Lower airway microbiota and decreasing lung function in young Brazilian cystic fibrosis patients with pulmonary Staphylococcus and Pseudomonas infection

Paulo Kussek¹, Dany Mesa²,³*, Thaïs Muniz Vasconcelos³, Luiza Souza Rodrigues³, Damaris Krul³, Humberto Ibanez², Helisson Faoro⁴, Jussara Kasuko Palmeiro⁵, Libera Maria Dalla Costa³*

¹Hospital Pequeno Príncipe, Curitiba, Paraná, Brazil, ²Big Data Center, Instituto de Pesquisa Pelé Pequeno Príncipe, Curitiba, Paraná, Brazil, ³Instituto de Pesquisa Pelé Pequeno Príncipe, Curitiba, Paraná, Brazil, ⁴Instituto Carlos Chagas, Curitiba, Paraná, Brazil, ⁵Departamento de Análises Clínicas, Centro de Ciências da Saúde, Universidade Federal de Santa Catarina, Florianópolis, Santa Catarina, Brazil

* lmdallacosta@gmail.com (LMDC); dmesaf7@gmail.com (DM)

Abstract

Cystic fibrosis (CF) is a genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator gene that leads to respiratory complications and mortality. Studies have shown shifts in the respiratory microbiota during disease progression in individuals with CF. In addition, CF patients experience short cycles of acute intermittent aggravations of symptoms called pulmonary exacerbations, which may be characterized by a decrease in lung function and weight loss. The resident microbiota become imbalanced, promoting biofilm formation, and reducing the effectiveness of therapy. The aim of this study was to monitor patients aged 8–23 years with CF to evaluate their lower respiratory microbiota using 16S rRNA sequencing. The most predominant pathogens observed in microbiota, Staphylococcus (Staph) and Pseudomonas (Pseud) were correlated with clinical variables, and the in vitro capacity of biofilm formation for these pathogens was tested. A group of 34 patients was followed up for 84 days, and 306 sputum samples were collected and sequenced. Clustering of microbiota by predominant pathogen showed that children with more Staph had reduced forced expiratory volume in one second (FEV₁) and forced vital capacity (FVC) compared to children with Pseud. Furthermore, the patients’ clinical condition was consistent with the results of pulmonary function. More patients with pulmonary exacerbation were observed in the Staph group than in the Pseud group, as confirmed by lower body mass index and pulmonary function. Additionally, prediction of bacterial functional profiles identified genes encoding key enzymes involved in virulence pathways in the Pseud group. Importantly, this study is the first Brazilian study to assess the lower respiratory microbiota in a significant group of young CF patients. In this sense, the data collected for this study on the microbiota of children in Brazil with CF provide a valuable contribution to the knowledge in the field.
Introduction

Cystic fibrosis (CF) is the most common life-shortening rare disease with an estimated incidence of 1 in every 6000 live births in Euro-Brazilians and 1/14000 in Afro-Brazilians [1]. The disease is caused by mutations in the CF transmembrane conductance regulator gene (CFTR), and the homozygous F508del is present in approximately 48% of all CF alleles [2]. Complications of CF disease begin in early life and over time, a combination of impaired mucociliary clearance, innate immune responses, inflammatory pulmonary process, chronic infection leads to bronchiectasis and respiratory failure [3].

The microbiota of the respiratory tract is recognized as an essential factor in the homeostasis of the respiratory system [4]. The respiratory microbiota is linked to progressive CF lung disease depending on many factors such as the time of diagnosis, patient age, chronic use of antibiotics, and mutation type of the CFTR gene [5]. Thus, the establishment of a community composed mainly of typical CF pathogens with other agents such as anaerobic bacteria, fungi, and viruses may cause dysbiosis of the respiratory system [5]. In addition, the pathophysiology of CF affects the respiratory microbiota, with the formation of biofilm and mucus plugging, making the pulmonary distal airways inaccessible by treatment agents [6].

A wide variety of bacterial species can be identified from patients with CF; the most frequently observed include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Burkholderia cepacia* complex [7, 8]. Other opportunistic bacterial species that are less frequently detected in CF patients include *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, *Ralstonia* spp., *Pandoraea* spp., *Cupriavidus* spp., and non-tuberculosis mycobacteria [9, 10]. In addition, CF patients experience short cycles of acute intermittent aggravations of symptoms called pulmonary exacerbations, characterized by a decrease in lung function and weight loss, generally caused by opportunistic pathogens which can promote biofilm formation and reduce the effectiveness of therapy [11].

Nevertheless, the composition of respiratory microbiota varies noticeably among individuals; some patients show marked changes in the bacterial community with alternating infectious agents, and others show community resilience [3]. We analyzed the microbiota of 306 sputum samples of patients with CF and evaluated correlations with clinical variables (mutation type and patient’s clinical status). In addition, we grouped CF patients in two groups by the dominant respiratory microbiota pathogens, Staph and Pseud, and analyzed these groups by their clinical variables using Pearson’s correlation analysis and non-metric multidimensional scaling. Furthermore, bacterial functional profiles were predicted for both pathogen groups.

Material and methods

Study setting

This study was performed at the Pequeno Príncipe Hospital, the largest pediatric hospital in Brazil. Currently, 390 pediatric beds are available in 32 pediatric specializations. The CF unit includes 80 pediatric patients who are followed until they are transferred to an adult unit.

