ORIGINAL ARTICLE
AIRWAY MICROBIOME

Protected sampling is preferable in bronchoscopic studies of the airway microbiome

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ABSTRACT The aim was to evaluate susceptibility of oropharyngeal contamination with various bronchoscopic sampling techniques.

67 patients with obstructive lung disease and 58 control subjects underwent bronchoscopy with small-volume lavage (SVL) through the working channel, protected bronchoalveolar lavage (PBAL) and bilateral protected specimen brush (PSB) sampling. Subjects also provided an oral wash (OW) sample, and negative control samples were gathered for each bronchoscopy procedure. DNA encoding bacterial 16S ribosomal RNA was sequenced and bioinformatically processed to cluster into operational taxonomic units (OTU), assign taxonomy and obtain measures of diversity.

The proportion of Proteobacteria increased, whereas Firmicutes diminished in the order OW, SVL, PBAL, PSB ($p<0.01$). The alpha-diversity decreased in the same order ($p<0.01$). Also, beta-diversity varied by sampling method ($p<0.01$), and visualisation of principal coordinates analyses indicated that differences in diversity were smaller between OW and SVL and OW and PBAL samples than for OW and the PSB samples. The order of sampling (left *versus* right first) did not influence alpha- or beta-diversity for PSB samples.

Studies of the airway microbiota need to address the potential for oropharyngeal contamination, and protected sampling might represent an acceptable measure to minimise this problem.

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Introduction

High-throughput sequencing has opened up a new window in microbial ecology, enabling the characterisation of microbial communities in biological compartments thought to be completely sterile only a few years ago. The implications for health and disease are widely unexplored, but are likely to be significant [1]. Recent studies have found compelling evidence for the lungs to have a distinct microbiome [2], providing a bacterial presence with which our immune system interacts [3, 4]. As almost all pulmonary diseases have a local inflammatory component, there is a possibility of a disrupted microbiome being integral to disease pathogenesis.

Thus, there is a current push to characterise the pulmonary microbiome, and its relation to different pulmonary diseases. However, sampling the pulmonary microbiome is difficult. Sputum is fraught with significant contamination from the oral cavity, and percutaneous sampling is unpractical with a high risk of complications like pneumothorax or bleeding. The emerging gold standard for sampling is bronchoscopy. But bronchoscopy also has its technical challenges, besides issues of discomfort, cost and sedation. The bronchoscope must pass through either the oral or nasal cavity in addition to the pharyngeal cavity, and might carry contaminants from the upper airways to the lower biomass compartment of the lower airways. Samples are collected through the same bronchoscope working channel through which fluid is suctioned up and out. The different modes of sampling (bronchoalveolar lavage (BAL) brushings, biopsies) might be carried through catheters, which may or may not have a wax-sealed tip to ensure sterility. Added to this is the conundrum caused by the constant influx of microbiota by microaspiration and inhalation that probably is responsible for maintenance and creation of a large fraction of the lung microbiome [5].

In 25 studies of the human lung microbiome sampling the airway microbiome by bronchoscopy of healthy subjects [2–4, 6–9] and patients with chronic obstructive pulmonary disease (COPD) [10–14], asthma [15, 16], interstitial lung disease [17, 18], cystic fibrosis (CF) [19], HIV [20–23] and lung-transplanted subjects [24–27]; only five used protected sterile brushes (PSB) to avoid contamination from the working channel [7, 8, 16, 19, 22]. Some authors reported that suction was not used prior to entering the trachea [2–4, 6–10, 20, 22], and three studies used separate bronchoscopes for anaesthesia and sampling of some or all participants [3, 4, 7]. No study performed bronchoalveolar lavage (BAL) through a protected catheter (protected BAL), and no study with more than 20 sampled subjects has compared protected with unprotected sampling methods.

In preparation for the analyses of a large, ongoing COPD microbiome study [28], we sought to reduce contamination as well as assess the performance of different sampling techniques. In the current paper we present analyses to examine the degree of oropharyngeal influence on the airway microbiome applying protected bronchoscopic sampling techniques. In addition we present an analysis on the effect of sampling the left or right lung first.

Material and methods

The design of the entire MicroCOPD study has been published previously [28]. The current analysis includes 58 control subjects, 64 subjects with COPD and three subjects with asthma. All participants were at least 35 years old and were recruited from previous longitudinal case–control studies in addition to a few volunteers [29]. Subjects had neither acute respiratory symptoms nor any reported use of antibiotics or oral corticorticosteroids within the last 14 days prior to bronchoscopy. Other inclusion/exclusion criteria are listed in the supplementary material.

