Cystic fibrosis microbiome: analysis of nasal middle meatus and sputum in different lung disease stages*

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Abstract

Background: Culture independent methods of molecular detection of microbiome have shown the polymicrobial nature of respiratory infections in cystic fibrosis, with pathogenic agents undetectable in conventional culture methods. Composition and diversity of the airway microbiome are still poorly understood.

Methodology: This study evaluated the airway microbiome in 31 adult cystic fibrosis patients via the analysis of 16S rRNA sequences by next generation sequencing.

Results: *Staphylococcus, Streptococcus* and *Corynebacterium* were the most abundant genera in the middle meatus, and *Pseudomonas, Haemophilus* and *Prevotella* were the most abundant in sputum. In patients with advanced disease (FEV1< 50%), there was an increase in the prevalence of *Pseudomonas* in both sample types when studied separately. In each patient, in a paired analysis, the sputum and middle meatus showed similar microbiome composition in mild or moderate disease (FEV1≥ 50%). In patients with severe lung disease, the relative abundance of *Pseudomonas* had a positive correlation in both collection sites.

Conclusions: This is the first Brazilian study to evaluate the airway microbiome in cystic fibrosis patients. Our findings agree with those in the international literature and indicate the role of *Pseudomonas* in the sputum and middle meatus in patients with advanced disease.

Key words: cystic fibrosis; microbiology, 16S rRNA, high-throughput nucleotide sequencing, forced expiratory volume.

Introduction

In Cystic fibrosis (CF), the mucosal surface of the airways exhibits increased mucus viscosity and reduced mucociliary clearance [1,2]. There is a predisposition toward infection by *Staphylococcus aureus* and *Pseudomonas aeruginosa*[3], which is associated with a decline in pulmonary function, the main cause of mortality [4,5]. Chronic colonization by *P. aeruginosa* occurs most often up until the third decade of life. During the intermittent colonization phase, approximately 25% of cases of recolonization occur due to a bacterium of the same genotype, indicating a persistent source of environmental contamination or an unidentified reservoir in the patient. Furthermore, when comparing bacteria, such as *S. aureus* and *P. aeruginosa*, present in the nasal lavage

and sputum of CF patients, there is a high concordance of genotypes, with evidence of cross-infection between the upper and lower airways [6].

CF patients subject to lung transplantation exhibit colonization by *Pseudomonas* with mutations and gene expression, similar to that found before transplantation [7]. CF patients subject to endonasal endoscopic surgery followed by antibiotic therapy attained a reduction in the occurrence of positive cultures for CF-related pathogens in the first year after surgery [8]. These and other studies [6,9] suggest that the paranasal sinuses function as reservoirs of pathogenic microorganisms.

Genetic sequencing platforms allow for more comprehensive analysis of the composition and relative abundances of microor-

ganisms in a given community. Such tools draw attention to the complexity of the microbial communities present in the airways of patients with chronic inflammatory diseases [10,11].

Extensive knowledge of the composition and diversity of the nasosinusal microbiome of CF patients is important in understanding the pathophysiology of the disease when considering the likely role of the paranasal sinuses as a reservoir of bacteria for lung infections and also considering that the interactions between species may influence the virulence of a bacteria in a polymicrobial community [12].

The objectives of this study were to compare the composition and diversity of the microbiome between the nasal middle meatus and the sputum of adult patients with cystic fibrosis and between groups with mild/moderate lung disease (forced expiratory volume in one second (FEV1 ≥ 50%) and severe lung disease (FEV1 < 50%).

Materials and methods

Study population

For this exploratory cross-sectional study, patients from the Clinics Hospital of São Paulo aged 18 years and older with a diagnosis of cystic fibrosis, confirmed by a compatible clinical condition, a sweat chloride above 60 mEq/l and genetic tests that identified at least 2 known mutations were recruited. The exclusion criteria were a) the occurrence of pulmonary exacerbation (5) and or the use of a systemic antibiotic in the last 4 weeks, or b) previous lung transplantation. There were 31 patients (17 patients with a forced expiratory volume in one second (FEV1) ≥ 50 and 14 patients with FEV1 < 50). A total of 22 middle meatus samples and 26 sputum samples were collected. Questionnaires validated in the Portuguese language were used, including the Sino-Nasal Outcome Test-20 (SNOT-20) [13] and the Nose Obstruction Symptom Evaluation (NOSE) [14]. A pulmonary function test was performed to determine the FEV1 and a nasal endoscopy was performed for Lund-Kennedy Endoscopic Classification [15]. This study was approved by the Ethics Committee for Analysis of Research Projects - CAPPesq (no. 1.453.561). All participants read and signed the informed consent form.

Sample collection

During endoscopic visualization, the swab (FLOQSwabs; COPAN Diagnostics, Inc., Murrieta, CA, USA) was inserted, covered by a sterile catheter in the middle meatus, the swab was uncovered for sample collection with at least 3 complete rotations within the meatus. The swab was covered again and pulled out of the nose. The tip of the swab was cut and stored in a sterile cryotube that was free of DNase and RNase. The sputum samples were collected in a sterile vial and transferred to a cryotube and both samples were stored at -80 °C.

Table 1. Demographic and clinical characteristics of subjects in both groups classified according to pulmonary disease severity.

		Severity of lung disease		
	Total	**Mild/ moderate FEV 1 ≥ 50**	**Severe FEV1 <50**	**p***
Patients (n)	31	17	14	-
Age (mean, DP)	30,2 (14,8)	29,5 (14,8)	31,1 (15,4)	0,33
Gender (m=male, f=female)	13 m, 18 f	8 m, 9 f	5 m, 9 f	0,7
Alpha dornase	58,10%	47,10%	71,40%	0,27
Inhaled antibiotic	41,90%	17,60%	71,40%	<0,01
Oral antibiotic	71,00%	64,70%	78,60%	0,46
Lund-Kennedy**	3,9	4,0 (2,6)	3,9 (3,1)	0,95
SNOT-20***	31,6	32,0 (21,3)	31,6 (17,2)	0,98
NOSE****	7	7,1 (6,1)	7,2 (5,4)	0,87

p* p-value; ** Endoscopic classification of Lund-Kennedy; *** Sino-Nasal Outcome Test-20; **** Nose Obstruction Symptom Evaluation.

Bacterial DNA extraction

The sputum samples were treated with a 0.1% dithiothreitol (DTT, Invitrogen)-sputolysin solution prior to the DNA extraction step [16,17]. The total DNA was extracted from the collected samples with the QIAamp DNA Blood kit (Qiagen) and stored at -20°C.