Study population and clinical data

In this study, a group of 34 CF patients aged between 8 and 23 years was followed for 84 d (Fig 1). Patients were diagnosed by a sweat test and CFTR gene screening. After each regularly scheduled clinic visit or hospital admission, clinical data including the use of broad- and narrow-spectrum antibiotics, body mass index (BMI), and lung function parameters such as forced expiratory volume in one second (FEV₁) and forced vital capacity (FVC) were collected. Lung function was assessed using a JAEGGER MasterScope® Spirometer (VIASYS Healthcare...
GmbH, Hoechberg, Germany) following the standardization of pulmonary function test (PFT) by ATS/ERS Task Force [12]. Patients with O\textsubscript{2} saturation $\geq$88% in room air and capacity to perform PFT were included. The clinical conditions were categorized as baseline, exacerbation, treatment, and recovery [10]. Baseline condition: no acute respiratory symptoms and no systemic antibiotic use for $>30$ d; patients may or may not be on maintenance antibiotics such as azithromycin or inhaled antibiotics. Exacerbation condition: the initiation of acute respiratory symptoms and use of antibiotics (oral or intravenous). Treatment condition: the use of intravenous or oral antibiotics for more than 7 d for pulmonary exacerbation treatment. Recovery: no systemic antibiotic use for $>7$ d; patients may or may not be on maintenance antibiotics and may or may not be back to the baseline clinical condition.

**Ethics statement**
The Institutional Review Board (IRB) of the participating center (IRB #2.405.167) approved this study, and informed consent was obtained from the parents or guardians of all participants. Research was conducted in a manner to ensure the confidentiality for each patient.

**Sample collection, processing, bacterial culture, and identification**
A triplicate of sputum samples of the patients were collected on days 0, 42, and 84 (a total of 306 samples). Inhalation of hypertonic sterile saline solution (7%) by nebulization was used for sputum collection, followed by coughing and expectoration of airway secretions. Sputum characteristics ranged from salivary to purulent. The collected sputum samples were transported to the microbiology laboratory for processing within 2 h. Sputum samples were transferred to 15 mL graduated Falcon tubes, free of DNase and RNase, and sterile phosphate-buffered saline was added to bring the total volume to 8 mL. After homogenization, 2 mL of purulent sputum was transferred to new tubes and treated with $\beta$-mercaptoethanol and DNase I (Sigma-Aldrich, St. Louis, United States) to remove proteins and other soluble DNA, such as mitochondrial DNA [13]. The obtained pellet after treatment of all purulent samples and 1 mL aliquots of saliva samples were stored in a freezer at -80$^\circ$C until DNA extraction. The remaining volume in the initial Falcon tubes was sent to the microbiology laboratory for bacterial culture identification [14]. *S. aureus* and *P. aeruginosa* isolated from sputum samples and identified by matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF MS) using a MicroflexTM LT instrument (Bruker Daltonics, Billerica, MA, USA) were stored at -80$^\circ$C in...
brain heart infusion broth (HIMEDIA, Mumbai, Maharashtra, India) with 20% (v/v) glycerol for further analysis [15].

**Phenotypic biofilm production detection**

Qualitative biofilm production was performed using the tube method previously described [16]. A loop of microorganisms collected from tryptone soya agar (OXOID, Basingstoke, Hampshire, England) was inoculated into a polystyrene tube (15 mL Falcon tube) containing 10 mL of tryptone soy broth (OXOID, Basingstoke, Hampshire, England) supplemented with glucose (final concentration of 8%). Tubes were incubated at 35 ± 2˚C for 24 h, and the broth was gently aspirated. The tubes were washed thoroughly with phosphate-buffered saline (pH 7.2) and dried. Cells in the dried tubes were stained with 0.1% crystal violet for 7 min, and excess dye was removed by washing the cells with distilled water [16]. After drying, the tubes were visually evaluated for biofilm formation (presence or absence). Biofilm formation was considered positive when a visible film coated the wall and bottom of the tube. The experiments were performed in duplicate, and biofilm production was evaluated independently by two different observers. The sterile tryptone soy broth supplemented with glucose and the non-biofilm producer *Candida albicans* were used as a negative control and the biofilm producer *Candida tropicalis* was used as a positive control in the assay.

**DNA extraction and 16S rRNA amplicon sequencing**

All samples (frozen pellet and saliva) were kept on ice until they were completely thawed when subjected to genomic DNA extraction. A volume of 750 μL of lysis buffer was added to the pellet of each purulent sample and homogenized until the pellet was dissolved. The same volume of lysis buffer (750 μL) was added to 250 μL of each saliva sample. The total volume of each mixture was transferred to a ZR BashingBead® Lysis tube (Zymo Research, CA, USA) for DNA extraction. DNA extraction was performed using the ZymoBIOMICS® DNA Miniprep Kit (Zymo Research, CA, USA), according to the manufacturer’s recommendations. The purity and quality of the DNA were verified using a NanoVue Plus spectrophotometer (GE Healthcare, Life Sciences, Marlborough, MA, USA). Subsequently, DNA was stored at -80˚C.

