TITLE:

Evaluation of the Airway Microbiome in Non-Tuberculous Mycobacteria

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Supplementary Methods

Study Design

This was a prospective observational study of 106 patients with a diagnosis of non-cystic fibrosis bronchiectasis enrolled over a two-year period.

Subjects

Subjects were enrolled from a non-HIV, non-cystic fibrosis bronchiectasis cohort at New York University. All subjects signed informed consent to participate in this study and the research protocol was approved by the New York University and Bellevue Hospital Center (New York, NY) institutional review boards (IRB# S14-01400). The inclusion criteria included: CT imaging abnormalities consistent with bronchiectasis (i.e. mucoid impaction) and symptoms consistent with bronchiectasis (i.e. cough). Exclusion criteria included: participants recently on antibiotics and/or steroids (within the last month) and/or a recent history of smoking (within the last year) as cigarette smoke has been shown to be associated with changes in the upper airway microbiota [1]. At the time of recruitment, clinical information and questionnaires were obtained.

Variables Collected

At recruitment clinical information was collected, including age, sex, ethnicity, BMI, symptoms, smoking history, CT thorax imaging reports, lung function, and sputum cultures. Patients were also asked to fill out questionnaires: St. George's Respiratory Questionnaire (SGRQ), Eating Assessment Tool (EAT-10), Frequency Scale for the symptoms of GERD (FSSG), and the Reflux Symptom Index (RSI) [2-5]

Sample Collection

Oral wash and induced sputum samples were collected from every patient at enrolment. In addition, over the two-year period, serial samples were obtained as per clinical need and sent for culture as well as 16S rRNA gene sequencing. Patients were asked to first rinse their mouth and back of throat with 10ml of sterile water, to provide an oral wash sample. Patients were then placed on a 7% hypertonic saline nebuliser for 10 to 15 minutes. Following this, patients would expectorate as much sputum as they could into a sterile cup, to provide a sputum sample. A portion of this sputum sample was sent to the clinical laboratory for sputum culture and sensitivity, acid-fast bacilli testing and mycobacterium culture. All remaining sputum was transferred to our lab on ice for 16S rRNA gene sequencing.

Bronchoscopy

In all patients who consented to the study we obtained induced sputum (paralleled with oral wash) and we offered participation in the bronchoscopy study. A subset of patients from this cohort underwent bronchoscopy (n=20) in order to evaluate whether the sputum was representative of the lower airway microbiota and to assess the immune profile of the lower airway mucosa. As per our protocol, we asked every patient enrolled in this cohort about their interest in participating in a bronchoscopy arm. A few patients agreed to a research bronchoscopy (n=6) [6]. Other 14 patients had a bronchoscopy done as per clinical indication (in general because of difficulties with obtaining three induced sputum or persistence of clinical suspicious of NTM) and agreed to have bronchoscopic samples obtained for this research. All subjects had a CT scan of the chest done prior to bronchoscopy. In all subjects who underwent bronchoscopy we had a similar topographical sampling approach that

included: oral wash samples, supraglottic samples (sampled using Yankauer), background/equipment samples (sterile saline, Yankauer and Bronchoscope), sputum samples and bronchioloalveolar lavage samples (BAL) from involved and non-involved segments (predefined based on CT scan). From the BAL fluid, cell count and differential were obtained. BAL fluid aliquots were frozen at -80°C.

Bacterial 16S rRNA-encoding genes quantification and sequencing

DNA was then extracted from all samples with an ion exchange column (Qiagen). Total bacterial DNA levels were determined by quantitative PCR (qPCR) as previously described.[6, 7] High-throughput sequencing of bacterial 16S rRNA-encoding gene amplicons encoding the V4 region [8] (150bp read length, paired-end protocol) was performed with MiSeq. The V4 region of the bacterial 16S rRNA gene was amplified in duplicate reactions, using primer set 515F/806R, which nearly universally amplifies bacterial and archaeal 16S rRNA genes [8, 9]. Each unique barcoded amplicon was generated in pairs of 25µl reactions with the following reaction conditions: 11µl Polymerase Chain Reaction (PCR)-grade H2O, 10µl Hot MasterMix (5 Prime Cat# 2200410), 2µl of forward and reversed barcoded primer (5µM) and 2µl template DNA. Reactions were run on a C1000 Touch Thermal Cycler (Bio-Rad) with the following cycling conditions: initial denaturing at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 58°C for 1 minute, and extension at 72 C for 90 seconds, with a final extension of 10 min at 72°C.

To evaluate the Mycobacteriome we re-extracted DNA from additional aliquots and performed a nested PCR approach to enrich for *Mycobacterium* DNA template prior to library preparation for sequencing in order to describe the Mycobacteriome as

previously published [10]. Given the concern of inadequate NTM cell lysis using standard DNA isolation methods, we utilized a recently published optimized cell lysis and DNA isolation method as described in Caverly et al. [11]. Briefly, we added zirconium bead beating step followed by DNA isolation. Then, during library preparation, the first amplification was performed with two *Mycobacterium* specific primers (MycF121 and Myc858R) that targeted the 16S rRNA gene. This approach generated a 737bp amplicon that contained the V4 region. We then proceeded with a second PCR using the bar coded 515F/806R primer set as described above to generate the final amplicon product for sequencing. This "Mycobacteriome" approach was performed in parallel with our previously mentioned 16S rRNA gene sequencing approach. These methods were compared using a mock mixture of bacterial DNA (obtained from *Mycobacteriun fortuitum* and *Streptococcus pneumoniae*) and on subject's samples.

Analysis of 16S rRNA gene sequences

The obtained 16S rRNA gene sequences were analyzed using the QIIME package (version 1.9) for analysis of community sequence data [12]. The operational taxonomic unit (OTU) sequence counts were picked based on Greengenes database (version 13-8) and normalized to obtain the relative abundances of the microbiota in each sample. These relative abundances at 97% OTU similarity and each of the 5 higher taxonomic levels (phylum, class, order, family, genus) were tested for univariate associations with clinical variables. To decrease the number of features, we only focused on major taxa and OTUs, defined as those having relative abundance >1% in at least one sample.

Measurement of in vivo cytokines in BAL fluid and Alveolar Macrophages.

In vivo inflammation was assessed by BAL cell count differential and cytokines. Since analytes in the epithelial lining fluid are diluted with sterile saline during BAL, a concentration step was performed via dialysis against Tris 10 mM pH 7.5, EDTA 1 mM and lyophilization, using albumin as an internal control as previously described[13, 14]. For this, the initial volume of acellular BAL fluid was 5mL. After lyophilization at -80 degrees Celsius sample was re-suspended in 60μL of Phosphate-buffered saline. Inflammatory biomarkers were measured using a Human High Sensitivity T Cell Luminex Panel (Millipore HSTCMAG-28SK). Cytokines included: Fractalkine, GM-CSF, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17A, IL-21, IL-23, ITAC, MIP-1α, MIP-1β, MIP-3α, TNF-α. *Ex vivo* cytokine production was assessed using BAL cell supernatant ($10x10^6$ cells in 1 mL of Roswell Park Memorial Institute medium in a 12 well plate) after 18hrs of culture with media alone or 10ng of LPS. *Ex vivo* cytokine production during toll like receptor 4 (TLR4) stimulation was expressed as fold change in levels of biomarkers comparing media alone with LPS.

Statistical Analysis

Since the distributions of microbiome data are non-normal, and no distribution-specific tests are available, we used non-parametric tests of association. For association with discrete factors, we used either the Mann-Whitney test (in the case of 2 categories) or the Kruskal-Wallis ANOVA (in case of > 2 categories). Wilcoxon signed-rank test were used for paired analysis. We used the ade4 package in R to construct Principal Coordinate Analysis (PCoA) based on weighted UniFrac distances [15, 16]. PCoA is a method of dimensionality reduction that uses the

distance between points and plots the variation of these distances across two axes. Therefore, the closer two points are the more similar they are in their microbial composition. Similar methods of analysis were used to examine differences in cell count and cytokines.

To cluster microbiome communities into exclusive ‘metacommunities’ we used a Dirichlet Multinomial Mixture Model [17]. In this method, for each sample, we impute the component most likely to have generated it, thus separating samples into groups it has the highest probability of belonging to. This allows for variable cluster sizes and a more rigorous means of choosing optimal cluster number. The R package DirichletMultinomial was used for this method of analysis [18].

To evaluate differences between groups of 16S data, we used linear discriminant analysis (LDA) Effect Size (LEfSe) [19]. Features significantly discriminating among groups with LDA score > 2.0 were represented as a cladogram, as produced by LEfSe with default parameters. For tests of association with continuous variables, we used non-parametric Spearman correlation tests and false discovery rate (FDR) was used to control for multiple testing [20]. Co-occurrence between bacterial genera with more than one percent relative abundance in any given sample were assessed using SparCC [21] with 20 iterations and 500 boostrap replicates. Significant correlations were selected ($p < 0.05$, $|\rho| > 0.4$, two-sided t-test) and visualized with Cytoscape v3.0.2 [22]. The network layout was selected as edge-weighted spring embedded metrics. Correlation of microbial genera with continuous immune markers was estimated using nonparametric Spearman correlation with a cutoff threshold of $p < 0.05$. All data is publicly available in Sequence Read Archive (SRA) under accession number PRJNA418131.