Microbiome sequencing

The amplification of the V3-V4 region of the 16S rRNA gene was performed with 25 cycles of PCR using primers and conditions that were previously described [18]. The amplicons were pooled and loaded into an Illumina MiSeq clamshell style cartridge kit (v2) for 500 cycles for 250 paired-end sequencing at a final concentration of 12 pM. The library was clustered at a density of approximately 80,000 k/mm². The raw read files were demultiplexed and then bioinformatics analysis were conducted with QIIME software [19], as previously described [20]. Then, using the Microbiome Analyst [21], principal coordinate analysis (PCoAs) were performed to determine the differences in beta diversity between the different sites and groups [22]. Nucleic acid sequences are available at the Sequence Read Archive (SRA) under accession PRJNA548026.Species classification was performed using SPINGO software [23] using default parameters.

Statistical analysis

For the demographic and clinical characteristics of the patients, the continuous variables were represented as the means and
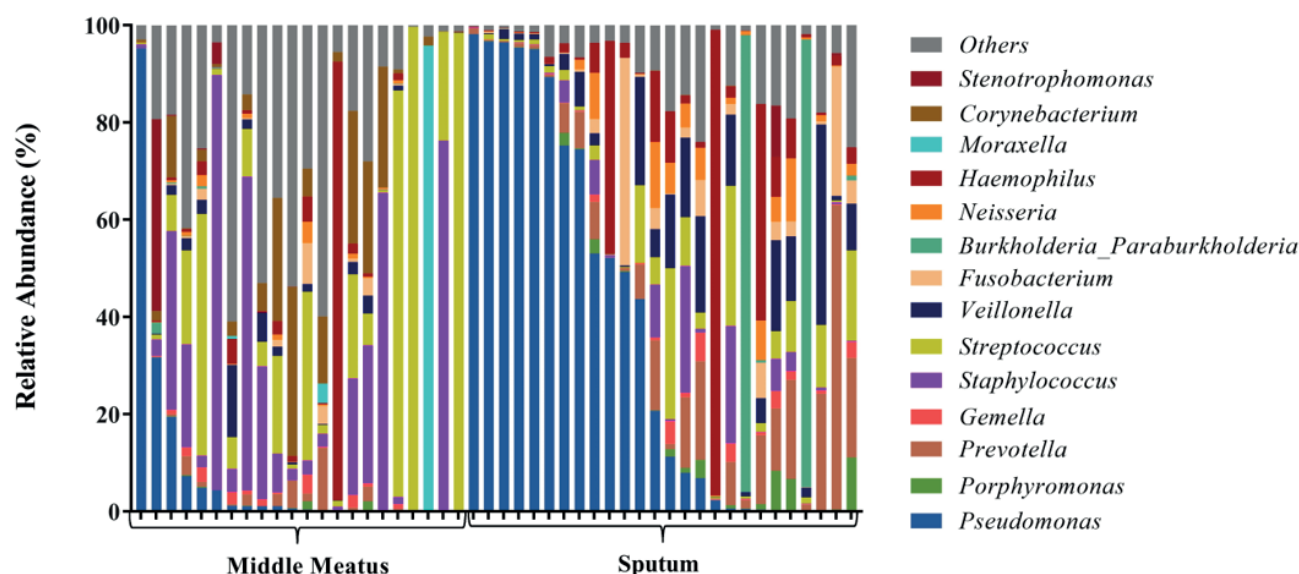
Figure 1. Relative abundance of main bacterial genera found in each patient, divided by sample site, showing differences in bacterial predominance. In the middle meatus samples, there is a higher prevalence of *Streptococcus* and *Staphylococcus*. In the sputum samples, there is a higher prevalence of *Pseudomonas* and *Prevotella*.

their respective standard deviations (SD), and the categorical variables were represented as percentages. After the verification of normality using the Shapiro-Wilk test, the ages, Lund-Kennedy scores, SNOT-20 scores and NOSE scores were compared between the groups using a nonparametric Wilcoxon-Mann-Whitney test. The comparisons based on sex and the use of alpha dornase and antibiotics were performed using Fisher's exact test. The statistical analysis of the microbiome was performed by using SPSS version 22. For all the analysis, the level of significance was p≤0.05. Analysis of group similarities (ANOSIM) were performed also using Microbiome Analyst [21], with Bray-Curtis distances, to observe the differences in beta diversity based on the sample type and the disease severity.

Results

Characterization of the sample group
The demographic and clinical data are summarized in Table 1. The groups did not differ in relation to age, sex, endoscopic scores (Lund-Kennedy), SNOT-20 and NOSE questionnaires, use of prophylactic oral antibiotics (azithromycin, 3 times a week) or alpha dornase (p>0.05). The group with severe lung disease used proportionally more inhaled antibiotics (tobramycin or colistin) (p <0.01).

Characterization of the microbiome
The main genera found were *Streptococcus* (22.30%), *Staphylococcus* (20.90%), *Corynebacterium* (8.60%) and *Pseudomonas* (6,88%) in the middle meatus and *Pseudomonas* (44.10%), *Haemophilus* (11.22%), *Veillonella* (6.85%) and *Prevotella* (6.40%)

in the sputum samples.
The individual patient results that refer to the relative abundance of the genera in the middle meatus and sputum samples (Figure 1) showed a difference in the microbial profiles between the collected materials, with a prevalence of *Streptococcus* and *Staphylococcus* in the middle meatus samples and *Pseudomonas* and *Prevotella* in the sputum samples.

Microbiome and pulmonary function - Severity of lung disease
Middle meatus
It was observed that the nasal middle meatus of patients with severe lung disease exhibited, on average, a greater abundance of *Staphylococcus*, *Streptococcus* and *Stenotrophomonas*, as well as a smaller abundance of *Pseudomonas*, *Haemophilus* and *Corynebacterium*, in comparison with those with mild to moderate lung disease (Figure S1). However, none of the results showed statistical significance (Table S1).
On the other hand, when we evaluated the relative abundance of the genera in each individual, we found a different microbiome profile. *Staphylococcus* and *Streptococcus* were present in both groups with similar abundances. In the mild to moderate lung disease group, only one patient exhibited colonization by *Pseudomonas*, but the abundance was greater than 90%. In the severe lung disease group, five patients were colonized with *Pseudomonas* but in smaller abundances.

Sputum
On average, the sputum of the patients with severe lung disease

Figure 2. Relative abundance of main bacterial genera observed in middle meatus and sputum in paired samples from each patient with mild to moderate and severe lung disease. (#: identification number of each patient. M: middle meatus samples; S: sputum samples). There is a difference in microbiome composition from both disease state, with *Streptococcus*, *Staphylococcus* and *Prevotella* being shared in both samples from each patient with mild to moderate lung disease, and a distinct microbiome composition in both samples from each patient with severe lung disease.

had a higher proportion of *Pseudomonas*, *Prevotella*, *Streptococcus* and *Veillonella* and a smaller proportion of *Haemophilus*, *Neisseria* and *Burkholderia-Paraburkholderia* compared to those with mild/moderate lung disease (Figure S2). The difference in the abundance of *Haemophilus* (p=0,05) was statistically significant. In the sputum analysis, the comparison of the mean abundance of each of the bacterial genera in each group (Table S1) was similar to the profile found in the individual analysis, which showed an increase in the abundance of *Pseudomonas* in patients with severe lung disease, as well as an increase in the

relative abundances of *Prevotella*, *Veillonella* and *Fusobacterium*.