Polymerase chain reaction (PCR) and universal primers (F515/R806) were used to amplify the V4 region of the 16S rRNA gene [17]. PCR consisted of 2.5 μL bovine serum albumin (3 mg/mL), 2.5 μL high-fidelity buffer (10x), 0.63 μL MgCl₂ (50 mM), 0.50 μL of dNTPs (10 mM), 0.625 μL primer mix (10 mM), 0.125 μL high-fidelity Taq polymerase (5 U/μL), 10 ng of template DNA, and 16.12 μL ultrapure water added to obtain a volume of 25 μL. The reaction conditions were as follows: 5 min at 95˚C, 25 cycles of 40 s at 95˚C, 2 min at 64˚C, 1 min at 72˚C, and 10 min at 72˚C. The amplicons were quantified with Qubit using an HS dsDNA kit (Invitrogen, Carlsbad, CA, USA), diluted to 500 pM, and pooled. Next, 16 pM of pooled DNA was sequenced using the MiSeq reagent 600V3 (llumina, San Diego, CA, USA). Sequencing was performed using a MiSeq® sequencer (Illumina) to obtain paired reads of 250 bp [18]. A negative control for sequencing was used to check contamination.

**Sequencing data and statistical analysis**

Sequencing data were analyzed using the QIIME2 Core 2021.8 pipeline [19]. Triplicate paired reads of the same collection of patients were joined in a single file (total of 102 samples). Next, the merged samples were filtered by quality, chimeras were removed and clustered into amplicon sequence variants using the DADA2 algorithm [20] in the QIIME2 program. Subsequently, taxonomic assignment was performed using the SILVA database, release 138 [21].
The reads output was normalized to 51,000 per sample, allowing a comparison of alpha and beta diversity between the groups.

Analysis of microbiota by the time of collection and clinical and genetic variables was compared using Welch’s t-test ($P < 0.05$) and Bonferroni correction in STAMP software. Next, the abundance of bacterial taxa was compared for different pathogen groups using Welch’s t-test ($P < 0.05$) and Bonferroni correction in STAMP software [22]. Clinical variables were analyzed using the Kruskal–Wallis and Mann-Whitney test ($P < 0.05$). Pearson’s correlation coefficients and non-metric multidimensional scaling (NMDS) plots were calculated using the psych and vegan packages included in R software [23], and functional profiles of Staph and Pseud were obtained using the Tax4Fun program [24]. Only statistically significant results were reported ($P < 0.05$).

**Data accessibility**

The dataset was submitted to the National Center for Biotechnology Information (NCBI) database under the BioSample accession code SAMN19760689.

**Results**

**Study population and clinical data**

Of the 34 study participants, sputum samples were collected on days 0, 42, and 84 (a total of 102 samples), 17 were male, with an average age of 15.3 years (range of 8–23 years), and 31 had an early diagnosis before age 2. All the patients underwent PFT (spirometry) with a wide range of the impaired pulmonary function with FVC ranging from 31% to 152% (mean = 84.3%) and FEV1 from 26% to 157% (mean = 75.9%). As for the nutritional status (BMI and Z score), the most repeated value in three collections was sought: 10 patients were considered eutrophic [25], 16 patients had grade I malnutrition, 8 patients had grade II malnutrition, and 2 patients had grade III malnutrition [26]; however, 12 individuals had score differences between collections for a higher or lower standard deviation according to their clinical condition at the time. Of the 34 patients, 26 had a negative Z-score (mean = -0.59), and 32 patients had pancreatic insufficiency. *S. aureus* was the most frequent microorganism (47%) identified by the culture-based methods, followed by *P. aeruginosa* (16%); both microorganisms were present in association from different culture media (25%), and in negative culture (14%). The metadata content of the clinical variables of the patients enrolled in this study is shown in supporting information ([S1 Table](#)).

**Phenotypic biofilm production detection**

Sputum samples that showed Staph and Pseud with relative abundance >50% in the 16S rRNA sequencing were stored in -80°C for further biofilm production (total 40 samples, 20 samples of each pathogen). Therefore, a total of 13 *S. aureus* and 10 *P. aeruginosa* strains were evaluated for their biofilm production capacity. All of these were in vitro biofilm producers. Only the dichotomous analysis, presence or absence, was used, considering that by visual analysis the interpretation of intensity (+/+ and ++++) can be subjective, especially without specific controls for each of these categories.

**Taxonomic classification of sputum microbiota**

This section presents the results of the analysis carried out at the genera and species levels. The analysis of the bacterial community at different collection times (days 0, 42, and 84) revealed 226 taxa, distributed among 178 genera and 48 species. The most abundant genera in the...
community were Veillonella, Prevotella, Haemophilus, Pseudomonas, Staphylococcus, Streptococcus, Serratia, Neisseria, and Porphyromonas (S1 Fig). A total of 155 taxa represented the core microbiota of the community on days 0, 42, and 84; these taxa were identified at all sampling periods and in all samples (S2 Fig).

Analyses of microbiota considering the time of collection, clinical variables or mutation did not identify any specific pattern in the lower respiratory microbiota of CF patients associated with the different clinical conditions. Thus, considering this result, the samples were clustered into two main groups Staph and Pseud, based on culture results and the most abundant pathogens identified in 16S rRNA sequencing (abundance >50%). Few samples with great abundance for other genera such as Veillonella and Haemophilus, among others were observed. Owing to the low number of samples, other microorganisms were not analyzed. The clinical variables of the patients were correlated with these two pathogen groups. Beta diversity analysis showed an adequate clustering of groups by pathogen type, which was reflected in the results of the principal component analysis (PCA), which highlighted that each group had a dominant organism, Staph or Pseud (Fig 2).