Supplementary Results

Comparing bacterial load of Sputum and Oral Wash Based on NTM status

There were no significant differences in bacterial load based on NTM status (Median [IQR]= 2,616[1,700-42,036] copies/μl vs. 70,846[7,659–100,617] copies/μl for sputum and 8,949[2,180–20,591] copies/μl vs. 13,406[5,169–46,679] copies/μl for oral wash comparing NTM- vs. NTM+ respectively, p=ns).

Evaluation of the Mycobacteriome

To test sensitivity of this approach we first utilized a mock community of *Mycobacterium fortuitum* and *Streptococcus pneumoniae* mixed at various gradient admixtures (Mycobacterium to Streptococcus ratio ranging from 100:1 to 1:1,000,000, **Supplementary Figure 6**). Standard 16S rRNA gene sequencing approach yielded detectable *Mycobacterium* reads up to a *Mycobacterium* to *Streptococcus* ratio of 1:10 but the nested mycobacteriome approach successfully biased the sequencing to a ratio of 1:10,000.

We further validated this approach using a larger number of sputum and oral wash samples (oral wash= 56 samples [52% NTM+], sputum= 54 samples [54% NTM+]) Using our standard 16S rRNA gene sequencing *Mycobacterium* was not abundant (>1% relative abundance) in either sputum or oral wash samples. This approach yielded *Mycobacterium* reads in only 2/56 (4%) oral wash samples (both NTM-) and 5/54 (9%) sputum samples (all NTM+ samples), which was 17% of NTM+ samples, a smaller proportion than that identified in NTM+ BAL samples. Blast analysis was utilized to characterize the *Mycobacterium* species identified by this method. The five NTM+ sputum samples with *Mycobacterium* reads had 100% similarity with *Mycobacterium avium*. In contrast, the two NTM- oral wash samples with

Mycobacterium reads had 100% similarity with *Mycobacterium aurum* (another species that has not been identified as pathogenic).

Clustering of Bronchoscopic Samples

Using DMM two clear clusters were identified within the bronchoscopy samples (**Supplementary Figure 9**) similar to previous description of the lower airway microbiota [6, 13]. A LEfSe analysis of these clusters showed a clear distinction of taxa, where Cluster 1 samples were enriched with *Alicyclobacillus*, *Acinetobacter* and *Bradyrhizobium* whereas Cluster 2 samples were enriched with oral commensals such as *Prevotella*, *Veillonella* and *Streptococcus*.

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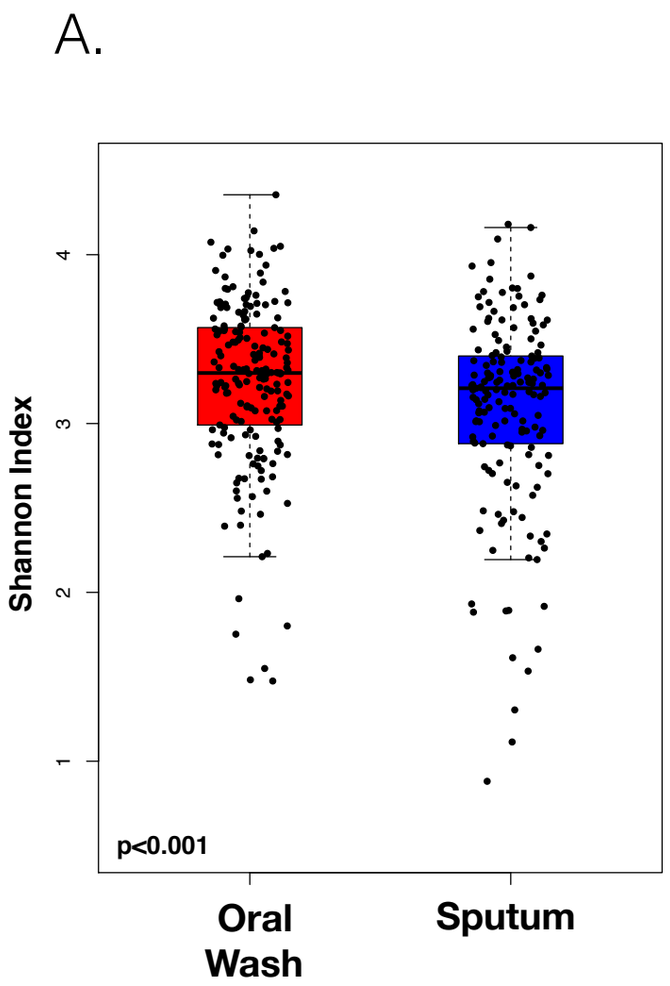
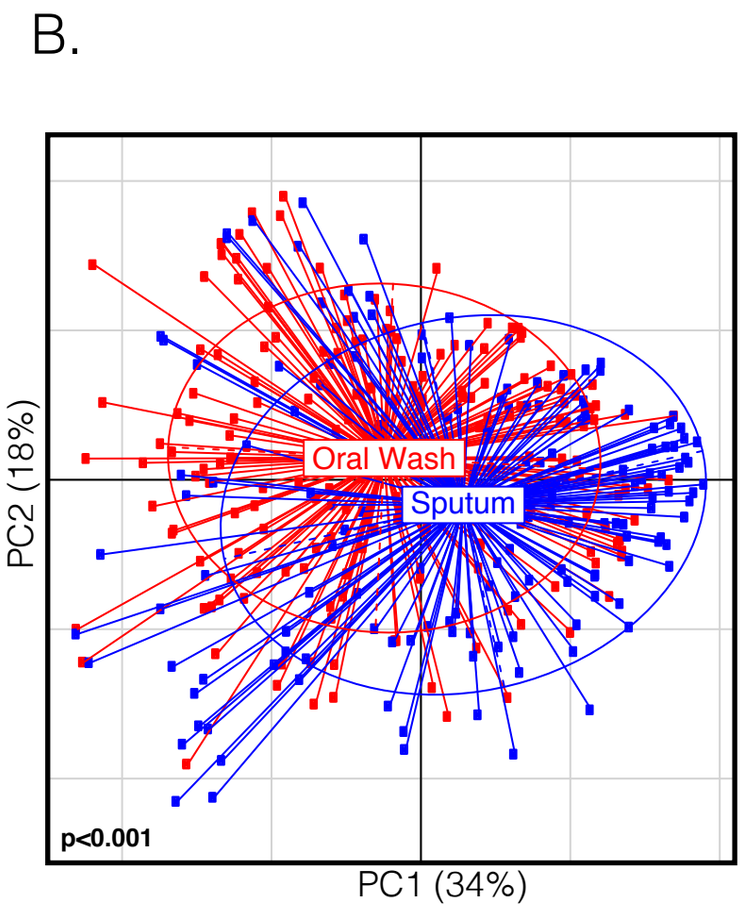
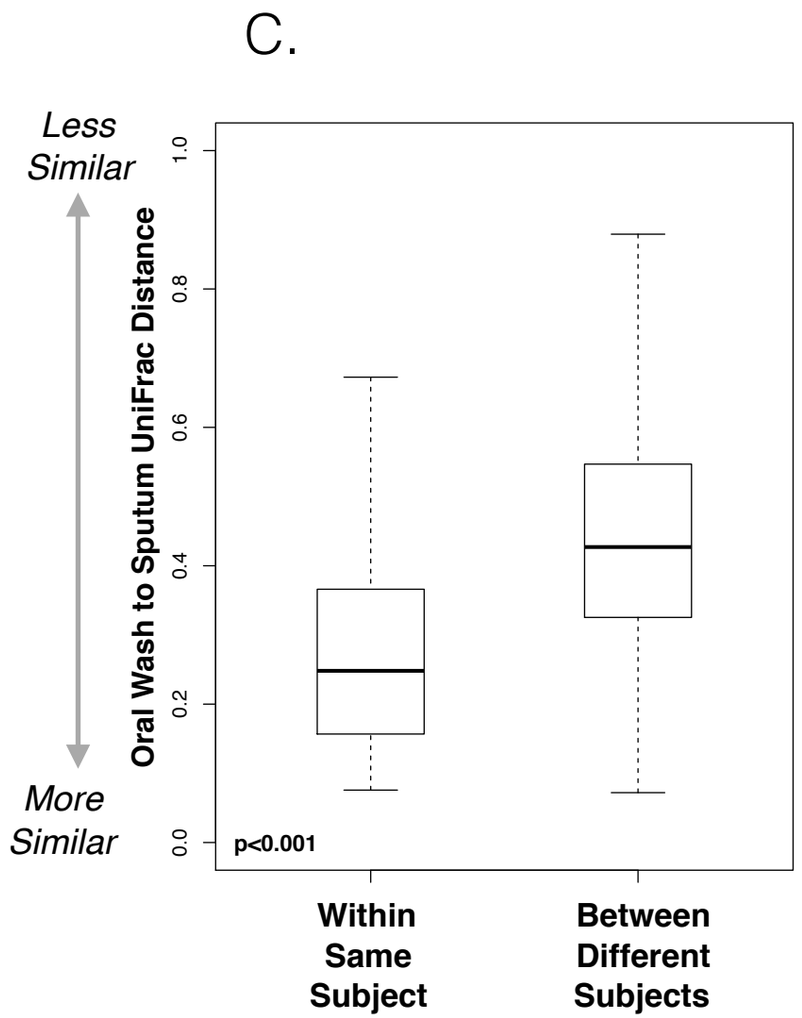
Supplementary Figures Legends:

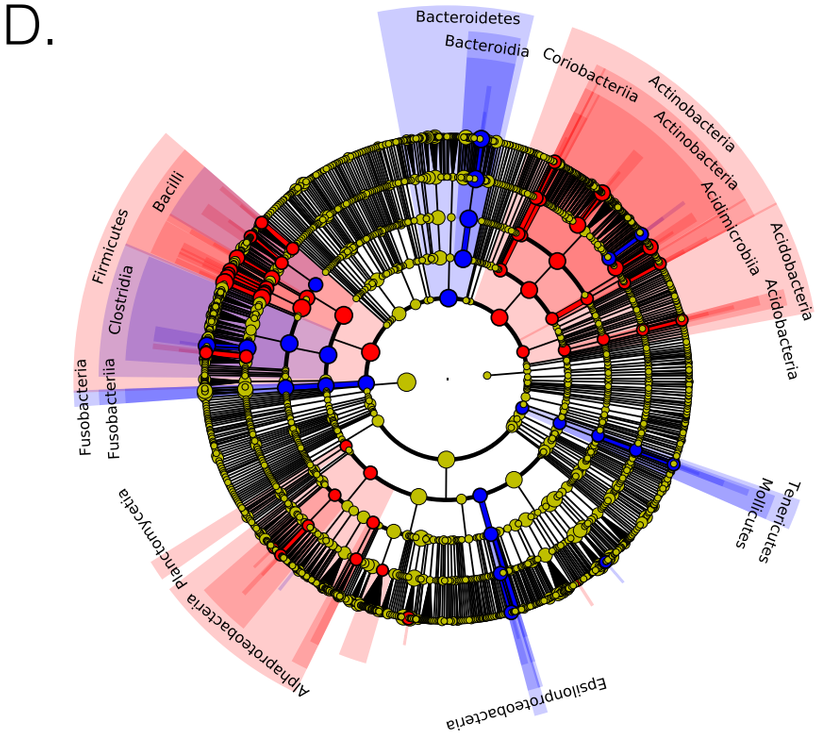
Supplementary Figure S1: Comparison of microbial diversity in oral wash and sputum samples. A. Alpha diversity based on Shannon index was higher in oral wash as compared to sputum. **B**. PCoA based on weighted UniFrac distance demonstrates significant differences between oral wash and sputum samples (PERMANOVA $p<0.001$). **C**. Comparison of degree of similarity between oral wash and sputum samples within the same subject vs. between different subjects. **D**. LEfSe analysis was utilized to identify taxa differentially enriched in oral wash and sputum samples. Multiple significant taxonomic differences were observed at different phylogenetic levels as represented in the cladogram, left panel. Bar plots in the right represents Linear Discriminant Analysis (LDA) effect size (left) and differences in relative abundance of differentially enriched taxa at a genus level (LDA>2).