Paired samples (same patient comparison)
It was possible to make paired analysis only in those patients who had both samples available, which were 17 patients (10 patients with mild/moderate disease and 7 with severe disease). The relative abundances of the bacterial genera were compared in the paired samples from each patient according to pulmonary function (Figure 2). It was noted that the middle meatus and sputum samples from almost all patients with mild/moderate
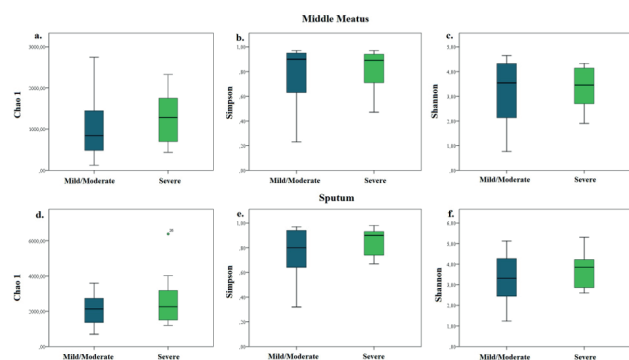
Figure 3. Alpha diversity in sample sites according to lung disease severity (blue: mild/moderate; green: severe). a. Chao1 richness estimator, b. Simpson´s dominance and c. Shannon´s diversity indexes from the middle meatus samples. d. Chao 1 richness estimator, e. Simpson´s dominance and f. Shannon´s diversity indexes from the sputum samples. Richeness, dominance and diversity were higher in both sample sites in the group with severe disease, although without statistical significance. Despite the differences in bacterial abundance in the sample sites according to the disease state in other analysis, there were no significative differences between alfa diversity indexes.

lung disease shared certain bacterial genera, mainly *Streptococcus*, *Staphylococcus* and *Prevotella*.

In patients with severe lung disease there was a very distinct profile in terms of relative abundance when comparing the two sites studied, which showed a higher prevalence of *Staphylococcus* in the samples from the middle meatus and of *Pseudomonas* in the sputum samples. In this case, only one patient (# 32) exhibited relative similarity in the microbiome profiles obtained from the samples. In terms of clinical information, this patient showed the highest SNOT-20 and NOSE scores among those with severe disease.

In the analysis of correlation between genera in middle meatus and sputum, using Pearson´s correlation, for some genera, the correlation reached statistical significance. In patients with mild/moderate disease, it was observed a positive correlation of *Prevotella* (p=0,02), *Veillonella* (p=0,03) and *Neisseria* (p=0,03) in the two collection sites. In patients with severe disease, it was found a positive correlation of *Pseudomonas* (p=0,02). In both groups, it was found a negative correlation of *Staphylococcus* (p=0,03 for mild/moderate disease and p=0,04 for severe disease) and *Corynebacterium* (p<0,01 for mild/moderate disease and p=0,01 for severe disease).

Species analysis

In the same patient, analysis of species revealed that *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Fusobacterium nucleatum* were present in both middle meatus and sputum in mild/moderate and severe lung disease patient´s samples. *Pseudomonas aeruginosa* were present in 80% of the sputum sample
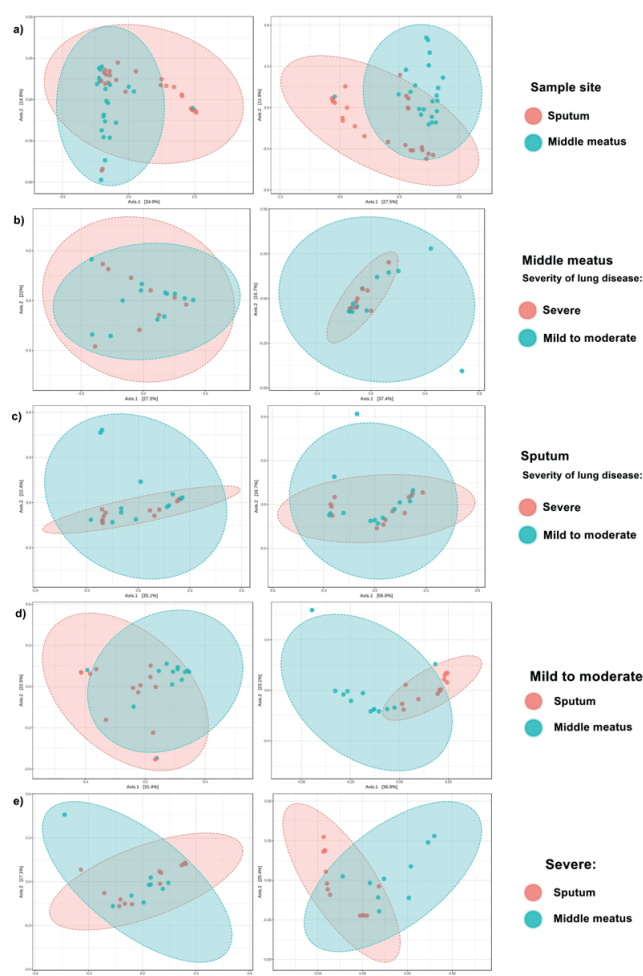

Figure 4. Principal coordinate analysis (PCoA) from sample site and from disease severity. Weighted analyses were plotted according to the more abundant genera (left) and unweighted analyses were plotted according to all bacteria genera (right): a) by sample site (weighted: p <0.001; unweighted: p <0.001); b) by disease severity in the middle meatus samples (weighted: p = 0.99; unweighted: p = 0.82); c) by disease severity in the sputum samples (weighted: p = 0.28; unweighted: p = 0.92); d) by sample site in mild/moderate disease (weighted: p < 0.001; unweighted: p < 0.001); and e) by sample site in severe disease (weighted: p < 0.001; unweighted: p < 0.001). This result expresses the microbial community structure in (a) each site, (b) and (c) sample site according to disease state, (d) and (e) disease state according to sample site. There were significant differences (p<0,001) in microbial community in each sample site (a), and in each sample site according to disease state (d and e).

of mild/moderate lung disease and in 57% of sputum samples from patients with severe disease (Figure S3 and S4). (Note that inhaled colistin or tobramycin was not suspended for the study).

Alpha and beta diversity

The alpha diversity (Shannon and Simpson indexes) and richness (Chao1 richness estimator) were observed in the middle meatus and sputum samples according to pulmonary disease

severity (Figure 3). However, none of the results showed statistical significance.

