling period was 72.0% (Range 60.9 – 92.2%). To

265   assess the effect of IV antibiotics on bacterial community stability the number of IV

266   antibiotic therapies was normalised against the total number of samples for each patient and

267   compared to the mean similarity. A significant negative correlation was observed suggesting

268   more IV interventions led to an increase in the turnover of species in the bacterial

269   communities (P = 0.02; Fig 3). Recent work has identified that there is little effect on

270   bacterial community structure with short term antibiotic administration (Daniels et al., 2013,

271   Tunney et al., 2011). However, a previous study has identified that antibiotic load is a

272   significant driver in the reduction of diversity in the bacterial communities of the CF lung

273   over time (Zhao et al., 2012). It may be the case that susceptible bacteria are removed from

274   the community by antibiotic therapy and are replaced in either location or niche by resistant

275   bacteria which spread over time causing the reduction in bacterial diversity seen with age.

276   From the cohort, 94% (17/18) of the patients had a fungal isolate detected from at least one of

277   the samples during the study period. Of the 149 samples collected, 42% (63/149) were

278   positive for fungal species. A total of 89 bands were identified in the positive samples.

279   Analysis of the fungal communities by DGGE found that all but one patient had fungal

280   colonisation on at least one sampling date; however, this patient was receiving antifungal

281   therapy for part of the study period. Previous studies using DNA based techniques for the

282   identification of fungi in CF sputum have found colonisation rates of 70% using a DNA

283   microarray approach and 100% using PCR and sequencing (Nagano et al., 2010). However,

284   there is very little information using molecular methods on the persistence of fungi in CF

285   sputum. Cluster analysis was performed and qualitative analysis of the gels was used to

286   examine fungal colonisation patterns. Cluster analysis found that patient 17 was colonised by

287 *C. parapsilosis* for the entire sampling period (Fig 4a). Similarly, patient 13 was colonised *C.*

288 *albicans* from the second sample date and this taxa persisted for the remaining samples

289 collected (Fig 4b). The cluster analysis from the remaining patients revealed that particular

290 taxa were present in the patient on at least one sampling date but then disappeared.

291 Interestingly, in eight of the patients, several taxa went through periods of presence and

292 absence throughout the sampling period. Ghannoum et al. (2010) have investigated the oral

293 mycobiome of healthy individuals and found that Candida species were frequently isolated

294 and that Aspergillus species were present in over a third of the study participants. This data

295 may suggest that these patients are being exposed to the same pathogen repeatedly, which

296 may implicate the oral cavity as a reservoir for fungal, as well as bacterial pathogens.

297 The Pearson product moment correlation of fungal species richness against the continuous

298 variables found that there were no significant correlations present. One-way ANOVA

299 analysis found that fungal species richness was significantly greater in patients who were

300 homozygous ΔF508 compared to heterozygotes ($P = 0.016$). The relationship between CFTR

301 genotype and fungal colonisation has been identified previously for a single fungal species as

302 well as for fungal diversity (Giraud et al., 2010, Nelson et al., 2013). Delhaes et al. (2012)

303 have found that lung function decline is associated with a reduction in fungal diversity which

304 supports the findings in bacterial community structure (Cox et al., 2010). However, we have

305 not found the same result in our cohort. Furthermore, we have found that ΔF508

306 homozygotes who usually have a worse clinical outcome have more diverse fungal

307 communities. The discrepancy between these findings could be due to methodological

308 differences or potentially due to inconsistent patient management or the environmental

309 distribution of fungal taxa.

310 In conclusion, we have found that a biomarker community state does not occur in the CF lung

311 at times of pulmonary exacerbation. We have found that acquisition of new bacterial taxa was

312 strongly linked with clinical status in one patient. This may mean that exacerbations in CF

313 can be borne by multiple aetiologies. We have also found that IV antibiotic load increases

314 bacterial turnover in the CF lung. With regards to fungal communities, we have shown that

315 although fungal species are not the cause of exacerbation, they are capable of chronically

316 colonising the CF lung.