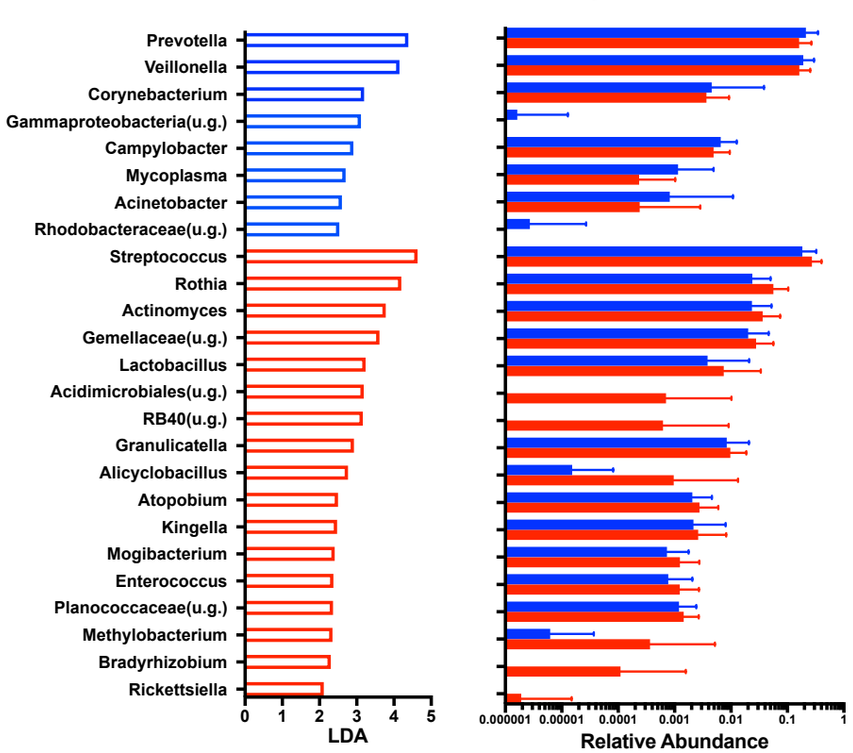
Supplementary Figure 1





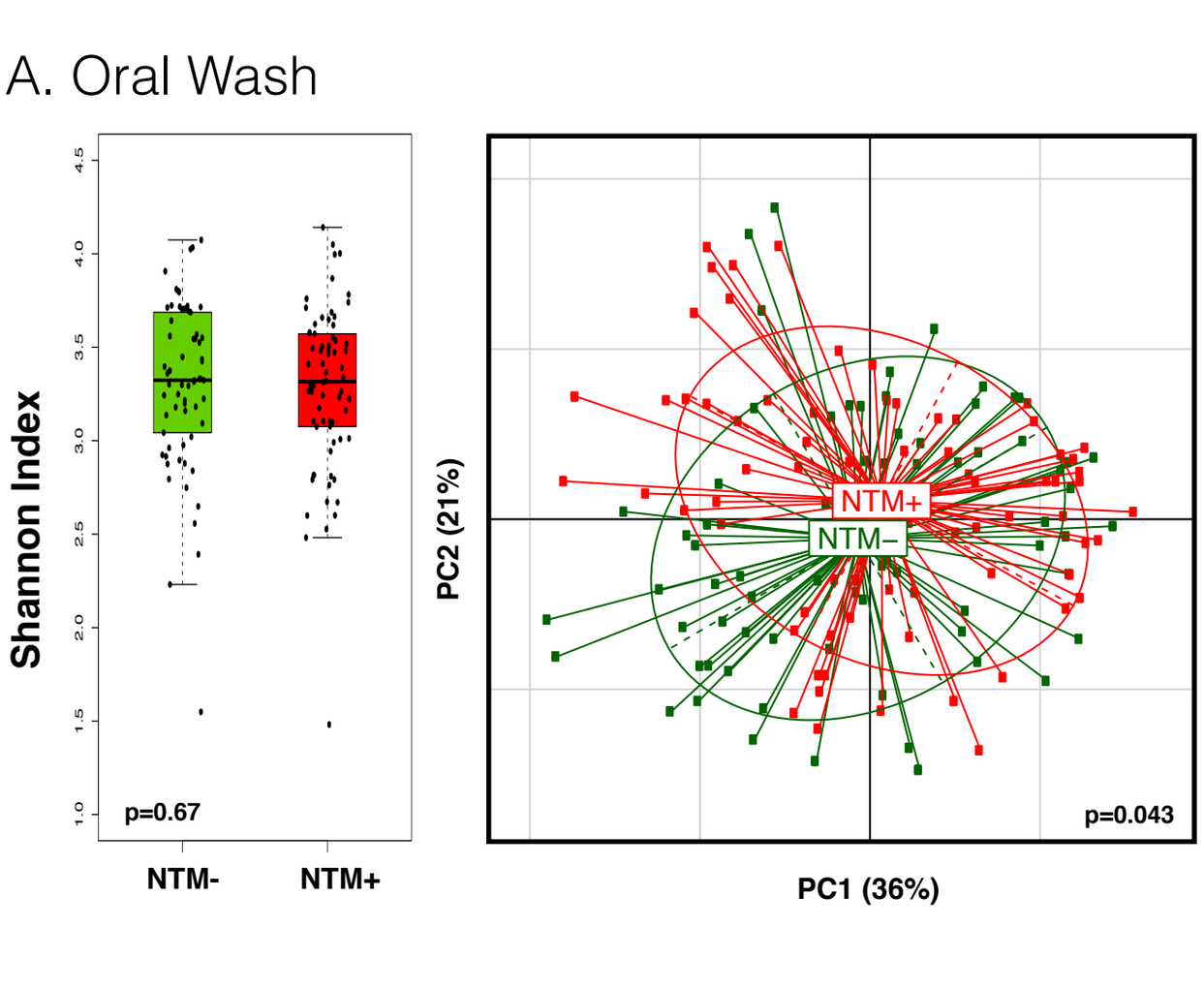


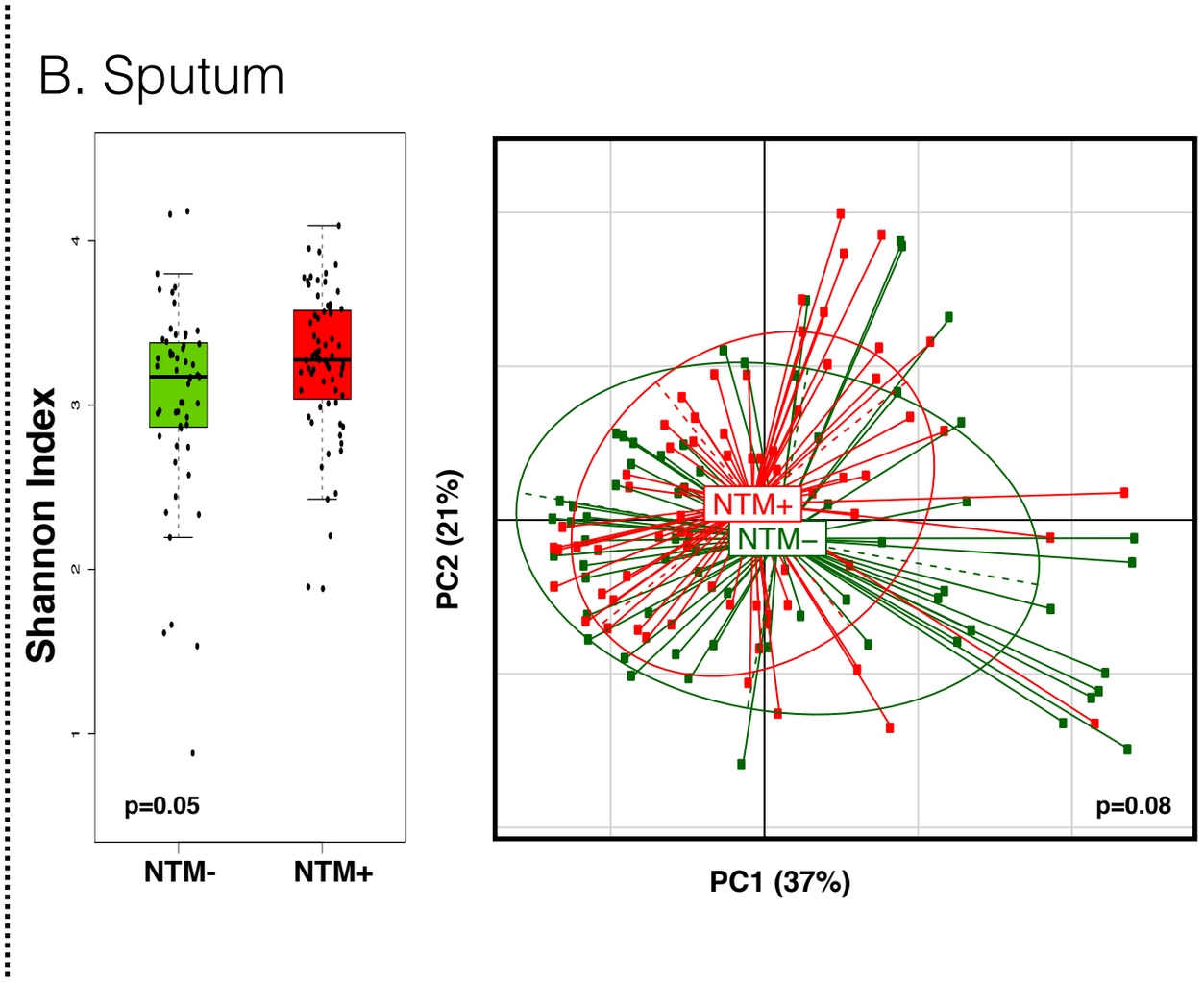




Supplementary Figure S2: Differences in diversity between all oral wash and sputum samples for the NTM+ and NTM- groups. For α diversity Shannon Diversity Index was used, for β diversity weighted UniFrac was used. **A**. For oral wash samples there were no significant differences in a diversity (Mann Whitney p=ns) but significant differences in β diversity were noted (PERMANOVA p=0.043). **B**. For sputum samples there were significant differences in α diversity, and a non-significant difference in β diversity.