To observe the differences in relation to beta diversity, PCoA chart was prepared based on the weighted and unweighted UniFrac distance matrices. In general, the spatial distribution of the sputum and middle meatus samples showed a significant difference between the samples in the weighted (p <0.001, F =4.90) and unweighted (p <0.001, F =7.45) analysis (Figure 4a). We observed no difference when comparing the mild/moderate lung disease group with the severe lung disease group (weighted: p =0.44, F =0.96; unweighted: p =0.84, F=0.55). The use of alpha dornase (weighted: p = 0.50, F = 0.91; unweighted: p =0.72, F=0.66) and the use of inhaled antibiotics (weighted: p =0.35, F =1.06; unweighted: p=0.78, F =0.60) were also factors that did not significantly alter the bacterial community structure in the groups studied. In relation to the use of prophylactic oral antibiotics, we observed a statistically significant difference between the patients who used them compared with those who did not in the weighted analysis (p =0.03, F =1.79), but no statistically significant difference in the unweighted analysis (p =0.32, F =1.08).

When we examined the middle meatus samples, we could not distinguish between the bacterial community profiles from patients with mild/moderate lung disease and those from patients with severe lung disease in the weighted (p =0.99, F =0.31) and in the unweighted analysis (p =0.82, F =0,66) (Figure 4b). When the sputum sample profile was analysed, there was no distinction statistically significant between groups with mild to moderate and severe lung disease (weighted: p =0.28, F =1.23; unweighted: p =0.92, F =0,32) (Figure 4c).

When comparing the profile results of bacterial communities from different collection sites (sputum and middle meatus) and the severity of the disease (Figure 4d and 4e), we were able to obtain interesting findings that corroborated the relative abundance results previously shown (Figure 2).

In the analysis of patients with severe lung disease (Figure 4e), the comparison of the spatial distribution of the sputum and middle meatus samples showed a difference between the collection sites that was larger than that seen in patients with mild to moderate lung disease (Figure 4d).

Using ANOSIM analysis, there were significant difference between sample types (R: 0.39773; p<0.001 - Figure S5a), sample types related to pulmonary function, middle meatus and sputum samples from patients with mild to moderate disease (R: 0.32154; p<0.001 - Figure S5d) and middle meatus and sputum samples from patients with severe disease (R: 0.4606; p<0.001 - Figure S5e). There was no statistically significant difference in middle meatus samples from patients with mild to moderate and severe disease (R:-0.091768; p=0.935 - Figure S5b), or in sputum samples from patients with mild to moderate and severe disease (R:-0.0028814; p=0.425 - Figure S5c).

Discussion

A significant difference was found in the composition of the microbiome between the middle meatus and the sputum. Goddard et al. found a difference between the compositions of the sputum and upper airway microbiomes; despite using an oropharyngeal swab instead of one from the middle meatus [24], the data were similar to that of the present study.

Despite the differences in bacterial abundance in the sample sites according to the disease state (Figure 1 and 2, Table S1), and the differences in microbial community structure (Figure 4) there were no significative differences between alfa diversity indexes (Figure 3) in the present study.

In evaluating the relative abundances of the genera found in the middle meatus, we observed that 5 patients in the group with severe lung disease had concomitant contamination with *Staphylococcus* and *Pseudomonas*, whereas *Pseudomonas* was almost absent in patients with mild disease, apart from a single patient who presented a relative abundance of *Pseudomonas* greater than 90%. The observation of the coexistence of these two bacteria within the same sample in the severe lung disease group is compatible with data from the literature that shows that the association of these two bacteria is related to a decline in pulmonary function [12]. Although we found higher mean alpha diversity in the middle meatus in patients with severe disease, that was not statistically significant, the interindividual difference was greater in the mild/moderate group. Some data from the literature indicates that lower diversity is related to worse pulmonary function [11,25–27]. Like us, Paganin et al. also found no statistically significant difference between sputum samples from patients that were classified by lung disease severity, even within a sample of 78 patients [28].

In a study by Fodor et al., the most prevalent bacteria in the sputum of adult CF patients represented *Pseudomonas* and *Burkholderia* [27]. In our sample, the main genera observed were *Pseudomonas*, *Haemophilus*, *Prevotella*, *Veillonella* and *Burkholderia*.

Pulmonary function is negatively influenced by the presence of *Pseudomonas* as the dominant genus, according to findings by Coburn et al. [26]. We found 4 patients in the group with mild/moderate lung disease and 6 patients in the group with severe disease in whom *Pseudomonas* was the dominant genus in the sputum.

Several studies suggested that middle meatus may be a reservoir for some pathogens [6–9,29–33]. In our findings, the microbial compositions of the middle meatus and the sputum were different in the study population, but certain genera colonized both of the evaluated sites, including pathogenic bacteria such as *Pseudomonas*, *Staphylococcus*, *Prevotella*, *Fusobacterium* and *Haemophilus*. However, these data are questionable when comparing the results from the middle meatus and sputum according to the severity of the lung disease. In patients with

mild/moderate lung disease, both sites presented with mainly *Streptococcus*, *Staphylococcus* and *Prevotella*. In the samples from patients with severe lung disease, it was observed that the patients presented a very distinct profile in terms of relative abundance when comparing the two sites.

These findings suggest that patients with mild/moderate lung disease share more genera between the middle meatus and sputum than those patients with severe lung disease. This information is corroborated by species analysis, where it was also observed. Data published in the literature have shown that the middle meatus is a source of lung infection; however, no distinctions between the levels of lung disease severity were shown in the existing studies [6–9,29–33]. Indeed, Pletcher et al. showed a loss of niche specificity of CF airway microbial communities in relation to control population, but they also did not take into account the levels of lung disease severity in the analysis [34]. On the other hand, Lucas et al., found a different result from all these studies. They found distinct bacterial communities between upper and lower airway. The bacterial communities clustered more closely by sampling site, rather than by patient. Therefore, as in the other studies, they did not perform separate pulmonary function analysis [35].

In the middle meatus, *Staphylococcus* and *Corynebacterium* were among the most prevalent genera between all patients studied. In severe disease group, *Corynebacterium* had a smaller relative abundance, compared to the other group. Besides that, pathogenic bacteria such as, *Prevotella*, *Veillonella*, *Neisseria* and *Pseudomonas* had a positive correlation between both collection sites, that is, the greater the relative abundance in the middle meatus, the greater also in the sputum, with statistical significance. And commensal bacteria, previously described as composing a healthy airway microbiome [36], such as *Staphylococcus* and *Corynebacterium*, had a negative correlation between the two sites, with statistical significance.

The microbiome may promote or maintain inflammation that was previously induced by a failure of mucosal integrity or by local immunity. It is unclear whether chronic inflammation changes the composition of the microbiota, if the overgrowth of certain species affects the abundance of other species and then promotes inflammation, or if the two phenomena occur simultaneously [37]. According to the findings of Mika et al. [38], dysbiosis of the airway microbiome can be observed as early as the second month of life in CF patients.