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428

Patient	Gender	Age	No. IV	F508	Stable mean Bacterial load	Exacerbation mean Bacterial load	Bacterial load v Clinical status (P-value)
1	F	30	5	HET	9.02	9.37	0.639
2	F	33	9	HOM	8.09	8.37	0.424
3	M	32	3	HOM	9.04	8.79	0.261
4	M	27	2	HOM	8.96	9.50	0.550
5	F	28	3	HET	9.15	9.25	0.809
6	M	34	5	HET	7.87	7.55	0.521
7	M	23	3	HOM	8.76	8.79	0.924
8	M	19	2	HOM	9.13	N/A	N/A
9	F	19	5	HOM	8.54	8.42	0.784
10	M	20	7	HET	7.84	8.57	0.255
11	M	26	4	HOM	9.39	8.81	0.625
12	F	56	2	HET	8.39	8.93	0.481
13	M	56	4	HET	8.67	8.73	0.671
14	F	19	1	HET	9.61	9.40	0.822
15	F	59	3	HET	9.62	9.97	0.404
16	F	23	3	HOM	8.90	8.92	0.905
17	F	39	2	HOM	9.00	9.15	0.388
18	M	39	1	HOM	9.10	N/A	N/A

Table 1. Patient demographics and bacterial load data

M=Male, F=Female, HET=ΔF508 heterozygous, HOM=ΔF508 homozygous. Bacterial load data is log copies/ml
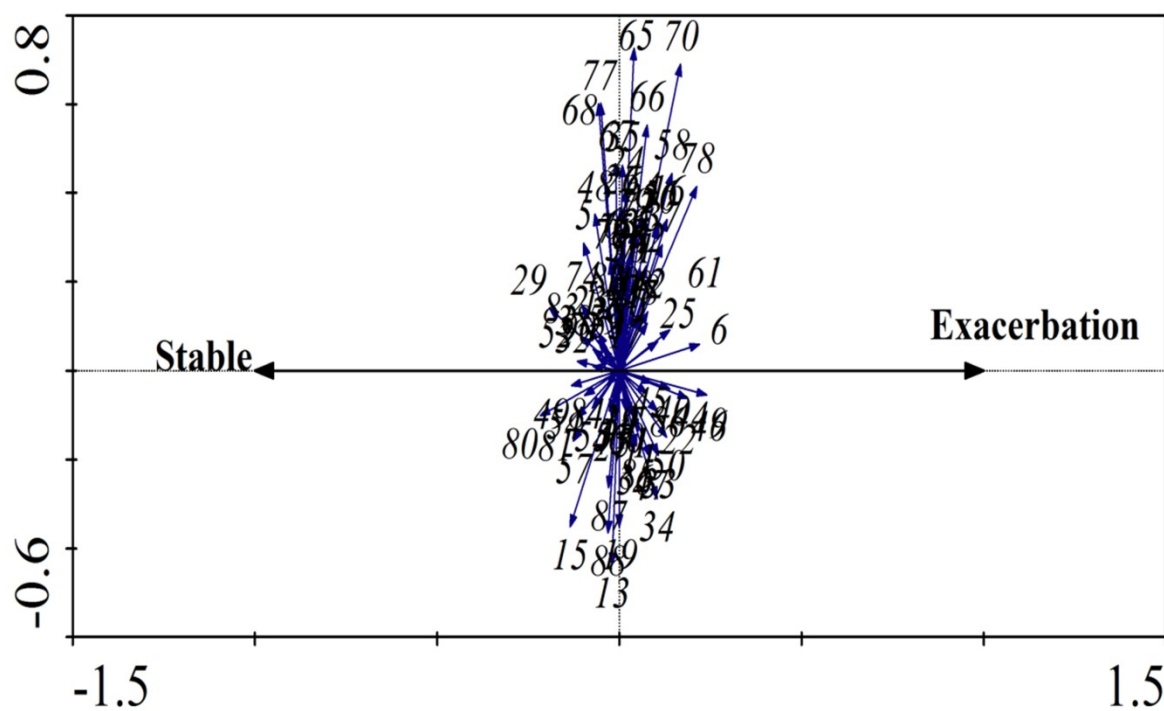
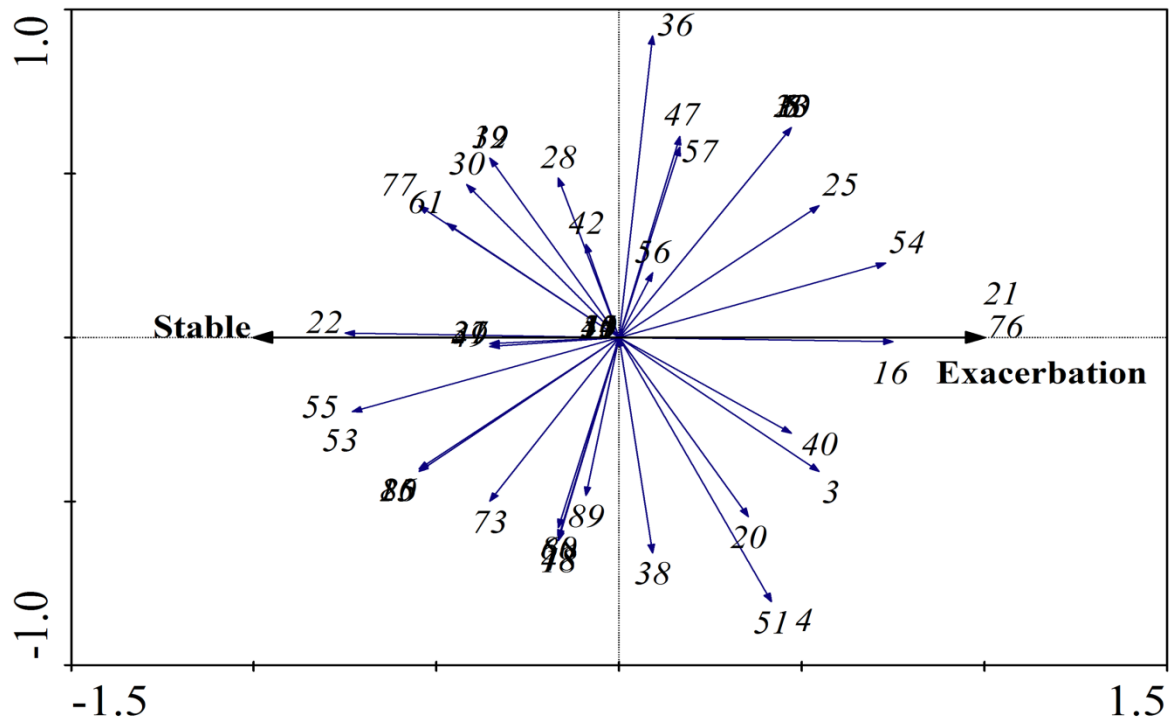Fig 1. RDA showing the relationship of bacterial taxa with clinical status