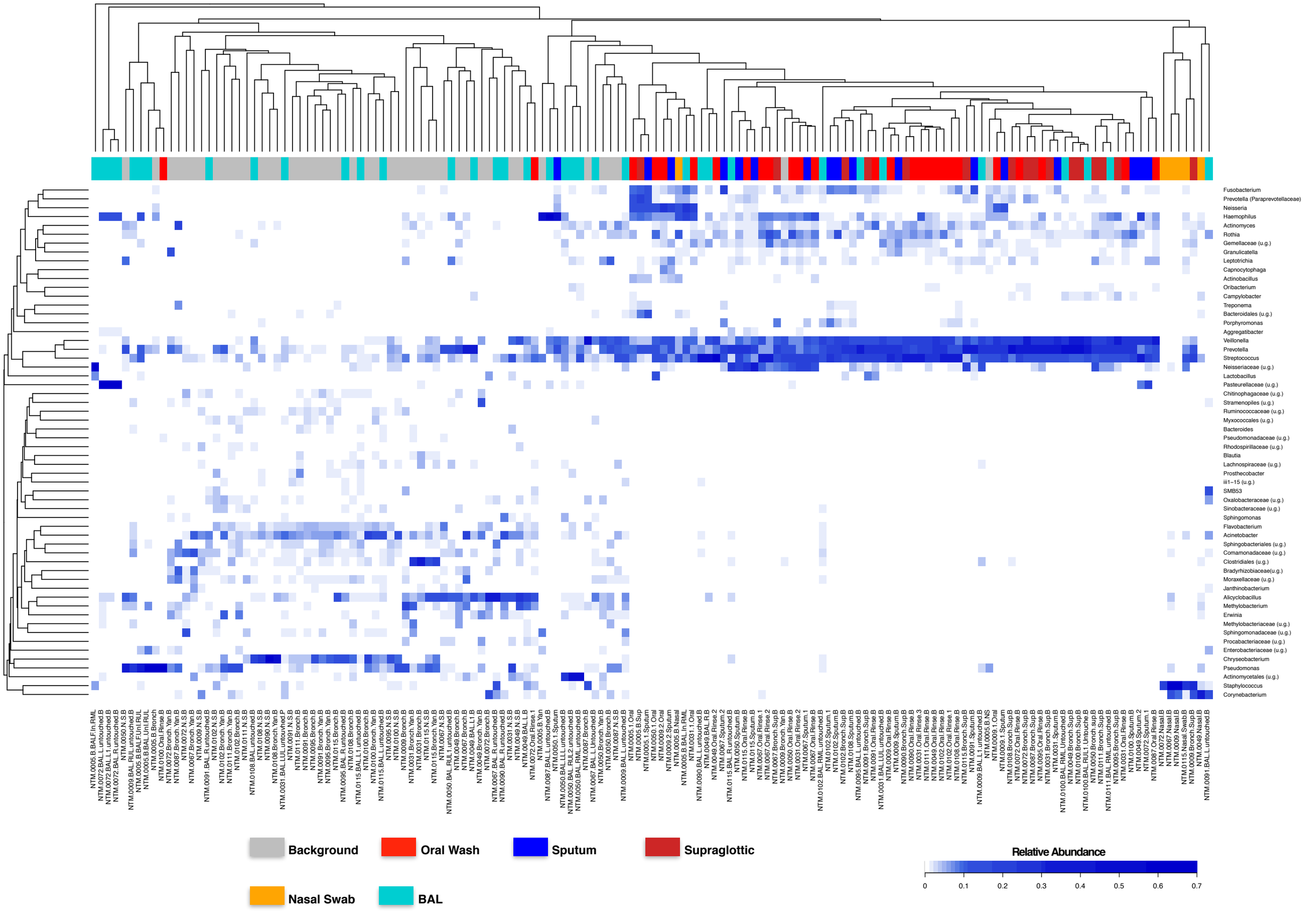
Supplementary Figure 2





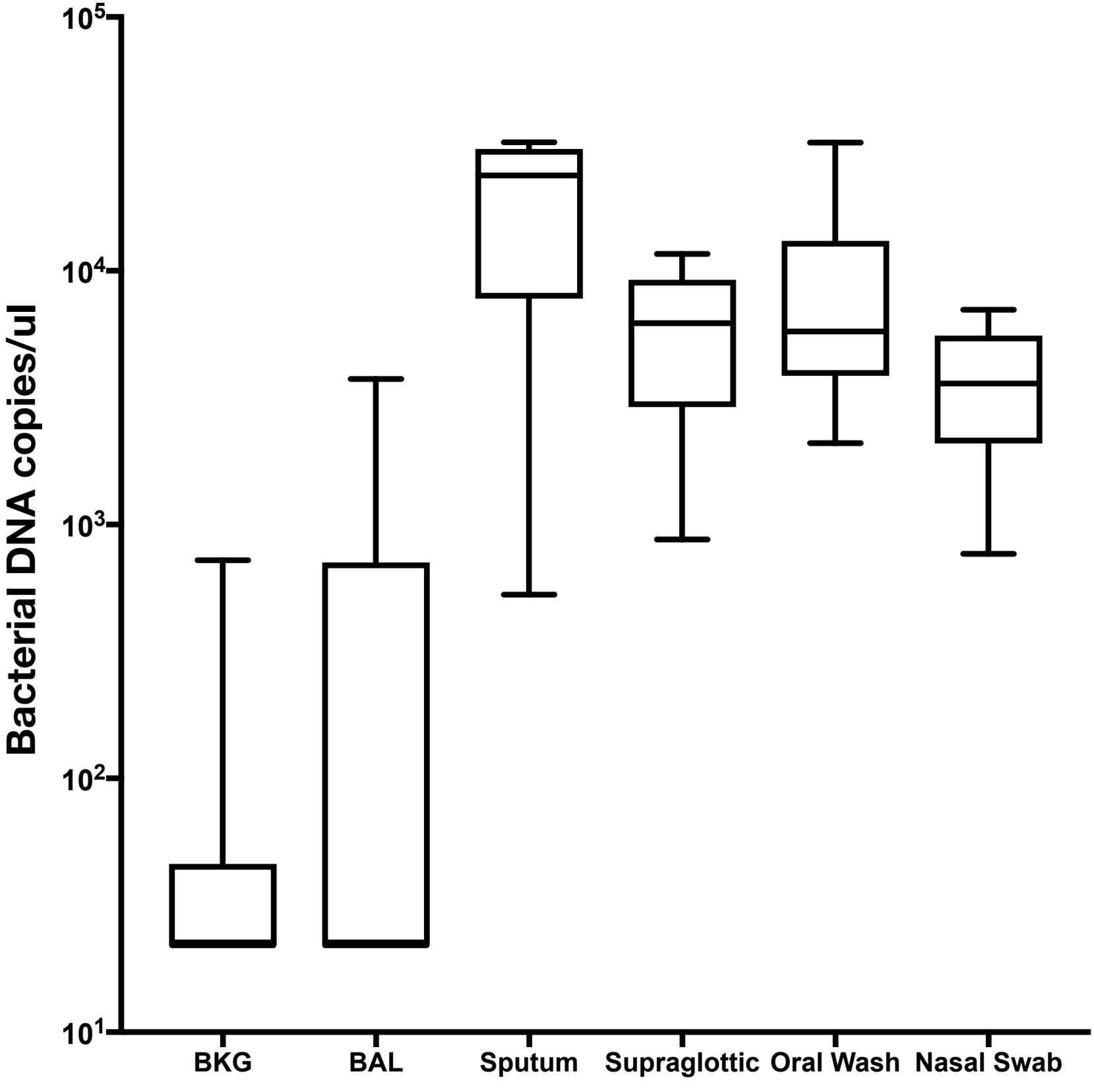
Supplementary Figure S3: Heat Map of 16S sequencing of all samples obtained during bronchoscopy. Unsupervised hierarchical clustering of most abundant taxa (relative abundance ≥ 1% in any sample) identified in Background, Nasal Swab, Oral Wash, Sputum, Supraglottic and Bronchoalveolar Lavage (BAL).

Supplementary Figure 3



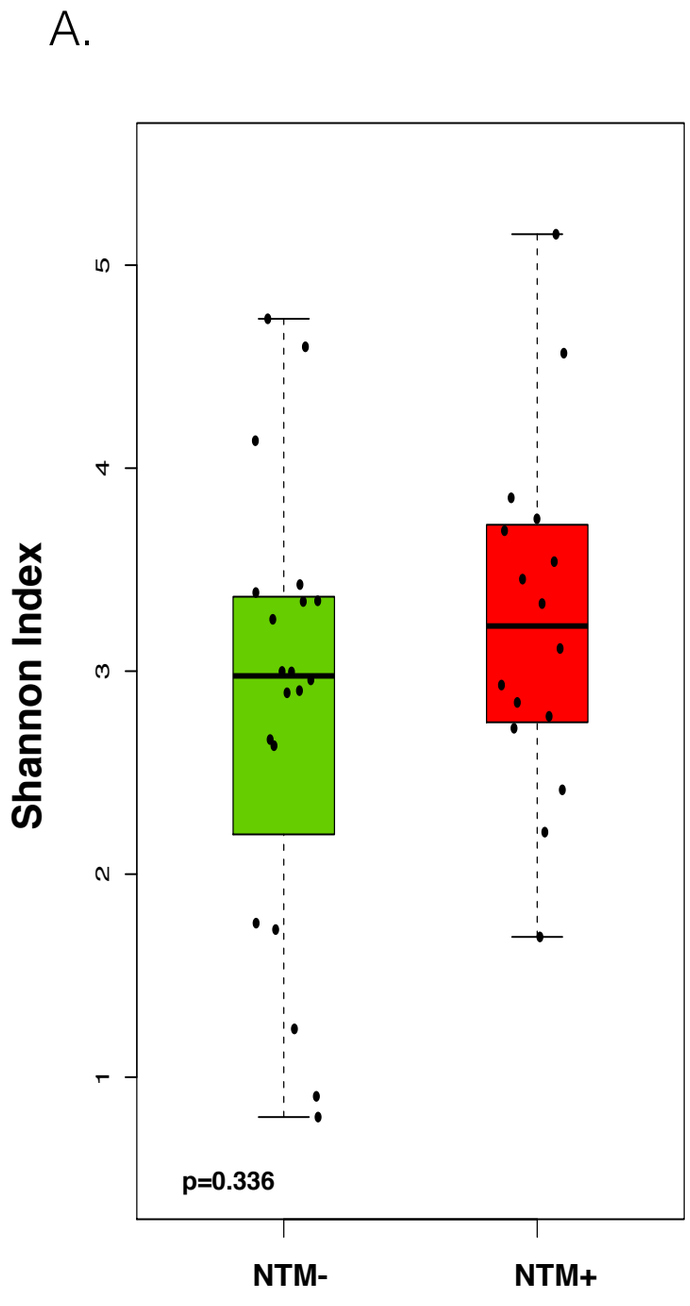
Supplementary Figure S4: Comparison of bacterial load in bronchoscopic samples. qPCR for 16S rRNA gene was used to compare bacterial load of background samples, lower airway samples (BAL), upper airway samples (oral wash and supraglottic) and sputum.