Microbiome study is a relatively new field of investigation and we must consider the limitation of this study. The nasal middle meatus is the drainage site for the maxillary, ethmoid and frontal sinuses and may be a good representative of the sinus microbiota, as several studies with evidence Level IIb have shown [33]. Surgical access to these sites limits the size of the sample and selects for more symptomatic or more severe cases of the disease. Collection of middle meatus material is less invasive, which facilitates the reproducibility of the method in clinical practice. As an observational study, it was not possible to interfere with the treatment of these patients, and specially in severe lung disease patients group using inhaled antibiotics against *Pseudomonas* (71,4%), this medication could reduce the abundance of this specific bacteria in middle meatus.

Certain measures were taken to reduce possible bias as the identification of prophylactic antibiotics in the sample. The middle meatus samples from 10 patients were lost during DNA extraction because they did not present satisfactory DNA quality or quantity. Six of these patients used inhaled antibiotics, seven used azithromycin three times a week as a prophylactic oral antibiotic, and eight used alpha dornase.

Despite being the study with the largest sample and the first study to evaluate the airway microbiome in CF patients from Sao Paulo, it is still a small one. This is a pilot cross-sectional study, however with an important goal. It is necessary to first understand the composition and then formulate new hypothesis for future studies. Other studies must be done to enrich knowledge in airway microbiome in CF to enable comparison with other populations around the world.

Conclusions

Our findings agree with those of the international literature, which indicate the role of *Pseudomonas* in advanced disease, present in the sputum and middle meatus.

Pseudomonas aeruginosa, *Staphylococcus aureus* and *Fusobacterium nucleatum* were present in both middle meatus and sputum in mild/moderate and severe disease patient´s samples. The results shown here indicate that the middle meatus and the sputum share some important genera involved in the pathophysiology of CF, in the mild or moderate stages of the disease.

In patients with severe lung disease, there was a positive correlation for the relative abundance of *Pseudomonas* between the two sites.

More studies need to be conducted to better understand the pathophysiology of colonization in these patients and to confirm the hypothesis of the middle meatus as a reservoir to lung infections.

Acknowledgments

Authorship contribution

FGOM, RRMP, RA and RLV conceived the study design. FGOM and CRT drafted the manuscript. FGOM, RRMP and RA enrolled

patients. FGOM collected clinical data and samples and processed samples for sequencing. LGS and CRT sequenced samples, processed sequence data, and analysed and interpreted the data. RVC prepare the sequences and submitted them to GenBank. LGS created all the figures. All authors reviewed the manuscript.

Conflict of interest

The authors declare that there are no competing financial or nonfinancial interest and there are no personal relationships with other people or organizations that could inappropriately influence (bias) their work.

Consent for publication

Not applicable

Availability of data and materials

Not applicable.

References

1. Cutting GR. Cystic fibrosis genetics: From molecular understanding to clinical application. Nat Rev Genet. 2015;16(1):45–56.
2. Stuart Elborn J. Cystic Fibrosis. Lancet. 2016;6736(16):00576–6.
3. Kang SH, Dalcin P de TR, Piltcher OB, Migliavacca R de O. Chronic rhinosinusitis and nasal polyposis in cystic fibrosis: update on diagnosis and treatment. J Bras Pneumol. 2015;41(1):65–76.
4. Ramsey BW. Management of pulmonary disease in patients with cystic fibrosis. N Engl J Med. 1996;335(6):179–88.
5. Silva Filho LVRF, Ferreira FDA, Reis JFC, et al. Pseudomonas aeruginosa infection in patients with cystic fibrosis: scientific evidence regarding clinical impact, diagnosis, and treatment. 2013;39(4):495–512.
6. Folkesson A, Jelsbak L, Yang L, et al. Adaptation of Pseudomonas aeruginosa to the cystic fibrosis airway: An evolutionary perspective. Nat Rev Microbiol. 2012;10(12):841–51.
7. Ciofu O, Johansen HK, Aanaes K, et al. P. aeruginosa in the paranasal sinuses and transplanted lungs have similar adaptive mutations as isolates from chronically infected CF lungs. J Cyst Fibros. 2013;12(6):729–36.
8. Aanaes K. Bacterial sinusitis can be a focus for initial lung colonisation and chronic lung infection in patients with cystic fibrosis. J Cyst Fibros. 2013;12(SUPPL.2):S1–20.
9. Boutin S, Graeber SY, Weitnauer M, et al. Comparison of microbiomes from different niches of upper and lower airways in children and adolescents with cystic fibrosis. PLoS One. 2015;10(1):1–19.
10. Lynch S V., Bruce KD. The Cystic Fibrosis Airway Microbiome. Cold Spring Harb Perspect Med. 2013 Jul 12;3(3):a009738.
11. Vickery TW, Kofonow J, Ramakrishnan VR. Characterization of sinus microbiota by 16S sequencing from swabs. Methods Mol Biol. 2017; 1616:23–38.
12. Maliniak ML, Stecenko AA, McCarty NA. A longitudinal analysis of chronic MRSA and Pseudomonas aeruginosa co-infection in cystic fibrosis: A single-center study. J Cyst Fibros. 2016;15(3):350–6.

13. Bezerra TFP, Piccirillo JF, Fornazieri MA, et al. Cross-Cultural Adaptation and Validation of SNOT-20 in Portuguese. Int J Otolaryngol. 2011;2011:1–5.
14. Bezerra TFP, Padua FGM, Pilan RR d. M, Stewart MG, Voegels RL. Cross-cultural adaptation and validation of a quality of life questionnaire: The nasal obstruction symptom evaluation questionnaire. Rhinology. 2011;49(2):227–31.
15. Lund VJ, Kennedy DW. Staging for rhinosinusitis. Otolaryngol Head Neck Surg. 1997;117:S35–40.
16. Hammerschlag MR, Harding L, Macone A, Smith AL, Goldmann DA. Bacteriology of sputum in cystic fibrosis: Evaluation of dithiothreitol as a mucolytic agent. J Clin Microbiol. 1980;11(6):552–7.
17. Sinha R, Weissenburger-Moser LA, Clarke JL, et al. Short term dynamics of the sputum microbiome among COPD patients. PLoS One. 2018;13(3).
18. Klindworth A, Pruesse E, Schweer T, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 2013;41(1):1–11.
19. Caporaso JG, Kuczynski J, Stombaugh J, et al. correspondence QIIME allows analysis of high- throughput community sequencing data Intensity normalization improves color calling in SOLiD sequencing. Nat Publ Gr. 2010;7(5):335–6.
20. Cortez R V., Petry T, Caravatto P, et al. Shifts in intestinal microbiota after duodenal exclusion favor glycemic control and weight loss: a randomized controlled trial. Surg Obes Relat Dis. 2018;14(11):1748–54.
21. Dhariwal A, Chong J, Habib S, King IL, Agellon LB, Xia J. Microbiome Analyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Res. 2017;45(W1):W180–8.
22. Lozupone CA, Hamady M, Kelley ST, Knight R. Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. Appl Environ Microbiol. 2007;73(5):1576–85.
23. Allard G, Ryan FJ, Jeffery IB, Claesson