The Regional Committee for Medical and Health Research Ethics approved the study (REK Nord, project number 2011/1307). All participants provided written informed consent.

All participants received at least 0.4 mg of salbutamol through a spacer before the bronchoscopy procedure. Flexible video-bronchoscopy was performed *via* the oral route in supine position. No suction was used prior to having entered the trachea. All subjects received local anaesthesia with lidocaine both before and during the procedure. All but 18 subjects received mild sedation (alfentanil) parenterally. Participants were monitored according to current guidelines, and were observed for at least 2 h after the procedure [30]. Six procedural samples, of which five were obtained during bronchoscopy, were analysed for each participant: oral wash (OW); three protected specimen brushes (PSBs) from the right lower lobe (right PSB) and three from the left upper lobe (left PSB); two 50-mL fractions of protected bronchoalveolar lavage of the right middle lobe (PBAL1 and PBAL2); and small-volume lavage (SVL) in the left upper lobe. In addition, we included negative control samples (NCSs) from the same bottle of phosphate-buffered saline that was used for the procedure of the corresponding individual. For 49 subjects, we examined the left lung before the right lung. BAL and SVL were always collected after obtaining PSB samples. Protected specimen brushes and protected bronchoalveolar lavage are illustrated in supplementary figures S1 and S2.

Bacterial DNA was extracted using a combination of enzymatic lysis with lysozyme, mutanolysin and lysostaphin, and mechanical lysis methods using the FastPrep-24 as described by the manufacturers of the FastDNA Spin Kit (MP Biomedicals, LLC, Solon, OH, USA).

Library preparation and sequencing of the V3-V4 region of the 16S rRNA gene was carried out according to the Illumina 16S Metagenomic Sequencing Library Preparation guide (Part no. 15044223 Rev. B). The V3-V4 region was PCR amplified (45 cycles) and prepared for a subsequent index PCR step using primers adapted from KLINDWORTH *et al.* [31] as follows. 16S amplicon PCR forward primer (overhang adaptor sequences are underlined): 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGG NGGCWGCAG. 16S amplicon PCR reverse primer (overhang adaptor sequences are underlined): 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. The samples were pooled and prepared for 2×300 cycles of paired-end sequencing on the Illumina Miseq sequencing platform using reagents from the Miseq reagent kit v3 (Illumina Inc., San Diego, CA, USA).

The chosen bioinformatic pipeline was Quantitative Insights Into Microbial Ecology (QIIME, http://qiime.org) v1.9.1. After creating a library of joined reads, operational taxonomic units (OTUs) were picked at a 97% similarity threshold, small OTUs and OTUs seen in negative control samples were removed, taxonomy was assigned to the OTUs and a phylogenetic tree was constructed after alignment. We used the GreenGenes version 13.8 as reference database [32]. Further details on the bioinformatic procedures can be found in the supplementary material.

Differences in relative abundance of taxa were evaluated by applying a beta distribution and non-parametric trend tests. Alpha-diversity was evaluated using Faith's phylogenetic diversity (PD), or "PD wholetree". Beta-diversity was estimated with unweighted UniFrac and visualised by principal coordinates analyses (PCoA) [33]. Diversity analyses require a similar number of sequences in each sample, which was ensured by rarefaction. Statistical significance for alpha-diversity and beta-diversity between sampling methods was evaluated by Bonferroni-corrected Wilcoxon matched-pairs test in Stata version 13.2 (Statacorp, Texas, USA) and permutational ANOVA (permanova) tests in QIIME, respectively.

Results

Only three subjects had asthma: two men and one woman. The 64 COPD subjects were slightly older, included more men and had a larger tobacco-smoking burden than the 58 control subjects (table 1).

For each of the 125 participants, seven samples were sequenced (negative control sample, OW, right PSB, PBAL1, PBAL2, left PSB, SVL). A total of 12.5 million sequences were obtained from the six procedural samples after bioinformatics clean-up, as described in the methods section. For alpha- and beta-diversity, we rarefied our data at 1000 sequences.

Taxonomy

Figure 1 shows the taxonomic classification by sampling method at the phylum level. As the degree of protection from influence of oral environment increased, the proportion of Proteobacteria increased, whereas Firmicutes diminished ($p<0.01$). At the genus level all sample types where dominated by streptococci, but the mean proportion of the largest *Streptococcus* OTU showed the same declining pattern by sample type (OW 14.5%, SVL 13.6%, PBAL1 11.8%, PBAL2 11.3%, right PSB 8,6% and left PSB 5.4%; non-parametric trend test $p<0.001$).