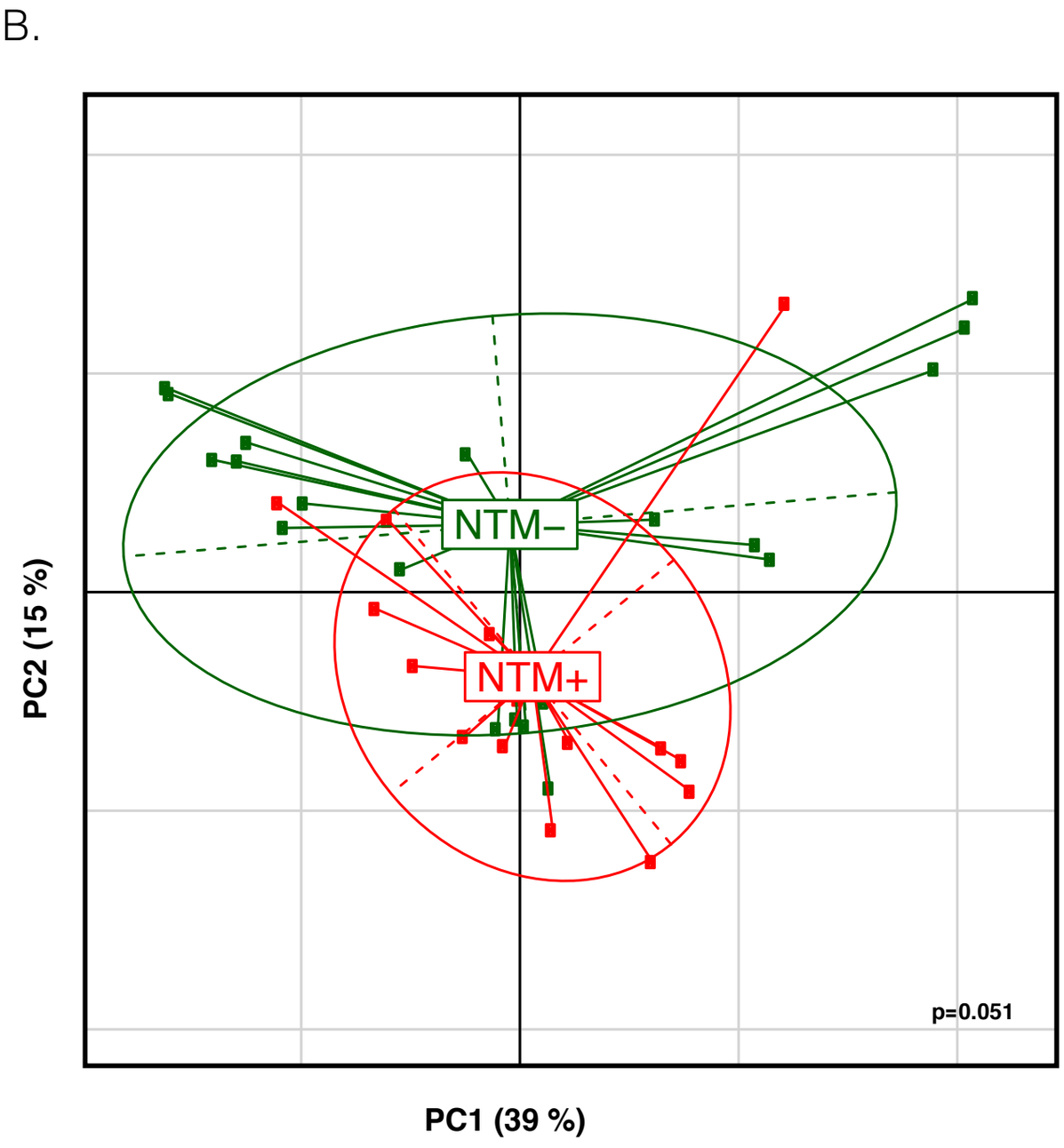
Supplementary Figure 4



Supplementary Figure S5: Differences in diversity between NTM+ and NTM- in lower airway microbiota. **A**. There were no significant differences in α diversity between NTM+ and NTM- groups. **B**. β diversity based on weighted UniFrac showed non-significant differences between NTM+ and NTM- groups.

Supplementary Figure 5

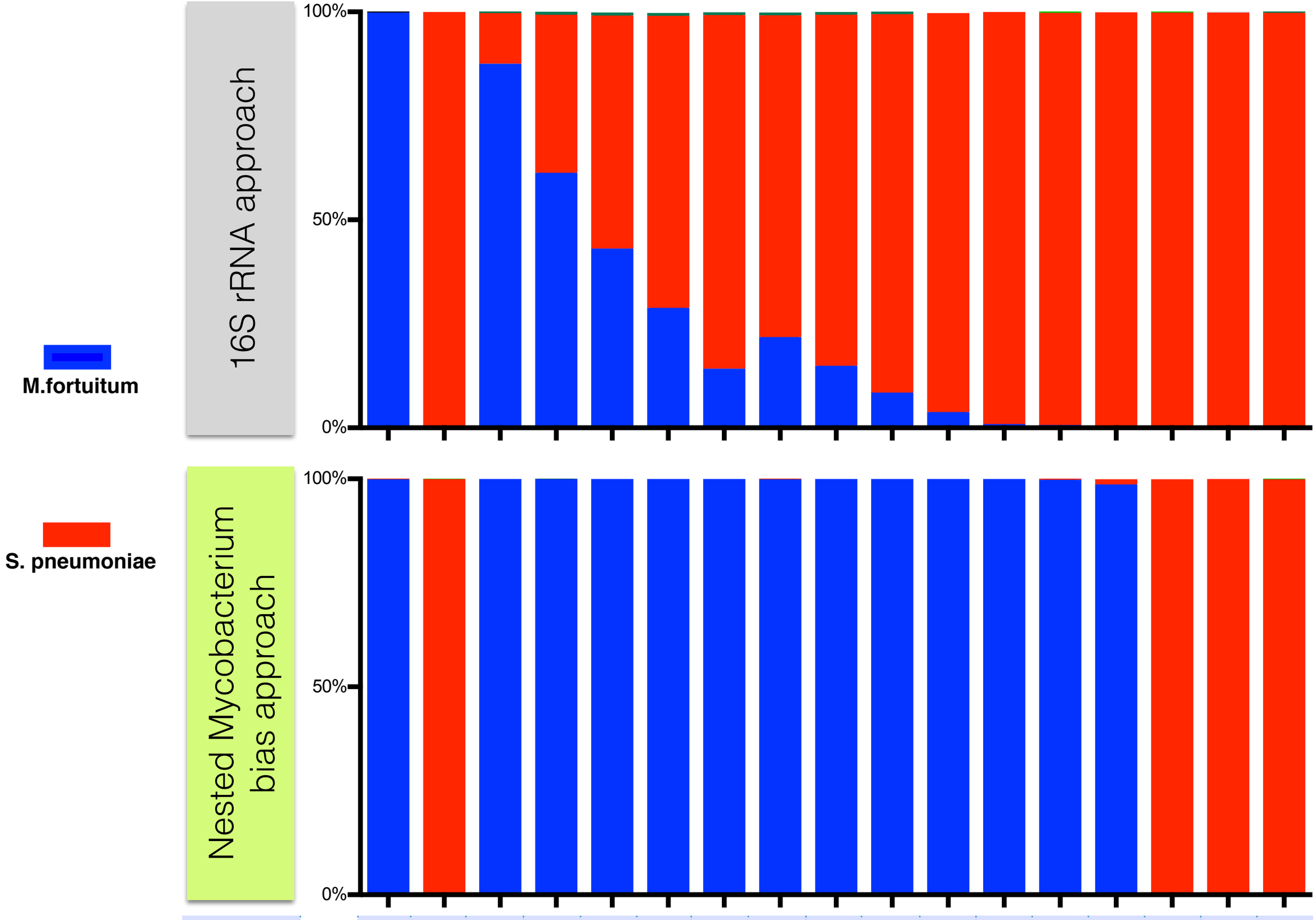




Supplementary Figure S6: Comparison between an unbiased 16S rRNA and a biased mycobacteriome approach using mock bacterial DNA mixture.

Mycobacterium fortuitum and *Streptococcus pneumoniae* isolates were used to extract DNA. DNA template was sequenced for each isolate and for a series of mixture ratios of *Mycobacterium*:*Streptococcus*. Mixing ratios started at 100:1 ratio (*Mycobacterium*:*Streptococcus* respectively) to a 1:10,000,000 ratio. Using a standard 16S rRNA sequencing approach, *Mycobacterium fortuitum* was identified until a ratio of 1:10, after which only *Streptococcus* could be identified. Using a nested mycobacterium bias approach, *Mycobacterium* was identified (with a relative abundance close to 100%) even in much lower dilution of its template, up to a ratio of 1:10,000.