MJ. SPINGO: A rapid species-classifier for microbial amplicon sequences. BMC Bioinformatics. 2015;16(1):1–8.
24. Goddard AF, Staudinger BJ, Dowd SE, et al. Direct sampling of cystic fibrosis lungs indicates that DNA-based analyses of upper-airway specimens can misrepresent lung microbiota. Proc Natl Acad Sci. 2012;109(34):13769–74.
25. Hoggard M, Biswas K, Zoing M, Wagner Mackenzie B, Taylor MW, Douglas RG. Evidence of microbiota dysbiosis in chronic rhinosinusitis. Int Forum Allergy Rhinol. 2017;7(3):230–9.
26. Coburn B, Wang PW, Diaz Caballero J, et al. Lung microbiota across age and disease stage in cystic fibrosis. Sci Rep. 2015;5:1–12.
27. Fodor AA, Klem ER, Gilpin DF, et al. The adult cystic fibrosis airway microbiota is stable over time and infection type, and highly resilient to antibiotic treatment of exacerbations. PLoS One. 2012;7(9).
28. Paganin P, Fiscarelli EV, Tuccio V, et al. Changes in cystic fibrosis airway microbial community associated with a severe decline in lung function. PLoS One. 2015;10(4):1–19.
29. Aanaes K, Johansen HK, Skov M, et al. Clinical effects of sinus surgery and adjuvant therapy in cystic fibrosis patients - Can chronic lung infections be postponed? Rhinology. 2013;51(3):222–30.
30. Godoy JM, Godoy AN, Ribalta G, Largo I. Bacterial pattern in chronic sinusitis and cystic fibrosis. Otolaryngol - Head Neck Surg. 2011;145(4):673–6.
31. Hansen SK, Rau MH, Johansen HK, et al. Evolution and diversification of Pseudomonas aeruginosa in the paranasal sinuses of cystic fibrosis children have implications for chronic lung infection. ISME J. 2012;6(1):31–45.
32. Johansen HK, Aanaes K, Pressler T, et al. Colonisation and infection of the paranasal sinuses in cystic fibrosis patients is accompanied by a reduced PMN response. J Cyst Fibros. 2012;11(6):525–31.
33. Fokkens WJ, Lund VJ, Mullol J, et al. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. Rhinology.

2012 Suppl.23:1-299.

34. Pletcher SD, Goldberg AN, Cope EK. Loss of Microbial Niche Specificity Between the Upper and Lower Airways in Patients With Cystic Fibrosis. Laryngoscope. 2019;129(3):544–50.

35. Lucas SK, Yang R, Dunitz JM, Boyer HC, Hunter RC. 16S rRNA gene Sequencing Reveals Site-specific Signatures of the upper and lower airways of cystic fibrosis patients. J Cyst Fibros. 2018;17(2):204–12.

36. Ramakrishnan, V. R., Feaze, L. M., Gitomer, S. A., Ir, D., Robertson, C. E., & Frank, D. N. (2013). The microbiome of the middle meatus in healthy adults. PLoS ONE, 8(12).

37. Mahdavinia M, Keshavarzian A, Tobin MC, Landay A, Schleimer RP. A comprehensive review of the nasal microbiome in chronic rhinosinusitis (CRS). Clin Exp Allergy. 2016;46(1):21–41.

38. Mika M, Korten I, Qi W, et al. The nasal microbiota in infants with cystic fibrosis in the first year of life: a prospective cohort study. Lancet Respir Med. 2016;4(8):627–35.

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Supplementary Material

Table S1. Relative abundances of the principal genera in the middle meatus and sputum samples according to lung disease severity. The microbiome composition of middle meatus from patients with severe lung disease exhibited an increase of *Burkholderia-Paraburkholderia*, in comparison with those with mild to moderate lung disease (p=0,05). The microbiome composition of the sputum from patients with severe lung disease had a decrease of *Haemophilus*, compared to those with mild/moderate lung disease (p=0,05).

Genera		Middle meatus		Sputum	
		Severity of lung disease		Severity of lung disease	
		Mild to moderate	Severe	Mild to moderate	Severe
Pseudomonas	Range	0 – 95,3	0 –31,7	0 – 96,7	0 –98,2
	Mean	8,24	6,91	33,72	40,9
	SD*	26,22	11,18	39,21	42,06
	p a		0,76		0,63
Porphyromonas	Range	0 – 2,0	0 – 0,2	0 – 2,0	0 – 11,10
	Mean	0,31	0,04	1,03	2,13
	SD*	0,68	0,07	1,87	3,6
	p [a]		0,16		0,16
Prevotella	Range	0 -5,7	0 -12,8	0,1 -20,4	0,5 -62,6
	Mean	1,51	1,66	6,3	12,9
	SD*	1,85	4,18	7,17	17,12
	p [a]		0,7		0,18
Gemella	Range	0 – 3,90	0 – 3,0	0 – 4,70	0 – 5,90
	Mean	0,9	0,88	0,72	1,51
	SD*	1,22	1,12	1,29	1,96
	p [a]		0,98		0,2
Staphylococcus	Range	0 – 76,3	0,1– 85,4	0 – 26,10	0– 24,20
	Mean	19,76	22,48	3,6	3,1
	SD*	25,13	32,42	7,46	6,8
	p [a]		0,81		0,85
Streptococcus	Range	0 – 98,3	0,5 – 99,5	0,1 – 31,0	0 – 28,8
	Mean	18,46	27,91	5,04	7,06
	SD*	26,35	39,45	8,57	9,15
	p [a]		0,47		0,54
Veillonella	Range	0 – 6,0	0 – 14,8	0,1 – 16,40	0 – 41,2
	Mean	1,59	2,36	5,5	10,2
	SD*	1,8	4,77	5,86	12,57
	p [a]		0,57		0,2
Fusobacterium	Range	0 – 8,40	0 – 3,70	0 – 7,30	0 – 42,7
	Mean	1,13	0,76	1,4	7,06
	SD*	2,4	1,31	2,24	12,87
	p [a]		0,65		0,1
Burkholderia-Paraburkholderia	Range	0 – 0,1	0 – 2,30	0 – 94,0	0 – 1,0
	Mean	0,007	0,37	14,36	0,07
	SD*	0,02	0,74	34,92	0,27
	p [a]		0,05*		0,12

| Genera		Middle meatus		Sputum	
		Severity of lung disease		Severity of lung disease	
		Mild to moderate	Severe	Mild to moderate	Severe
Neisseria	Range	0 – 4,40	0 – 2,32	0 – 13,60	0 – 9,50
	Mean	0,66	0,46	3,8	2,06
	SD*	1,2	0,72	4,98	3,07
	p ᵃ		0,65		0,26
Haemophilus	Range	0 – 90,2	0 – 5,20	0,1 – 95,8	0,1 – 8,20
	Mean	8	1,18	17,13	2,25
	SD*	24,74	1,75	28,37	2,49
	p ᵃ		0,39		0,05*
Moraxella	Range	0 -95,8	0 – 4,0	0 - 0	0 – 0,1
	Mean	7,36	0,51	0	0,01
	SD*	26,57	1,32	0	0,03
	p ᵃ		0,42		0,12
Pseudomonas	Range	0,1 – 34,8	0 – 24,9	0 – 0,1	0 – 0,1
	Mean	10	6,65	0,01	0,007
	SD*	12,61	8,58	0,03	0,02
	p ᵃ		0,46		0,53
Stenotrophomonas	Range	0 – 0,1	0 – 39,5	0 – 0	0 – 10,60
	Mean	0,007	4,95	0	0,86
	SD*	0,02	13,03	0	2,92
	p ᵃ		0,14		0,26