TABLE 1 Characteristics of 125 subjects of the MicroCOPD study

	COPD	Asthma	Control
Subjects	64	3	58
Males	34 (53.1%)	2 (67.7%)	34 (58.6%)
Current smokers	15 (23.4%)	0	16 (27.6%)
Ex-smokers	48 (75.0%)	2 (67.7%)	35 (60.3%)
Never-smokers	1 (1.6%)	1 (33.3%)	7 (12.1%)
Smoking exposure pack-years	28.49±16.08	20.88±24.22	22.83±18.55
FEV_1 % predicted	56.83±16.30	88.31±11.37	100.71±11.00
Age years	68.73±7.23	64.41±9.1	64.89±8.43
Use of inhaled corticosteroids	44 (68.8%)	1 (33.3%)	1 (1.7%)

Data are presented as mean±SD unless otherwise stated. COPD: chronic obstructive pulmonary disease; FEV_1: forced expiratory volume in 1 s.

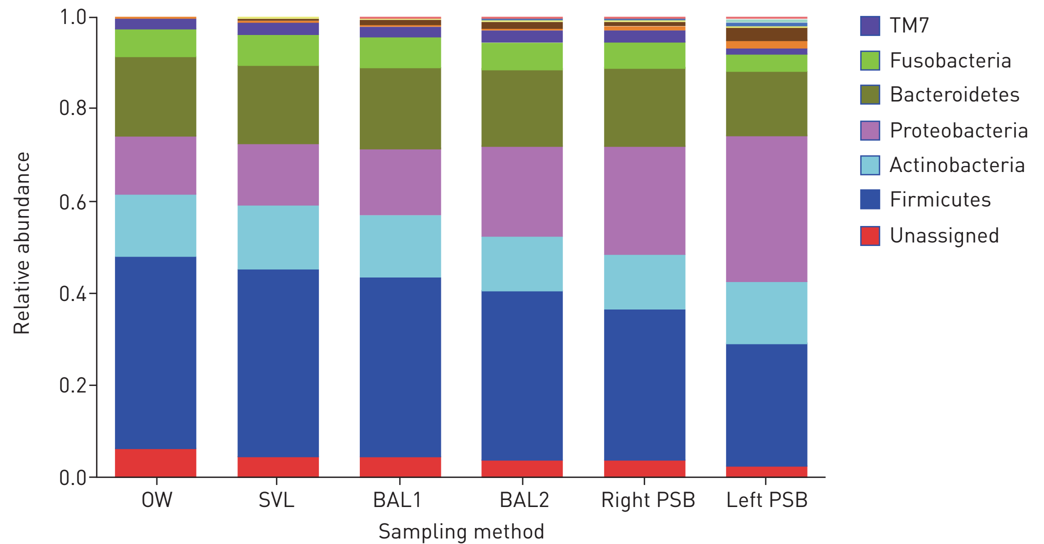

FIGURE 1 Mean taxonomic distribution at the phylum level, by sampling method, for all 125 individuals (unrarefied). OW: oral wash; SVL: small-volume lavage in the left upper lobe; BAL1: first fraction of protected bronchoalveolar lavage (BAL) from right middle lobe; BAL2: second fraction of protected BAL from right middle lobe; PSB: protected specimen brush from right lower lobe and left upper lobe. No legend for smallest phylae.

Alpha-diversity

Figure 2 shows a boxplot of the alpha-diversity metric, Faith's phylogenetic diversity, by sampling method and by disease category, excluding the three asthma subjects. The phylogenetic diversity within a sample is an indication of richness as the diversity increases both when a higher number of different OTUs are present, and when the phylogenetic distance is larger within the phylogenetic tree (less genetically similar). Bonferroni-corrected Wilcoxon matched-pairs signed-ranks tests showed that the oral wash samples were more alpha-diverse than all other sampling methods ($p<0.001$). The diversity was lower in COPD patients than controls, for most all sample types (figure 2). Importantly, the diversity decreased as the samples