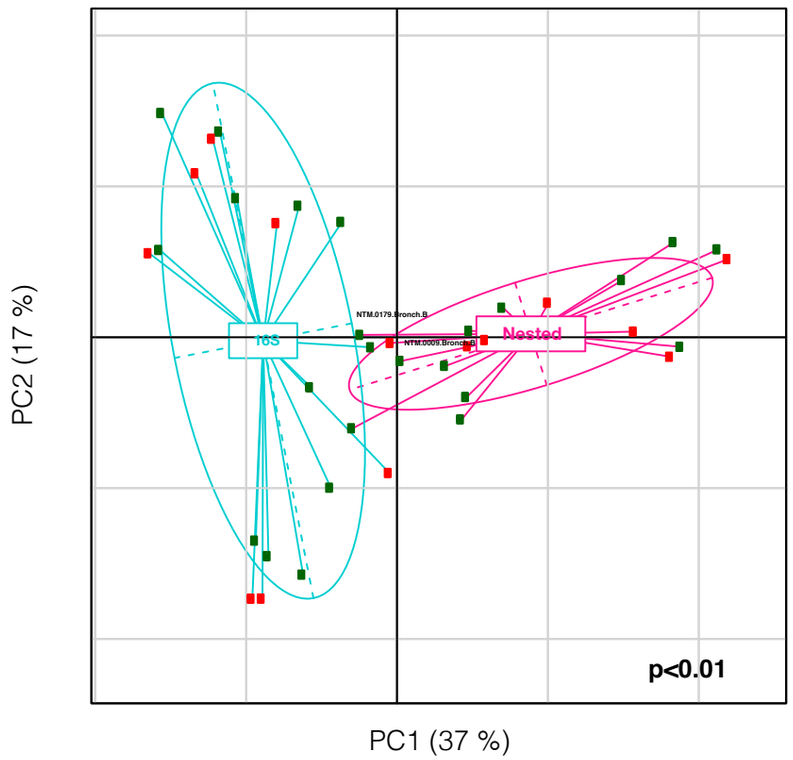
Supplementary Figure 6

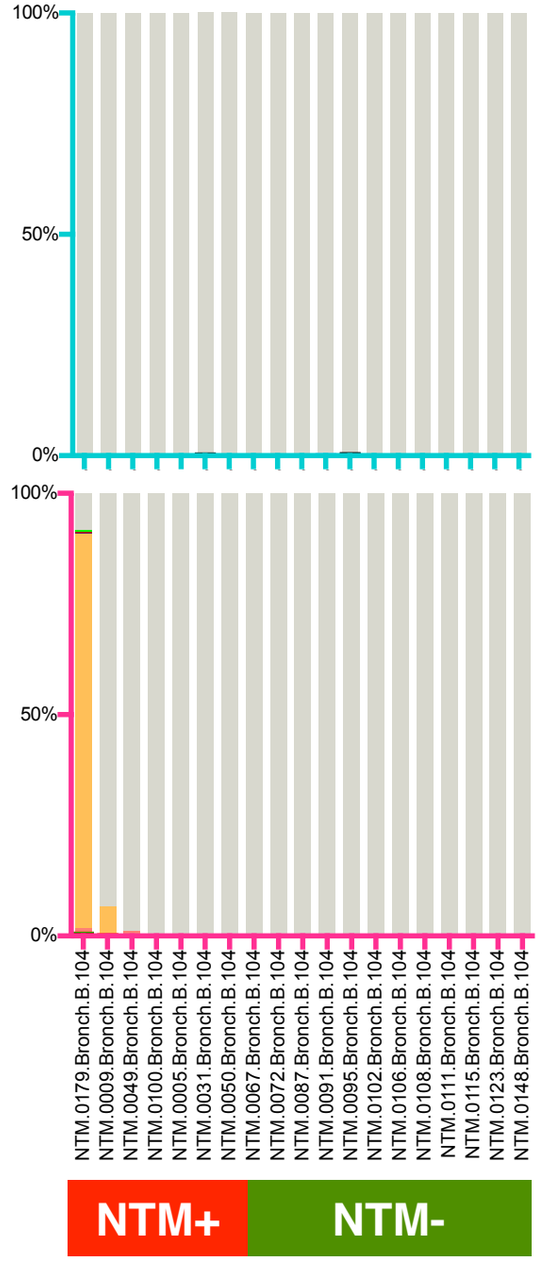


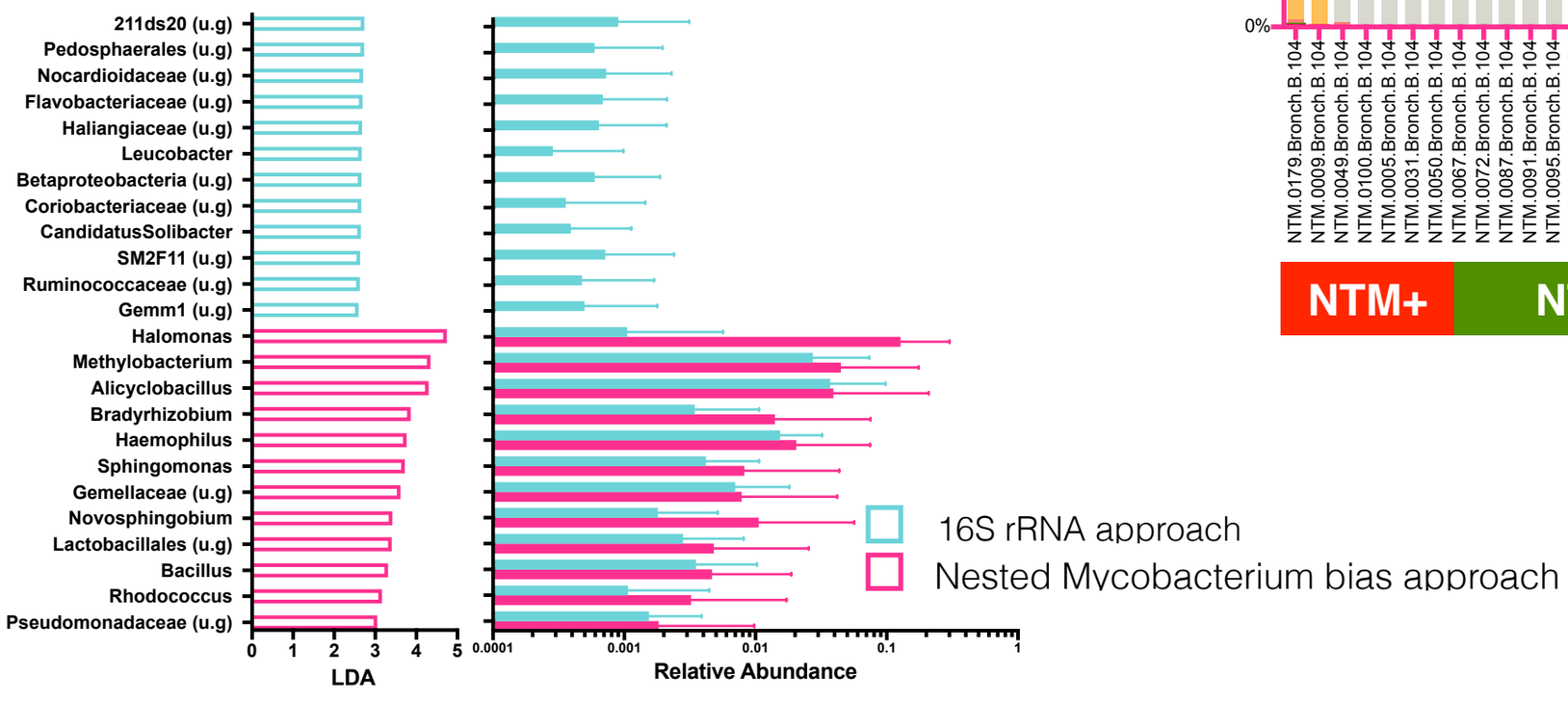
M. fortuitum	100%		10^2	10	4	2	1	1	1	1	1	1	1	1	1	1
S. pneumoniae		100%	1	1	1	1	1	1	2	4	10	10^2	10^3	10^4	10^5	10^6	10^7

Supplementary Figure S7: Comparison between sequence data obtained using an unbiased 16S rRNA approach and a biased mycobacteriome approach for equipment background samples. A. Shows the differences in β diversity (based on weighted UniFrac distance) between samples processed with unbiased 16S vs. biased mycobacteriome approach. **B.** LEFSE analysis showed enrichment of taxa identified through 16S rRNA and those identified through the biased mycobacteriome approach. **C.** Bar charts show relative abundance of OTUs annotated to Mycobacterium and their annotation based on BLAST on the two datasets.

Supplementary Figure 7

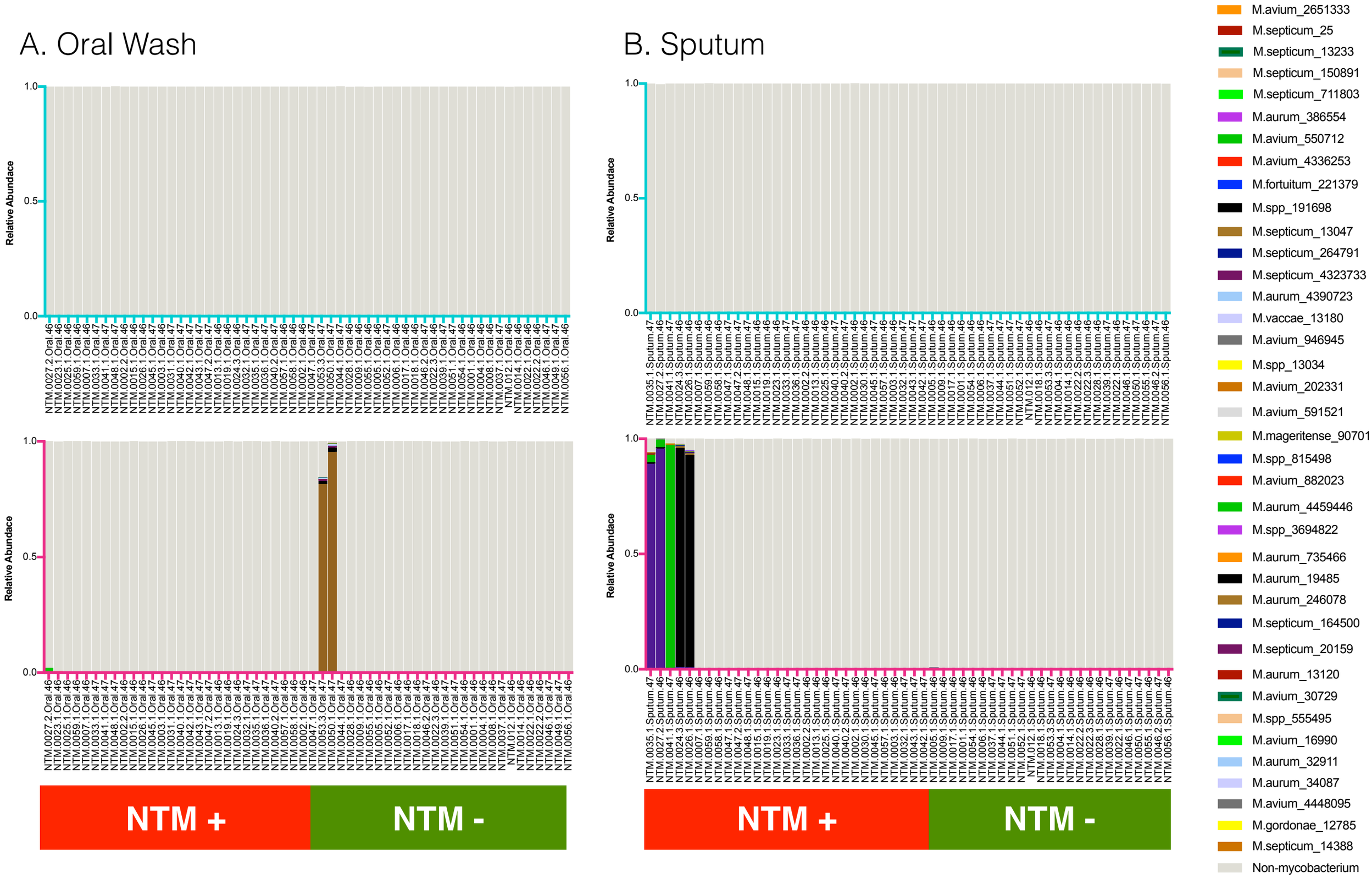






Supplementary Figure S8: Comparison between sequence data obtained using an unbiased 16S rRNA approach and a biased mycobacteriome approach for oral wash and sputum samples. A. In oral wash two samples were enriched with *Mycobacterium aurum*, a non-pathogenic *Mycobacterium* strain. Both of these samples were NTM negative on culture. **B.** In sputum 5 samples were enriched with *Mycobacterium avium*. All 5 samples were NTM positive on culture.