Correlations among clinical variables and microbiota

We analyzed the clinical data by grouping patients according to the type of predominant pathogen in the sputum microbiota (the selection criterion was patients with pathogen abundance >50% in microbiota). We identified two dominant groups, Staph and Pseud, with a genetic mutation frequency of F508del in 67% of alleles, followed by G542x in 20%, 1078delT in 6%, and others (R334W and 2184delA) in 7% of alleles. The analyses revealed the following results:
The BMI was obtained by comparing patients of the same age; the BMI values were 18.09 and 19.41 for the Staph and Pseud groups, respectively. In other words, the patients in the Staph group were underweight (BMI < 18.5 kg/m² is considered underweight). On the other hand, the patients in the Pseud group had normal weight (BMI = 18.5–25.0). All patients in the Pseud group (except one patient) received antimicrobial treatment against *Pseudomonas* spp., in other words, chronically inhaled antibiotic to reduce bacterial growth and the frequency of exacerbations. In addition, we chose BMI values because this parameter had positive values. Besides, BMI Z-scores had negative values, which made it difficult to use Pearson’s correlation analysis or any other statistical analysis.

The clinical condition of the patients was as follows: in the Staph group, nine patients were classified in the baseline clinical condition, five in the exacerbated, six in the treatment, and one in the recovery group. In the Pseud group, 11 patients were classified in the baseline clinical condition, one in exacerbated, and five in the treatment group. Due to chronic lung infection, most CF patients had reduced lung function with significant differences (*P* < 0.05) between the two groups; in general, the Staph group had reduced FEV₁ and FVC compared to the Pseud group (Fig 3).

**Pearson’s correlation analysis between clinical variables and microbiota**

Pearson’s correlation coefficient showed significant relationship (*P* < 0.05) between microbiota and clinical variables. In the Staph group, there were significant negative correlations between the variables (FVC and FEV) and prevalence of Staph and *S. aureus* (Fig 4). In contrast, positive correlations were observed between clinical variables (BMI and age; FVC and FEV). Similarly, positive correlations were observed between the pathogens Staph and *S. aureus*. These results highlight the veracity of the correlation matrix. On the other hand, in the Pseud group, there was no significant positive or negative relationship between microbiota and clinical variables.

**Non-metric multidimensional scaling (NMDS)**

To represent the behavior of the variables in a multivariate system, we used an NMDS plot. This analysis reveals the pairwise dissimilarity between objects in a two-dimensional space, in this case, microbiota and clinical variables (plotted as vectors). NMDS results confirmed the clustering of the pathogens in two groups with different taxonomic compositions. The first
group, the Staph group, had higher abundance of Staph and *S. aureus*, and the second group, the Pseud group, had higher abundance of Pseud and *P. aeruginosa* (Fig 5). In addition, vectors in the plot representing the clinical variables of patients were in the opposite direction to the Staph group, showing an inverse correlation of these parameters with bacteria present in high quantities in this group (Figs 4 and 5).

**Prediction of bacterial functional profiles**

Functional profiles were predicted from the 16S rRNA data obtained using the software package Tax4Fun. The aim of this analysis was to highlight the different profiles among pathogens groups in an unbiased manner. The complete profiles are shown in the Supporting Information (S2 Table). Genes encoding key enzymes involved in virulence pathways were identified in the resulting profiles using their KEGG orthologs (Table 1). Thus, key genes related to antibiotic resistance were identified in the Staph group, such as: K01467, beta-lactamase; K03327, multidrug resistance protein, MATE family; K08218, MFS transporter-PAT family, beta-lactamase induction signal transducer AmpG, and genes related to horizontal gene transfer, such as: K07481, transposase, IS5 family; K07485, transposase; and K07489, transposase. In the Pseud group, key genes related to secretion systems were: K02456, general secretion pathway protein G; K02459, general secretion pathway protein J; K03195, type IV secretion system protein VirB10; K07344, type IV secretion system protein TrbL; K11891, type VI secretion system protein ImpL; K11896, type VI secretion system protein ImpG; and a gene related to biofilm synthesis: K11937, biofilm PGA synthesis protein PgaD (Table 1).

**Discussion**

The bacterial taxa detected in the present study are in agreement with previous studies [27, 28]. Khanolkar et al. [29] and Raghuvanshi et al. [30] showed shifts in the composition of
respiratory microbiota in patients with CF, such as the enrichment of *Staphylococcus* spp., *Haemophilus* spp., *Pseudomonas* spp., *Streptococcus* spp., *Serratia* spp., *Neisseria* spp., and *Porphyromonas* spp. We classified patients into two groups based on the dominant pathogens observed in the respiratory microbiota: the Staph and Pseud groups. These taxa are consistent with the results obtained from microbiological cultures, clinical practice, and scientific articles on CF, especially in our target age range [31]. In older patients, other bacteria, such as *Burkholderia* spp., and some emerging bacteria, such as *Stenotrophomonas* spp. and *Acinetobacter* spp. are observed [32].