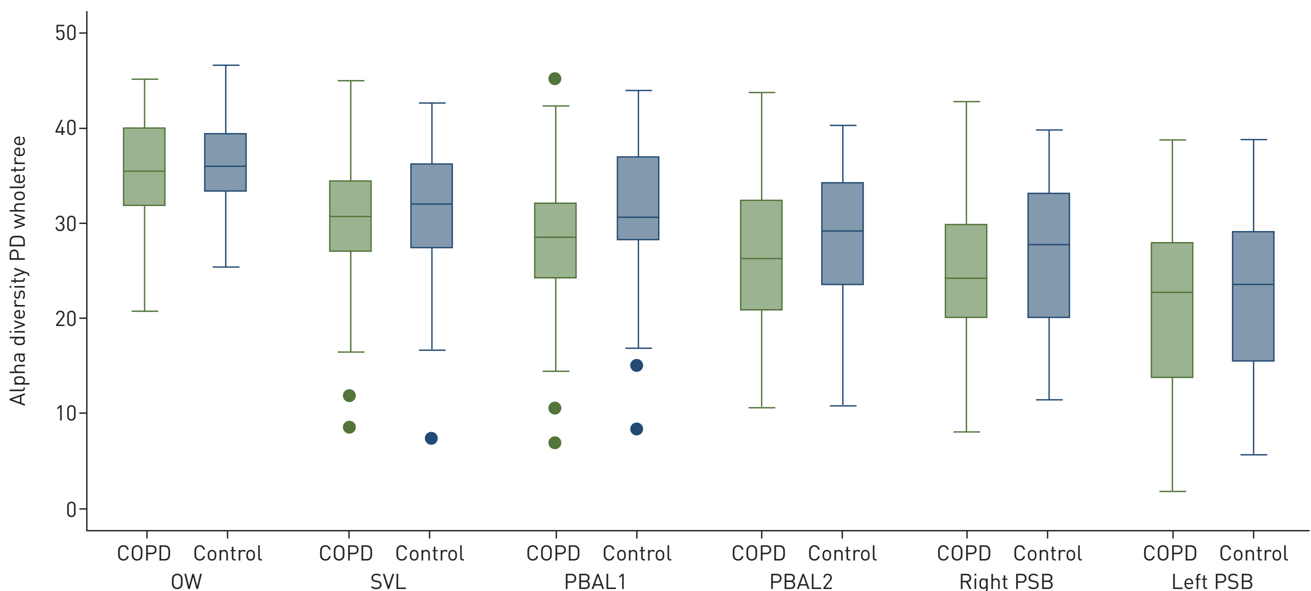

FIGURE 2 Box-plot of alpha-diversity measured by wholetree phylogenetic differences grouped according to sampling method and chronic obstructive pulmonary disease (COPD) status. Rarefied at 1000 sequences. OW: oral wash sample; SVL: small-volume lavage from left upper lobe; PBAL1: first fraction of protected bronchoalveolar lavage (BAL); PBAL2: second fraction of protected BAL; right PSB: protected specimen brush from right lower lobe; left PSB: protected specimen brush from left upper lobe.

were less exposed to potential oral and bronchoscope contamination (OW>SVL>PBAL1>PBAL2>rightPSB>leftPSB, non-parametric trend test p<0.01).

Beta-diversity

To compare between sample compositions (beta-diversity), we constructed principal coordinates analysis (PCoA) plots of unweighted UniFrac distances including all procedural samples. Figure 3 shows the PCoA plots for the oral wash *versus* each of the other sampling methods. Each dot represents a diversity measurement for one sample, and the OW sample is always shown in green. As can be seen, most respiratory tract samples clustered differently from the OW samples, but the visual impression is that the differences in diversity were smaller between OW and SVL and OW and PBAL samples than for OW and the PSB samples. Another way of comparing the beta-diversity was employed using a permanova test; estimating the beta-diversity between OW samples and each of the other sampling methods. This method tests to which degree the variation in a matrix of UniFrac distances can be explained by an imposed categorisation (*i.e.* sampling method). Overall permanova test confirmed that the beta-diversity differed by sampling method (pseudo F 8.73, p=0.001, 999 permutations). When the distance matrix was split according to the comparisons in figure 3, all were significant (p<0.01, permanova, corrected for multiple comparison), with the permanova pseudo F-statistic gradually increasing for the comparison of OW with SVL, PBAL1, PBAL2, right PSB and left PSB respectively, again indicating that PSB samples were more clearly separated from OW samples than SVL and PBAL.

Finally we investigated whether the order of sampling (left *versus* right lung first) influenced alpha- and beta-diversity in PSB samples. We found no significant difference in alpha- or beta-diversity for the right or the left PSBs as judged by phylogenetic diversity and unweighted UniFrac (supplementary figures S3 and S4).

Discussion

We have shown that protected BAL and protected brush samples differed more from oral wash samples than unprotected lavage through the bronchoscope working channel. Thus, unprotected sampling of the airway microbiome might convey an image of a microbiome that is more similar to the oral microbiome, than it would have been with protected sampling.