SD*: standard deviation; p ᵃ: p-value
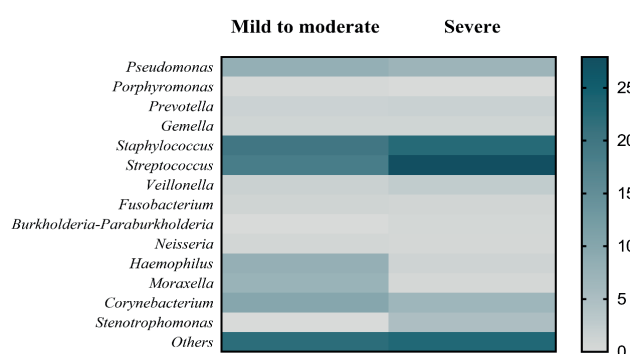
Figure S1. Heat map of bacterial genera in middle meatus according to lung disease severity. Patients with severe lung disease exhibited a greater abundance of *Staphylococcus*, *Streptococcus* and *Stenotrophomonas*, and a smaller abundance of *Pseudomonas*, *Haemophilus* and *Corynebacterium*, in comparison with those with mild to moderate lung disease.
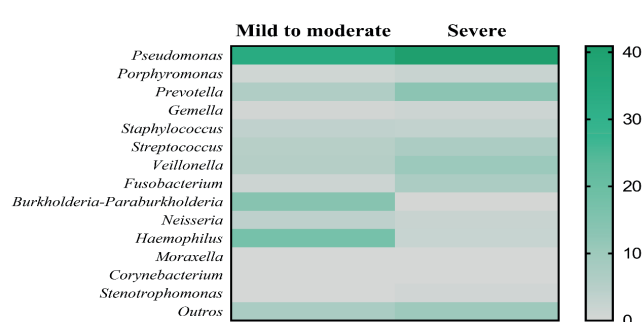

Figure S2. Heat map of bacterial genera in sputum according to lung disease severity. Patients with severe lung disease had a higher proportion of *Pseudomonas*, *Prevotella*, *Streptococcus* and *Veillonella* and a smaller proportion of *Haemophilus*, *Neisseria* and *Burkholderia-Paraburkholderia* compared to those with mild/moderate lung disease.
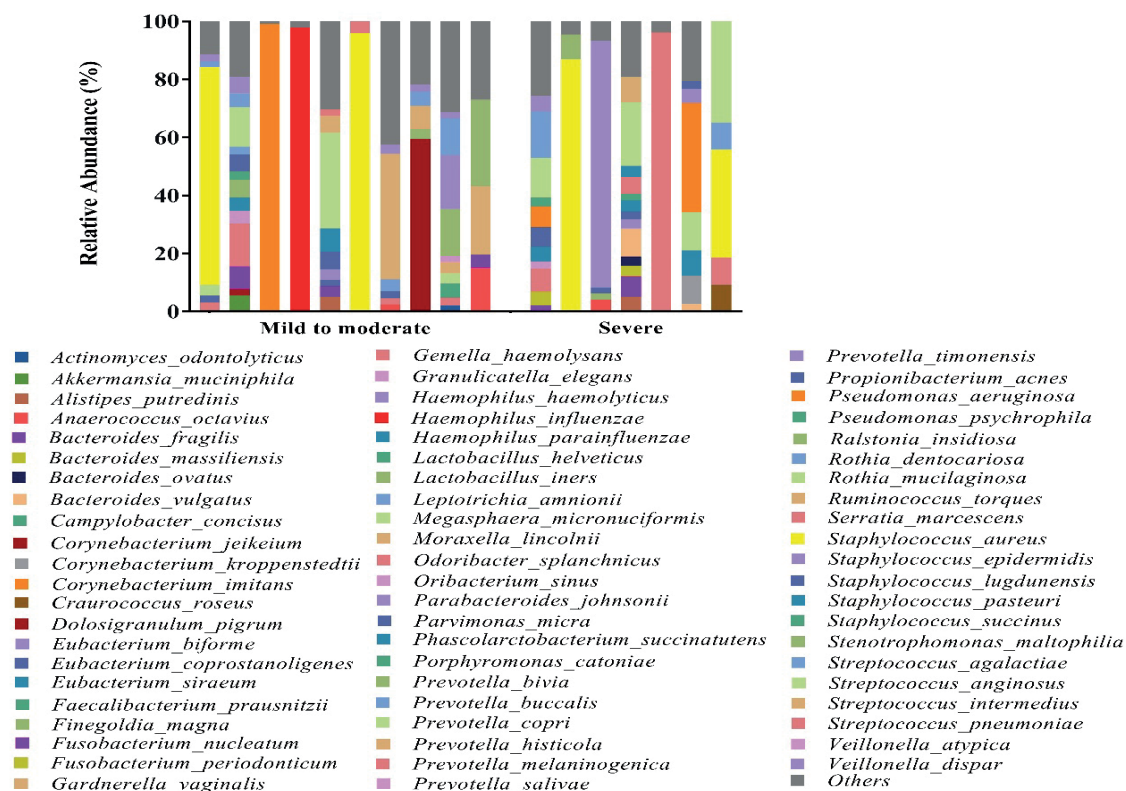
Figure S3. Main bacterial species found in middle meatus samples in each patient. *Pseudomonas aeruginosa, Staphylococcus aureus* and *Fusobacterium nucleatum* were present in both disease state.
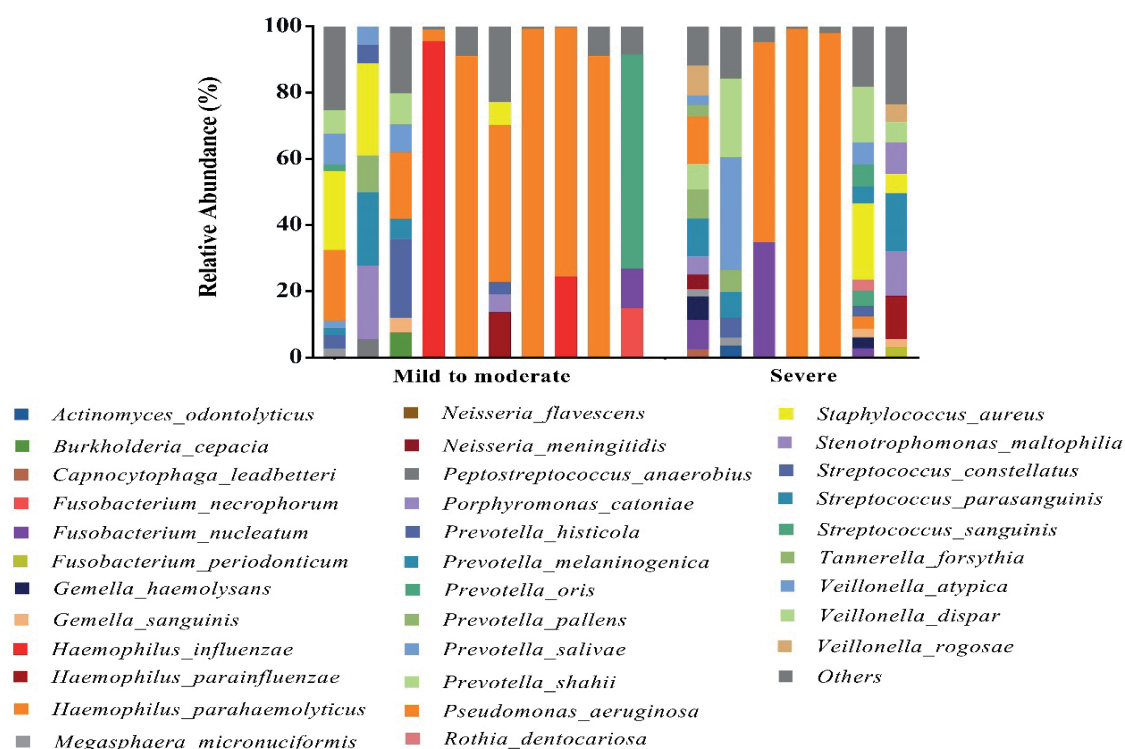