In Brazil, the diagnosis of CF follows the Brazilian guidelines for the diagnosis and treatment of cystic fibrosis [33]. Thus, the algorithm of newborn screening for cystic fibrosis used in Brazil is based on two tests of immunoreactive trypsinogen levels, the second of which is performed within 30 days of life. If screening is positive (i.e., two positive tests), sweat testing is performed to confirm or rule out cystic fibrosis. Sweat chloride concentrations ≥ 60 mmol/L, as measured by quantitative methods, in two samples, confirm the diagnosis. Diagnostic alternatives are detection of two cystic fibrosis-related mutations and CFTR functional tests.

Children in the Staph group showed lower BMI than the Pseud group (18.09 versus 19.41); that is, children in the Staph group were underweight. Overall, children and young patients infected with *Pseudomonas* usually have a lower BMI than children infected with genus *Staphylococcus*, but this does not occur when the patient is already being monitored and receiving medication; in Pseud patients, treatment includes chronic use of inhaled antibiotics, such as tobramycin (TOBI®) and azithromycin, which have immunomodulatory and antiviral effects [34]. However, it is not common to use prophylactic treatment in patients with chronic infection by *Staphylococcus* spp.; those patients use “off label” antibiotics [35]. This could explain...
the decreased BMI in patients with *Staphylococcus* spp.; however, this information should be used with caution because *S. aureus* is more prevalent at an earlier age and there are no reliable tools to measure lung function in children under six years of age.

Our results showed that patients’ clinical condition agreed with the results of pulmonary function (FVC and FEV₁). In clinical practice, this observation reflects the definition of disease exacerbation, which means worsening of symptoms, changes in sputum color, loss or cessation of weight gain, and worsening of lung function [10]. Greater patient numbers were observed in the Staph exacerbation group than in the Pseud group, a finding that was confirmed by the lower values of BMI and pulmonary function. Limoli *et al.* [36] observed that co-infection with *S. aureus* and *P. aeruginosa* was associated with decreased lung function and increased numbers of pulmonary exacerbations. Polymicrobial dynamics may be a better indicator of CF patient outcomes, as opposed to the presence of a single pathogen [37, 38]. Pearson’s correlation analysis revealed significant relationships between the Staph group and parameters of pulmonary function (FVC and FEV₁).

In this study, biofilm formation capacity was observed in *S. aureus* and *P. aeruginosa* isolates. Thus, there is increasing evidence that biofilm-mediated infections facilitate the

---

**Table 1. Prediction of bacterial functional profiles.**

| KEGG functions                                              | Staphylococcus group                                                                 |
|-------------------------------------------------------------|-------------------------------------------------------------------------------------|
| K01467; beta-lactamase                                       |                                                                                     |
| K02028; polar amino acid transport system ATP-binding protein |                                                                                     |
| K02029; polar amino acid transport system permease protein   |                                                                                     |
| K02030; polar amino acid transport system substrate-binding protein |                                                                                     |
| K03327; multidrug resistance protein, MATE family           |                                                                                     |
| K06994; putative drug exporter of the RND superfamily        |                                                                                     |
| K07481; transposase, ISS family                              |                                                                                     |
| K07485; transposase                                         |                                                                                     |
| K07489; transposase                                         |                                                                                     |
| K07668; two-component system, OmpR family, response regulator VicR |                                                                                     |
| K08138; MFS transporter, SP family, xylose: H⁺ symporter    |                                                                                     |
| K08191; MFS transporter, ACS family, hexuronate transporter  |                                                                                     |
| K08218; MFS transporter, PAT family, beta-lactamase induction signal transducer AmpG |                                                                                     |
| K11068; hemolysin III                                       |                                                                                     |
| K11070; spermidine/putrescine transport system permease protein |                                                                                     |
| K11071; spermidine/putrescine transport system permease protein |                                                                                     |
| K15342; CRISP-associated protein Cas1                       |                                                                                     |

| Pseudomonas group                                                                                          |
|-------------------------------------------------------------|-------------------------------------------------------------------------------------|
| K02456; general secretion pathway protein G                 |                                                                                     |
| K02459; general secretion pathway protein J                 |                                                                                     |
| K02657; twitching motility two-component system response regulator PilG                                    |                                                                                     |
| K03195; type IV secretion system protein VirB10              |                                                                                     |
| K03808; paraquat-inducible protein A                        |                                                                                     |
| K07344; type IV secretion system protein TrbL               |                                                                                     |
| K11891; type VI secretion system protein ImpL                |                                                                                     |
| K11896; type VI secretion system protein ImpG                |                                                                                     |
| K11937; biofilm PGA synthesis protein Pgd                    |                                                                                     |
| K12516; putative surface-exposed virulence protein           |                                                                                     |
| K13735; adhesin/invasin                                      |                                                                                     |

https://doi.org/10.1371/journal.pone.0273453.t001
development of chronic infectious diseases and recurrent infections [39]. Biofilms are often considered a survival strategy for bacteria, which are facilitated by numerous factors in CF lungs, including mucus accumulation [11]. Previous studies have suggested that antibiotic resistance of bacteria in CF lungs is due to biofilm formation [40]. In addition, multiple species of lung biofilm producers such as Pseud in CF patients are affected by specific treatments; thus, competitiveness among different species is harmful, promoting the survival of the most abundant pathogen [41]. However, the clinical significance of in vitro biofilm production remains unclear and biofilm detection by laboratory techniques does not necessarily indicate in vivo production because biofilms are a community of multiple bacterial species that coexist in a specific environment [42].