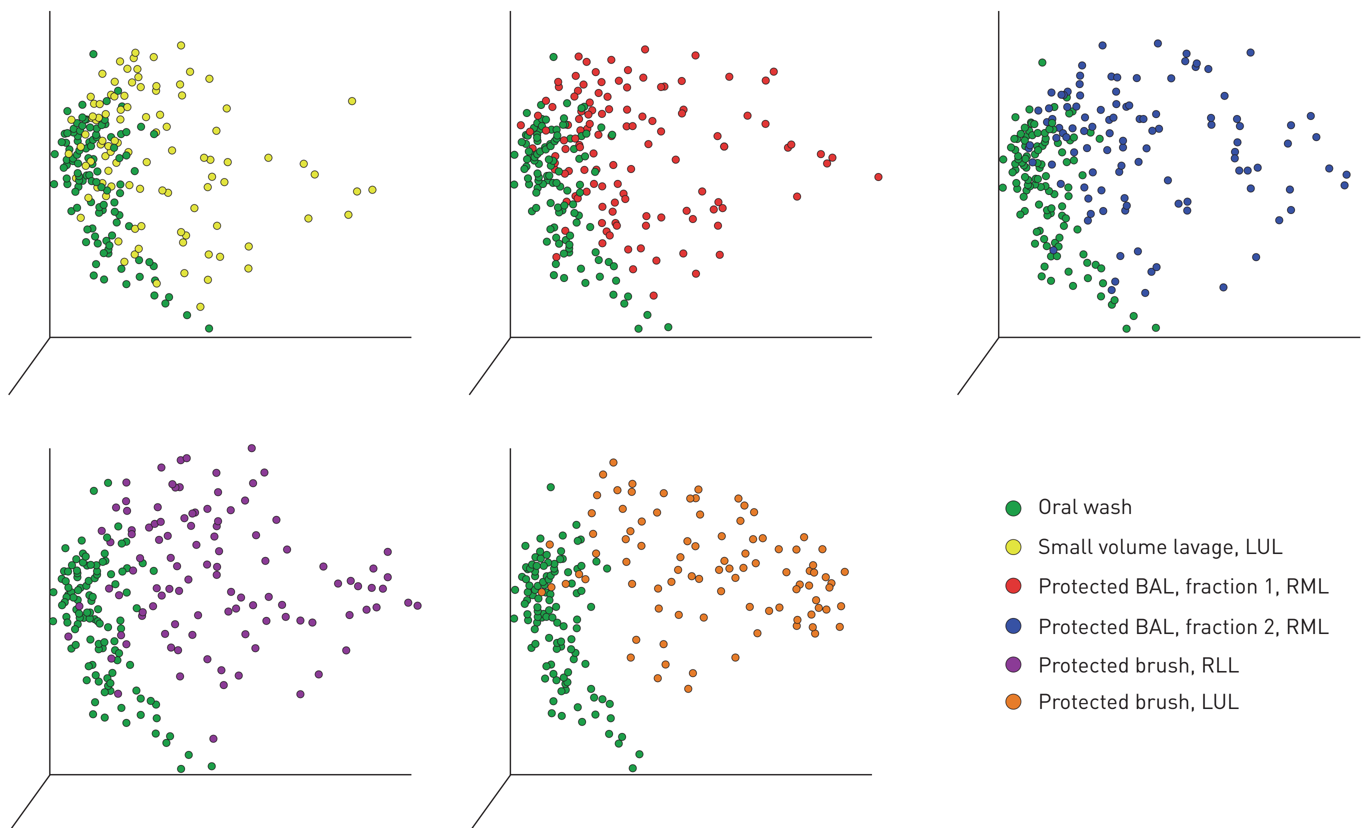

FIGURE 3 Principal coordinates analyses on unweighted UniFrac distance matrix comparing sampling methods in the MicroCOPD to oral wash samples. Rarefied at 1000 sequences. LUL: left upper lobe; BAL: bronchoalveolar lavage; RML: right middle lobe; RLL: right lower lobe.

To our best knowledge this is the first study that presents both protected brush *and* protected lavage sampling as compared with both the oral microbiome and unprotected sampling. With more than 120 examined subjects it is by today the largest single site bronchoscopy study of the lung microbiome.

As other authors we find evidence of a lung microbiome separated from the oral microbiome by a larger fraction of Proteobacteria and a proportionately lower fraction of Firmicutes [2, 8, 15, 20]. However, SEGAL and associates [3, 4] mainly found that the airway microbiome was characterised by enrichment from supraglottic areas of the respiratory tract, and in particular by *Prevotella* and *Veillonella* OTUs, which are Bacteriodetes and Firmicutes, respectively. They examined 49 subjects, with supraglottic brushes and BAL through the working channel, and observed that two clusters dominated airway samples: one dominated by OTUs present in negative control samples, and one dominated by OTUs present in supraglottic brushes. One interpretation might be that these two clusters represent two different modalities of contamination, the first one from laboratory procedures and the second from bronchoscopic carryover. SEGAL *et al.* argue that if it was bronchoscopic carry-over, they would have observed a dilutional effect when they compared a first BAL of the lingula, with the second BAL of the right middle lobe. However, this comparison was done for only 15 individuals, and anatomically one might expect lower biomass in the lingula than the right middle lobe.