Figure S4. Main bacterial species found in sputum samples in each patient. *Pseudomonas aeruginosa* were present in 80% of sample of mild/moderate lung disease and in 57% of samples of severe disease.
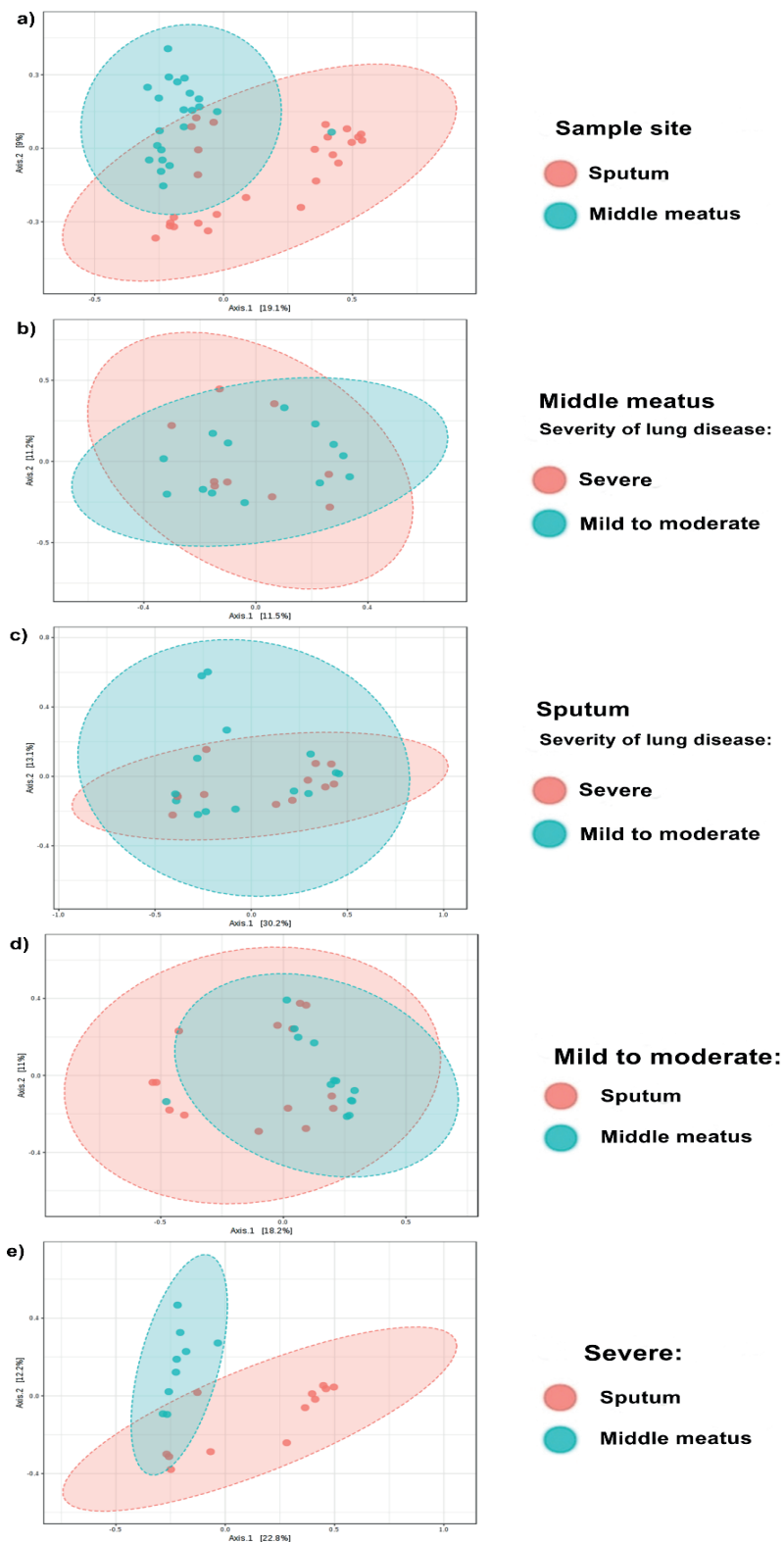
Figure S5. PCoA Analyzes of group similarities (ANOSIM) plots of Bray-Curtis distances between microbial communities from a) middle meatus and sputum samples (R: 0.39773; p<0.001), b) middle meatus samples from patients with mild to moderate and severe disease (R: -0.091768; p=0.935), c) sputum samples from patients with mild to moderate and severe disease (R: -0.0028814; p=0.425), d) middle meatus and sputum samples from patients with mild to moderate disease (R: 0.32154; p<0.001) and e) middle meatus and sputum samples from patients with severe disease (R: 0.4606; p<0.001). The two sites showed different community diversity (a). This was also observed when analysed separately by severity of lung disease (d and e).