Functional inference of communities showed that the presence of key genes in each pathogen group was possible because of the low biodiversity of each group, which was dominated by a single bacterial genus (Staph or Pseud). Thus, in the Staph group, a functional profile determined by antimicrobial resistance genes was observed. In the case of our isolates, this resistance profile was not identified, and all S. aureus isolates were sensitive to oxacillin and vancomycin. As in Voronina et al. [32], the presence of the mecA gene in sputum samples from pediatric patients with CF was not identified in this study; the mecA gene confers resistance to methicillin in S. aureus strains. The in silico inference profile is based on genomes deposited in the database; thus, these genomes may represent strains that carry genes of antibiotic resistance, and the result will depend on the database used as a comparison [43]. In the Pseud group, a dominant profile by secretion systems was identified which is expected because gram-negative bacteria carry several of these systems [44].

There are some limitations in this study. First, the cohort size is too small and heterogeneous. However, it represents the largest pediatric hospital in Brazil, and thus is an interesting clinical cohort from Brazil. In addition, this work is novel, as the only published work on the respiratory microbiota of Brazilian CF patients was recently published by Vasco et al. [45], where the authors evaluated the microbiota of 10 children under 6 years old with pancreatic insufficiency who underwent pancreatic enzyme replacement therapy with Creon®. In this sense, this preliminary work is totally different from ours.

The second limitation is regarding the over-simplification of the microbiota data. Initially, we used longitudinal data and triplicates, but this information was not used at all in the manuscript. This information would be relevant to answer important questions such as the longitudinal relationship between microbiome and lung function or the heterogeneity of sputum at a single timepoint. However, analyses of microbiota by the time of collection or by related clinical variables did not identify any specific microbiota pattern from the respiratory tract of the patients. Thus, considering this result, the microbiota was clustered into two main groups, Staph and Pseud. Microbiota samples with abundance >50% of either Staphylococcus or Pseudomonas were further analyzed. This grouping parameter may seem to skew the data to the expected result. However, this approach, using a cutoff in the data, yielded results that had not been observed in previous studies. Using a cutoff in the data allows the creation of a reference for future studies, and, importantly, this approach helped us to better understand the relationship between opportunistic microbiota pathogens and lung function.

Finally, in patients with CF, the composition of respiratory microbiota varies noticeably between individuals; some patients show marked changes in the bacterial community with alternating infectious agents. Besides, different types of CF mutation did not show unique microbiota. Thus, some points require further research: (i) How can we promptly identify the agent in patients with acute exacerbation with negative classical culture? (ii) How can we make better use of next-generation sequencing and other techniques to identify low-abundance microorganisms that are likely to be responsible for exacerbation in patients?
Supporting information

S1 Table. Clinical variables of the patients enrolled in this study. (XLSX)

S2 Table. Complete prediction profiles. (XLSX)

S1 Fig. Relative frequency of the most abundant genera of the bacterial community by data collection. (A) day 0; (B) day 42; and (C) day 84 of collection. (EPS)

S2 Fig. Venn diagram representing the core microbiota of the community by sampling time. 155 taxa were identified as the core microbiota. (EPS)

Acknowledgments

We thank GoGenetic for amplicon sequencing. We would like to thank Editage (www.editage.com) for English language editing.

Author Contributions

Conceptualization: Paulo Kussek, Helisson Faoro, Jussara Kasuko Palmeiro, Libera Maria Dalla Costa.

Data curation: Dany Mesa, Humberto Ibanez.

Formal analysis: Dany Mesa, Damaris Krul.

Funding acquisition: Libera Maria Dalla Costa.

Investigation: Damaris Krul.

Methodology: Thaís Muniz Vasconcelos, Luiza Souza Rodrigues.

Project administration: Libera Maria Dalla Costa.

Writing – original draft: Dany Mesa.

Writing – review & editing: Dany Mesa, Luiza Souza Rodrigues, Helisson Faoro, Jussara Kasuko Palmeiro, Libera Maria Dalla Costa.

References

1. Silva Filho LV, Castaños C, Ruiz HH. Cystic fibrosis in Latin America-Improving the awareness. Journal of cystic fibrosis: official journal of the European Cystic Fibrosis Society. 2016; 15(6):791–3. https://doi.org/10.1016/j.jcf.2016.05.007 PMID: 27262748

2. Raskin S, Pereira-Ferrari L, Reis FC, Abreu F, Marostica P, Rozov T, et al. Incidence of cystic fibrosis in five different states of Brazil as determined by screening of p.F508del mutation at the CFTR gene in newborns and patients. Journal of cystic fibrosis: official journal of the European Cystic Fibrosis Society. 2008; 7(1):15–22. https://doi.org/10.1016/j.jcf.2007.03.006 PMID: 17544945

3. Bevivino A, Bacci G, Drevinek P, Nelson MT, Hoffman L, Mengoni A. Deciphering the Ecology of Cystic Fibrosis Bacterial Communities: Towards Systems-Level Integration. Trends in Molecular Medicine. 2019; 25(12):1110–22. https://doi.org/10.1016/j.molmed.2019.07.008 PMID: 31439509