Fig 2. RDA analysis of bacterial taxa for patient 16 showing the association band 21 and 76 with exacerbation
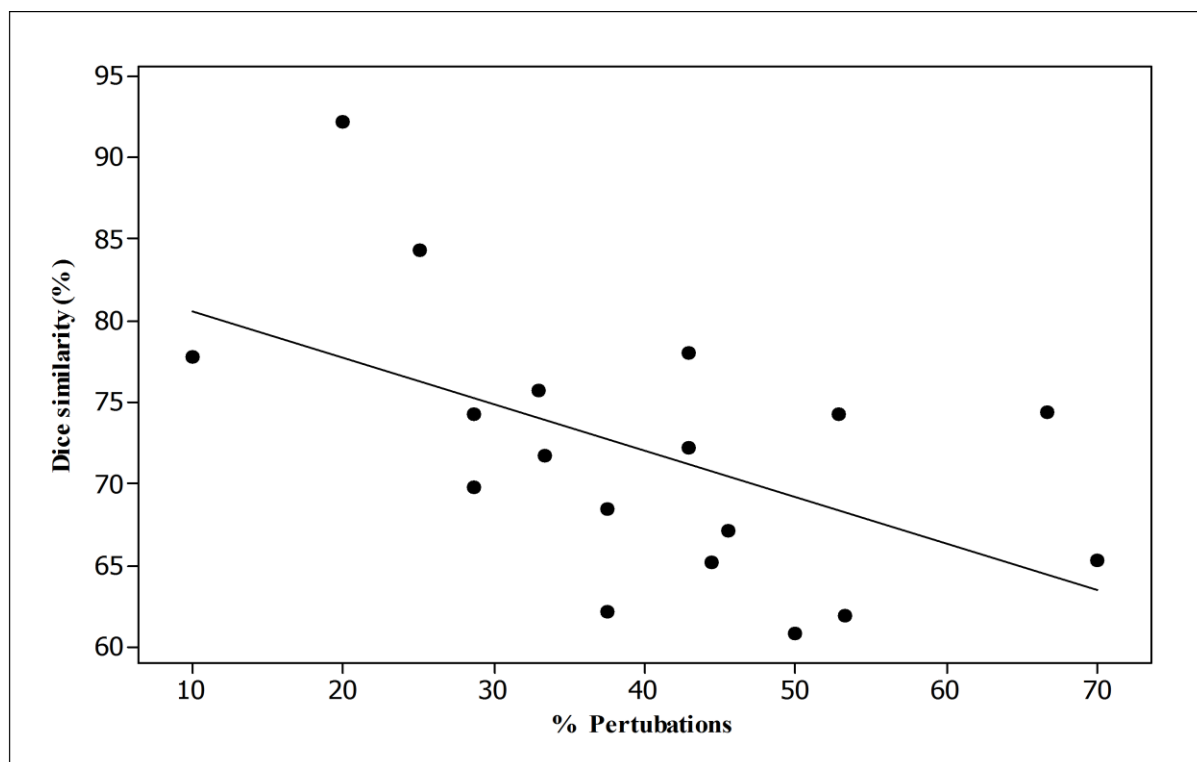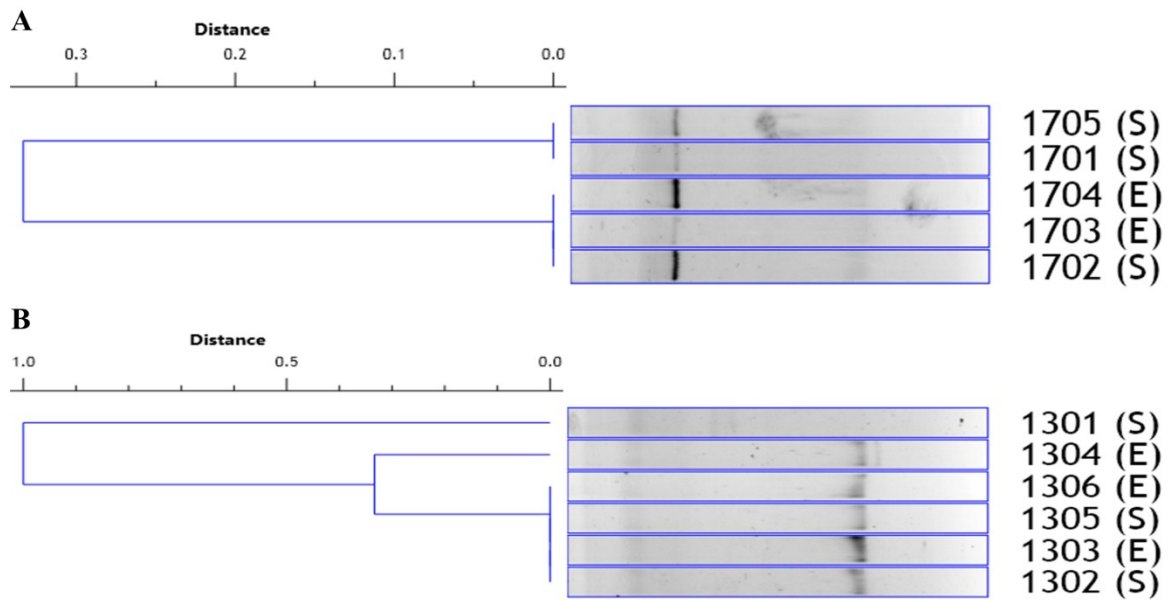
Fig 3 Relationship between IV antibiotics and bacterial community similarity

Fig 4. Cluster analysis for (a) patient 17 who was chronically colonised by *C. parapsilosis* (b) patient 13 who acquired *C. albicans*

S = stable, E = exacerbation