Other authors have also investigated the possibility of bronchoscopic carryover. BASSIS *et al.* examined oral wash samples of 12 subjects and compared them with a first BAL of the lingula and a second BAL of the right middle lobe [6]. They did not find any difference in quantitative PCR between the first and second BAL, and no difference in beta-diversity when comparing the OW with the two BALs. Their interpretation was that if there was significant carryover, there should have been observed some sort of dilutional effect. Nevertheless, the two sampled sites are separated by the carina, and the bronchoscope must be repositioned between sampling, and these two sites are indeed in different communication with the outside world, possibly leading to an *a priori* larger biomass in the right lung. Also, DICKSON *et al.* compared supraglottic brushes with PSB and BAL through the working channel [8]. In principal component analyses of beta-diversity they found no clustering by sample type, except that the supraglottic samples differed from the intrapulmonary sample communities. However, by performing unprotected BAL before PSB, residual BAL fluid might have affected the brush areas making them more similar to the BAL sample sites. Finally, 15 sampled subjects might not be sufficient to detect the differences we observed in the current study with more than 100 participants.

It is quite plausible that microbes migrate from the oropharyngeal cavity to the airways, generating a normal overlap between the oropharyngeal and airway microbiomes [5]. But as we have shown, co-existing sample contamination likely also is an issue. The oropharyngeal microbiome has a known large biomass, with a high diversity. By passing through this cavity, contamination to the outside of the bronchoscope including its tip is inevitable. Use of suction will contaminate the working channel [7]. Since the oral biomass is much greater than the airway biomass, even a small contamination will have a disproportionate effect on the supposed airway microbiome if the unprotected measurements are performed through the working channel. Using the working channel for unprotected lavage repeatedly at different lobes will lead to contamination from one lobe to another. Using larger volume lavage may negate this effect to some degree, but not eliminate the problem.

Results from the current study suggest that protected sheet sampling is the superior sampling methodology. Comparing unprotected SVL and PSB both taken from the upper left lobe in our study, SVL was most similar to the oral sample by visual assessment of the 10 most abundant taxa, and likewise both by alpha- and beta-diversity. A direct comparison of protected and unprotected lavage from the same lobe is impossible, as any washing will impact the contents of later washings. However, the diversity of PBAL from the right middle lobe was intermediate between that found in OW and that found in the PSB.

Besides the above-mentioned study by DICKSON and colleagues [8], only two other studies have compared PSBs to other sampling methods [7, 19]. CHARLSON *et al.* [7] sampled laboratory reagents, the bronchoscope itself during various parts of the procedure, and the oropharyngeal microbiome in addition to BAL through the working channel and PSBs. They concluded that the microbiome from the lower respiratory tract was indiscriminate from the oropharyngeal microbiome irrespective of sampling method. However, the study included only one PSB per sampling, had lower sequencing depth than the current study, included only six healthy individuals and there were no adjustments made for OTUs seen in the negative control samples [7]. HOGAN *et al.* compared PSB, and SVL samples of nine CF patients [19]. For eight CF patients who had PSB and SVL taken from the same lobe, diversity was consistently higher in the PSB samples [19], the opposite of our findings. HOGAN *et al.* employed the PSB only at visible mucus plugs, and the airways of adult CF patients are perhaps no longer representing a low biomass environment. In addition the number of study subjects was limited.

The main strength of our study was comprehensive sampling of a large, heterogeneous sample of subjects with and without COPD, while taking precautions to avoid excessive influence from laboratory and bronchoscopic contamination. However, some potential weaknesses should be acknowledged. First, we have not performed quantitative PCR, and thus cannot conclude regarding the amount of 16S rRNA gene copies in the samples before amplification. Second, our analyses do not include a mock community, and we are therefore not able to provide sequencing error rates for the current study. We could also have spiked our samples with bacteria that would have indicated the efficiency of our DNA extraction. Third, pre-bronchoscopy all participants received 0.4 mg salbutamol. This was done for obtaining pre-bronchoscopy post-bronchodilator lung function values, but had the added benefit of protecting against procedural bronchospasm. Salbutamol was given as an aerosol through large volume spacers that are cleaned daily, and we are not aware of reports on contamination through metered dose inhalers. Furthermore, since both patients and controls received salbutamol, our conclusions should not be affected. Fourth, some results are difficult to compare with those of other authors because of differences in DNA extraction, PCR amplification, sequencing and bioinformatic approach. This is the result of a field where standards for 16S rRNA gene amplicon studies of microbial communities currently do not exist. To facilitate reproducibility we have used well-documented analytic approaches and mostly default settings for our bioinformatic pipeline (QIIME), in addition to using primers and PCR recommendations from a major next-generation sequencing provider (Illumina). Regardless of this, we cannot rule out that some of our findings only pertain to the current set of methodological choices such as the choice of sequencing hypervariable region V3V4 [34]. To minimise the influence of small/spurious OTUs we have excluded singletons by using default settings in our OTU picking, and removed OTUs that constituted less than 0.005% of the total number of sequences.