4. Man WH, de Steenhuijsen Pijpers WAA, Bogaert D. The microbiota of the respiratory tract: gatekeeper to respiratory health. Nature Reviews Microbiology. 2017; 15(5):259–70. https://doi.org/10.1038/nrmicro.2017.14 PMID: 28316390

5. Blanchard AC, Waters VJ. Microbiology of Cystic Fibrosis Airway Disease. Semin Respir Crit Care Med. 2019; 40(06):727–36. https://doi.org/10.1055/s-0039-1698464 PMID: 31887768
6. Van der Gast CJ, Walker AW, Stuessmann FA, Rogers GB, Scott P, Daniels TW, et al. Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. The ISME journal. 2011; 5 (5):780–91. https://doi.org/10.1038/ismej.2010.175 PMID: 2115003

7. Zhao J, Schloss PD, Kalikin LM, Carmody LA, Foster BK, Petrossino JF, et al. Decade-long bacterial community dynamics in cystic fibrosis airways. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109(15):5809–14. https://doi.org/10.1073/pnas.1105771109 PMID: 22451929

8. Carmody LA, Zhao J, Schloss PD, Petrossino JF, Murray S, Young VB, et al. Changes in cystic fibrosis airway microbiota at pulmonary exacerbation. Annals of the American Thoracic Society. 2013; 10 (3):179–87. https://doi.org/10.1513/AnnalsATS.201211-107OC PMID: 23802813

9. Mahboubi MA, Carmody LA, Foster BK, Kalikin LM, VanDevanter DR, LiPuma JJ. Culture-Based and Culture-Independent Bacteriologic Analysis of Cystic Fibrosis Respiratory Specimens. Journal of clinical microbiology. 2016; 54(3):613–9. https://doi.org/10.1128/JCM.02299-15 PMID: 26699705

10. Cuthbertson L, Rogers GB, Walker AW, Oliver A, Green LE, Daniels TW, et al. Respiratory microbiota resistance and resilience to pulmonary exacerbation and subsequent antimicrobial intervention. The ISME journal. 2016; 10(5):1081–91. https://doi.org/10.1038/ismej.2015.198 PMID: 26555248

11. Pallett R, Leslie LJ, Lambert PA, Milic I, Devitt A, Marshall LJ. Anaerobiosis influences virulence properties of Pseudomonas aeruginosa cystic fibrosis isolates and the interaction with Staphylococcus aureus. Scientific Reports. 2019; 9(1):6748. https://doi.org/10.1038/s41598-019-42952-x PMID: 31043640

12. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardization of spirometry. The European respiratory journal. 2005; 26(2):319–38. https://doi.org/10.1183/09031936.05.0004805 PMID: 16055882

13. Lim YW, Evangelista JS 3rd, Schmiede R, Bailey B, Haynes M, Furlan M, et al. Clinical insights from metagenomic analysis of sputum samples from patients with cystic fibrosis. Journal of clinical microbiology. 2014; 52(2):425–37. https://doi.org/10.1128/JCM.02204-13 PMID: 24478471

14. Burns J, Rolain JM. Culture-based diagnostic microbiology in cystic fibrosis: can we simplify the complexity? Journal of cystic fibrosis: official journal of the European Cystic Fibrosis Society. 2014; 13(1):1–9. https://doi.org/10.1016/j.jcf.2013.09.004 PMID: 24094376

15. Miller JM, Binnicker MJ, Campbell S, Carroll KC, Chapin KC, Gilligan PH, et al. A Guide to Utilization of the Microbiology Laboratory for Diagnosis of Infectious Diseases: 2018 Update by the Infectious Diseases Society of America and the American Society for Microbiology. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America. 2018; 67(6):e1–e94.

16. Christensen GD, Simpson WA, Bisno AL, Beachey EH. Adherence of slime-producing strains of Staphylococcus epidermidis to smooth surfaces. Infect Immun. 1982; 37(1):318–26. https://doi.org/10.1128/IAI.37.1.318-326.1982 PMID: 6179880

17. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic acids research. 2012; 41(1):e1–e. https://doi.org/10.1093/nar/gks908 PMID: 22933715

18. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, et al. Global patterns of 16S rDNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci USA. 2011; 108(Supplement 1):4516–22.

19. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology. 2019; 37(10):1116–23. https://doi.org/10.1038/s41587-019-0209-9 PMID: 31341288

20. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods. 2016; 13(7):581–3. https://doi.org/10.1038/nmeth.3869 PMID: 27214047

21. Rideout JR, He Y, Navas-Molina JA, Walters WA, Ursell LL, Gibbons SM, et al. Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. PeerJ. 2014; 2:e545. https://doi.org/10.7717/peerj.545 PMID: 25177538

22. Parks DH, Tyson GW, Hugenholtz P, Beiko RG. STAMP: statistical analysis of taxonomic and functional profiles. Bioinformatics. 2014; 30(21):3123–4. https://doi.org/10.1093/bioinformatics/btu494 PMID: 25061070

23. R-Core-Team. R: A Language and Environment for Statistical Computing. Vienna, Austria2015.

24. Aßhauer KP, Wernheuer B, Daniel R, Meinicke P. Tax4Fun: predicting functional profiles from metagenomic 16S rRNA data. Bioinformatics. 2015; 31(17):2882–4. https://doi.org/10.1093/bioinformatics/btv287 PMID: 25957349
25. Duggan MB. Anthropometry as a tool for measuring malnutrition: impact of the new WHO growth standards and reference. Annals of tropical paediatrics. 2010; 30(1):1–17. https://doi.org/10.1179/146532810X1263774541834 PMID: 20196929
26. Weir CB, Jan A. BMI Classification Percentile And Cut Off Points. StatPearls. Treasure Island (FL) 2022.
27. Cuthbertson L, Walker AW, Oliver AE, Rogers GB, Rivett DW, Hampton TH, et al. Lung function and microbiota diversity in cystic fibrosis. Microbiome. 2020; 8(1):45. https://doi.org/10.1186/s40168-020-00810-3 PMID: 32238195
28. Hahn A, Burrell A, Anususinha E, Peng D, Chaney H, Sami I, et al. Airway microbial diversity is decreased in young children with cystic fibrosis compared to healthy controls but improved with CFTR modulation. Heliyon. 2020; 6(6):e04104–e. https://doi.org/10.1016/j.heliyon.2020.e04104 PMID: 32514485
29. Khanolkar RA, Clark ST, Wang PW, Hwang DM, Yau YCW, Waters VJ, et al. Ecological Succession of Polymicrobial Communities in the Cystic Fibrosis Airways. mSystems. 2020; 5(6):e00809–20. https://doi.org/10.1128/mSystems.00809-20 PMID: 33262240
30. Raghuvanshi R, Vasco K, Vázquez-Baeza Y, Jiang L, Morton JT, Li D, et al. Azithromycin and the microbiota of cystic fibrosis sputum. BMC microbiology. 2021; 21(1):96. https://doi.org/10.1186/s12866-021-02159-5 PMID: 33784986
31. Castellani C, Duff AJA, Bell SC, Heijerman HGM, Munck A, Ratjen F, et al. The evolving dynamics of the microbial community in the cystic fibrosis lung. Environmental microbiology. 2015; 17(2):153–78. https://doi.org/10.1017/jem.2015.100 PMID: 25906920
32. Limoli DH, Yang J, Khansaheb MK, Helfman B, Peng L, Steenken AA, et al. Staphylococcus aureus and Pseudomonas aeruginosa co-infection is associated with cystic fibrosis-related diabetes and poor clinical outcomes. European Journal of Clinical Microbiology & Infectious Diseases. 2016; 35(9):947–53. https://doi.org/10.1007/s10096-016-2621-0 PMID: 26993289
33. Paganini P, Fiscarelli EV, Tuccio V, Chiancianesi M, Bacci G, Morelli P, et al. Changes in Cystic Fibrosis Airway Microbial Community Associated with a Severe Decline in Lung Function. PLoS One. 2015; 10(4):e0124348. https://doi.org/10.1371/journal.pone.0124348 PMID: 25938134
34. McGuigan L, Callaghan M. The evolving dynamics of the microbial community in the cystic fibrosis lung. Environmental microbiology. 2015; 17(1):16–28. https://doi.org/10.1011/1146-2492.12504 PMID: 24801013
35. Rasamiravaka T, Labtani Q, Dlez P, El Jaziri M. The formation of biofilms by Pseudomonas aeruginosa: a review of the natural and synthetic compounds interfering with control mechanisms. Biomed Res Int. 2015; 2015:759348. https://doi.org/10.1155/2015/759348 PMID: 25866808
36. Moreau-Marquis S, Stanton BA, O'Toole GA. Pseudomonas aeruginosa biofilm formation in the cystic fibrosis airway. Pulmonary pharmacology & therapeutics. 2008; 21(4):595–9. https://doi.org/10.1016/j.pupt.2007.12.001 PMID: 18234534
37. Vandeplassee E, Sass A, Ostyn L, Burnelle M, Kragh KN, Bjarnsholt T, et al. Antibiotic susceptibility of cystic fibrosis lung microbiome members in a multispecies biofilm. Biofilm. 2020; 2:100031. https://doi.org/10.1016/j.biofilm.2020.100031 PMID: 33447816
38. Woods PW, Haynes ZM, Mina EG, Marques CNH, Maintenance of S. aureus in Co-culture With P. aeruginosa While Growing as Biofilms. Frontiers in Microbiology. 2019; 9(2321).
39. Alcock BP, Raphenya AR, Lau TTY, Tsang KK, Bouchard M, Edalatmand A, et al. CARD 2020: antibiotic resistance surveillance with the comprehensive antibiotic resistance database. Nucleic acids research. 2020; 48(D1):D517–d25. https://doi.org/10.1093/nar/gkz935 PMID: 31665441
44. Filloux A. Protein Secretion Systems in Pseudomonas aeruginosa: An Essay on Diversity, Evolution, and Function. Frontiers in Microbiology. 2011; 2(155).

45. Vasco J, Riedi C, Marconi C, Nogueira K, Rodrigues LS, Omori WP, et al. Respiratory and Gut Microbiota of Children with Cystic Fibrosis: A Pilot Study. Annals of Clinical and Medical Microbiology. 2021; 5(2).