Insights concerning the airway microbiome in disease and health might provide vital understanding of disease mechanisms and provide new targets for treating lung diseases such as COPD, asthma, cystic fibrosis and interstitial lung diseases. However, to date only a minority of studies have performed protected sampling, and might have been affected by exposure to exposure to microbiota encountered before reaching the sampled sites. We have shown that unprotected sampling is likely to be affected by this phenomenon, and we encourage the use of protected specimen brushes when sampling the airway microbiota.

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R. Grønseth was the guarantor of the study and all authors had full access to all of the data in the study and take full responsibility for the integrity of the data and the accuracy of the data analysis. R. Grønseth, H.G. Wiker, M. Aanerud, P. Bakke and T. Eagan designed the study. R. Grønseth, H.G. Wiker, G.R. Husebø, Ø. Svanes, S. Lehmann, M. Aardal, T. Kalananthan, E.M. Hjellestad Martinsen, E. Orvedal Leiten, E. Nordeide, I. Haaland, I. Jonassen, P. Bakke and T. Eagan took part in the data collection. T. Hoang, C. Drengenes, H.G. Wiker and T. Kalananthan performed DNA extraction and high-throughput sequencing analyses. R. Grønseth, Y. Xue, S. Tangedal, T. Eagan and I. Jonassen. performed statistical and bioinformatic analyses. Data were interpreted by R. Grønseth, C. Drengenes, H.G. Wiker, S. Tangedal, Y. Xue, I. Haaland, I. Jonassen and T. Eagan. R. Grønseth, C. Drengenes, S. Tangedal and T. Eagan drafted the paper. All authors revised the draft and approved the version to be published.

References

1 Cho I, Blaser MJ. The human microbiome: at the interface of health and disease. *Nat Rev Genet* 2012; 13: 260–270.
2 Morris A, Beck JM, Schloss PD, *et al.* Comparison of the respiratory microbiome in healthy nonsmokers and smokers. *Am J Respir Crit Care Med* 2013; 187: 1067–1075.
3 Segal LN, Alekseyenko AV, Clemente JC, *et al.* Enrichment of lung microbiome with supraglottic taxa is associated with increased pulmonary inflammation. *Microbiome* 2013; 1: 19.
4 Segal LN, Clemente JC, Tsay JC, *et al.* Enrichment of the lung microbiome with oral taxa is associated with lung inflammation of a Th17 phenotype. *Nat Microbiol* 2016; 1: 16031.
5 Dickson RP, Erb-Downward JR, Martinez FJ, *et al.* The microbiome and the respiratory tract. *Annu Rev Physiol* 2016; 78: 481–504.
6 Bassis CM, Erb-Downward JR, Dickson RP, *et al.* Analysis of the upper respiratory tract microbiotas as the source of the lung and gastric microbiotas in healthy individuals. *MBio* 2015; 6: e00037–15.
7 Charlson ES, Bittinger K, Haas AR, *et al.* Topographical continuity of bacterial populations in the healthy human respiratory tract. *Am J Respir Crit Care Med* 2011; 184: 957–963.
8 Dickson RP, Erb-Downward JR, Freeman CM, *et al.* Spatial variation in the healthy human lung microbiome and the adapted island model of lung biogeography. *Ann Am Thorac Soc* 2015; 12: 821–830.

9 Venkataraman A, Bassis CM, Beck JM, *et al.* Application of a neutral community model to assess structuring of the human lung microbiome. *MBio* 2015; 6: e02284-14.
10 Cabrera-Rubio R, Garcia-Nunez M, Seto L, *et al.* Microbiome diversity in the bronchial tracts of patients with chronic obstructive pulmonary disease. *J Clin Microbiol* 2012; 50: 3562–3568.
11 Einarsson GG, Comer DM, McIlreavey L, *et al.* Community dynamics and the lower airway microbiota in stable chronic obstructive pulmonary disease, smokers and healthy non-smokers. *Thorax* 2016; 71: 795–803.
12 Erb-Downward JR, Thompson DL, Han MK, *et al.* Analysis of the lung microbiome in the "healthy" smoker and in COPD. *PLoS One* 2011; 6: e16384.
13 Pragman AA, Kim HB, Reilly CS, *et al.* The lung microbiome in moderate and severe chronic obstructive pulmonary disease. *PLoS One* 2012; 7: e47305.
14 Zakharkina T, Heinzel E, Koczulla RA, *et al.* Analysis of the airway microbiota of healthy individuals and patients with chronic obstructive pulmonary disease by T-RFLP and clone sequencing. *PLoS One* 2013; 8: e68302.
15 Hilty M, Burke C, Pedro H, *et al.* Disordered microbial communities in asthmatic airways. *PLoS One* 2010; 5: e8578.
16 Huang YJ, Nelson CE, Brodie EL, *et al.* Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally controlled asthma. *J Allergy Clin Immunol* 2011; 127: 372–381.
17 Garzoni C, Brugger SD, Qi W, *et al.* Microbial communities in the respiratory tract of patients with interstitial lung disease. *Thorax* 2013; 68: 1150–1156.
18 Molyneaux PL, Cox MJ, Willis-Owen SA, *et al.* The role of bacteria in the pathogenesis and progression of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2014; 190: 906–913.
19 Hogan DA, Willger SD, Dolben EL, *et al.* Analysis of lung microbiota in bronchoalveolar lavage, protected brush and sputum samples from subjects with mild-to-moderate cystic fibrosis lung disease. *PLoS One* 2016; 11: e0149998.
20 Beck JM, Schloss PD, Venkataraman A, *et al.* Multicenter comparison of lung and oral microbiomes of HIV-infected and HIV-uninfected individuals. *Am J Respir Crit Care Med* 2015; 192: 1335–1344.
21 Cribbs SK, Uppal K, Li S, *et al.* Correlation of the lung microbiota with metabolic profiles in bronchoalveolar lavage fluid in HIV infection. *Microbiome* 2016; 4: 3.
22 Lozupone C, Cota-Gomez A, Palmer BE, *et al.* Widespread colonization of the lung by *Tropheryma whipplei* in HIV infection. *Am J Respir Crit Care Med* 2013; 187: 1110–1117.
23 Twigg HL, Knox KS, Zhou J, *et al.* Effect of advanced HIV Infection on the respiratory microbiome. *Am J Respir Crit Care Med* 2016; 194: 226–235.
24 Borewicz K, Pragman AA, Kim HB, *et al.* Longitudinal analysis of the lung microbiome in lung transplantation. *FEMS Microbiol Lett* 2013; 339: 57–65.
25 Dickson RP, Erb-Downward JR, Freeman CM, *et al.* Changes in the lung microbiome following lung transplantation include the emergence of two distinct *Pseudomonas* species with distinct clinical associations. *PLoS One* 2014; 9: e97214.
26 Dickson RP, Erb-Downward JR, Prescott HC, *et al.* Cell-associated bacteria in the human lung microbiome. *Microbiome* 2014; 2: 28.
27 Willner DL, Hugenholtz P, Yerkovich ST, *et al.* Reestablishment of recipient-associated microbiota in the lung allograft is linked to reduced risk of bronchiolitis obliterans syndrome. *Am J Respir Crit Care Med* 2013; 187: 640–647.
28 Grønseth R, Haaland I, Wiker HG, *et al.* The Bergen COPD microbiome study (MicroCOPD): rationale, design, and initial experiences. *Eur Clin Respir J* 2014; 1: 26196.
29 Eagan TM, Ueland T, Wagner PD, *et al.* Systemic inflammatory markers in COPD: results from the Bergen COPD Cohort Study. *Eur Respir J* 2010; 35: 540–548.
30 Du Rand IA, Blaikley J, Booton R, *et al.* British Thoracic Society guideline for diagnostic flexible bronchoscopy in adults: accredited by NICE. *Thorax* 2013; 68: Suppl. 1, i1–i44.
31 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: e1.
32 McDonald D, Price MN, Goodrich J, *et al.* An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J* 2012; 6: 610–618.
33 Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* 2005; 71: 8228–8235.
34 Hiergeist A, Reischl U, Priority PIMCQAP, *et al.* Multicenter quality assessment of 16S ribosomal DNA-sequencing for microbiome analyses reveals high inter-center variability. *Int J Med Microbiol* 2016; 306: 334–342.