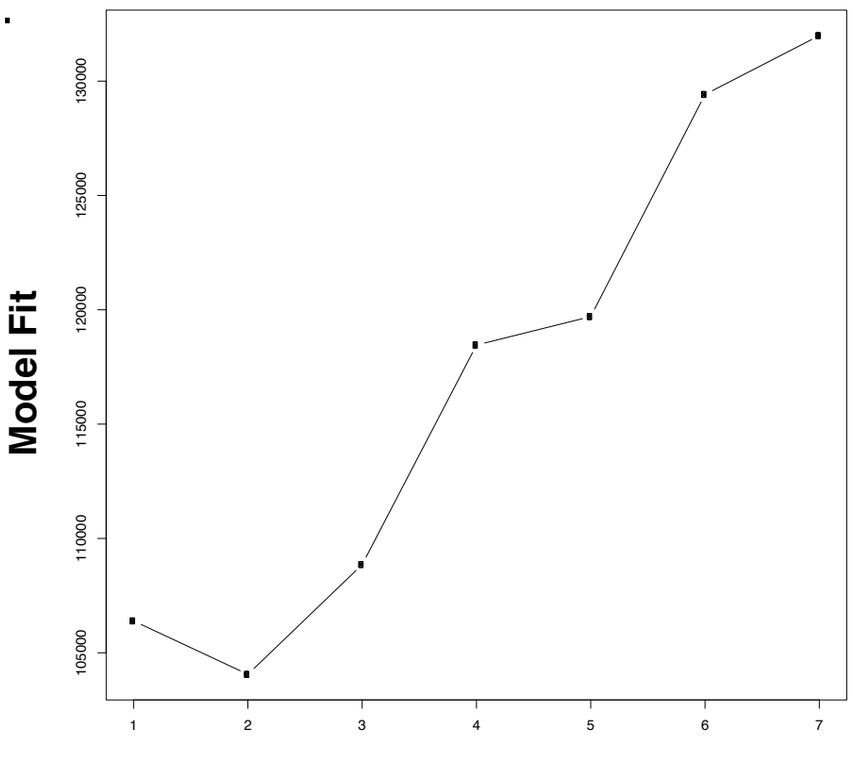
Supplementary Figure 8



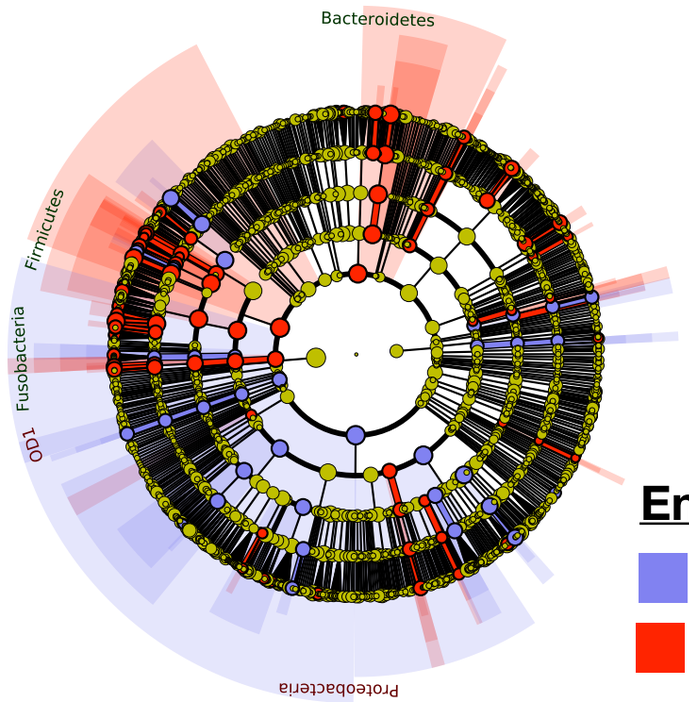
Supplementary Figure S9: Clustering of Bronchoscopy Samples by a Dirichlet Multinomial Model. **A.** Model fitness was plotted against number of clusters. Lower model fitness indicates best fitness. Two clusters were identified as having the best model fit. **B.** LEfSe analysis identified taxonomic differences in lower airway samples (BAL) between cluster 1 and cluster 2 and represented in Cladogram. **C.** LDA and differences in relative abundance of taxa at genera level found differentially enriched in BAL samples between cluster 1 and cluster 2.

Supplementary Figure 9

A.



B.

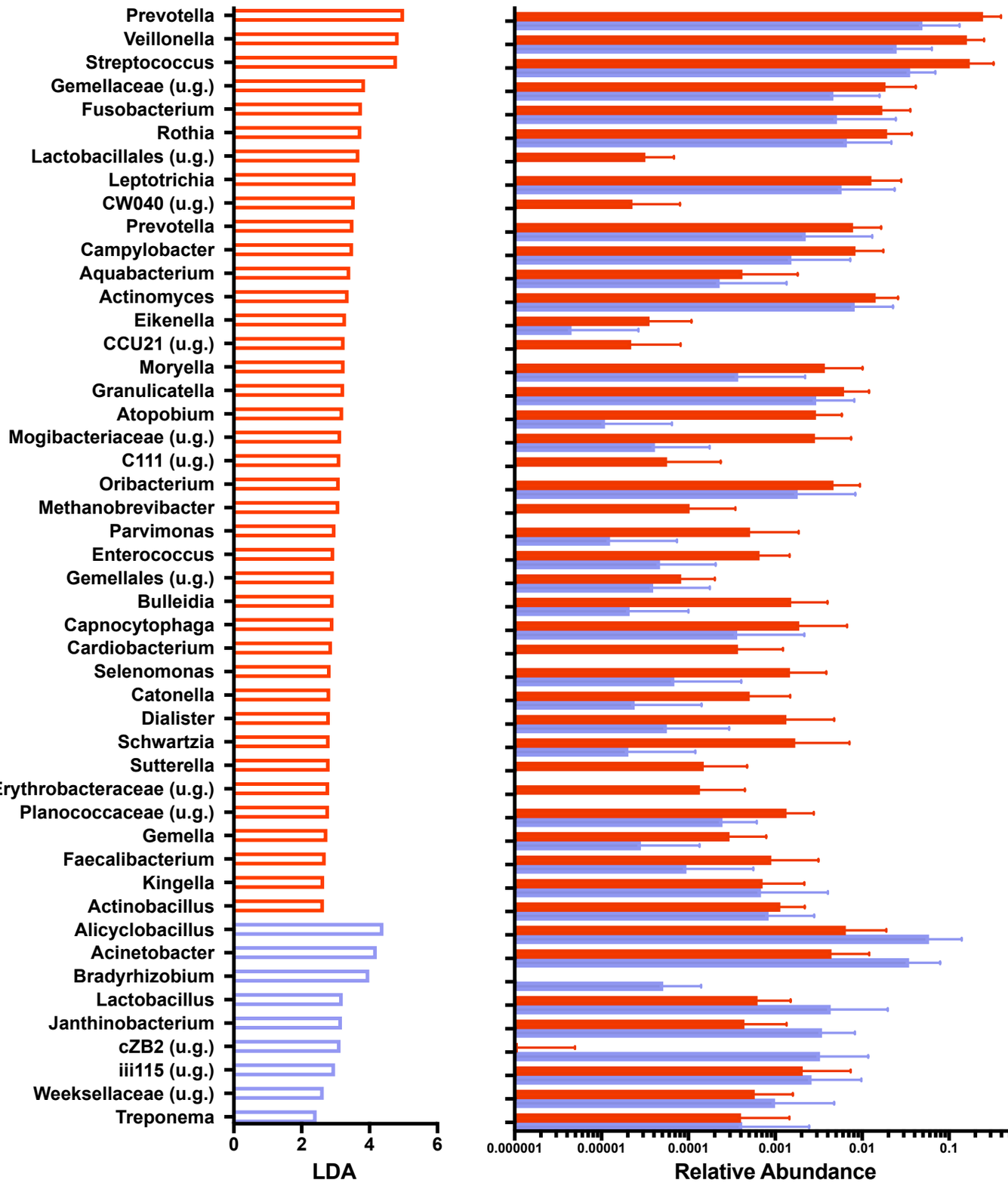


Enriched in:

Cluster 1

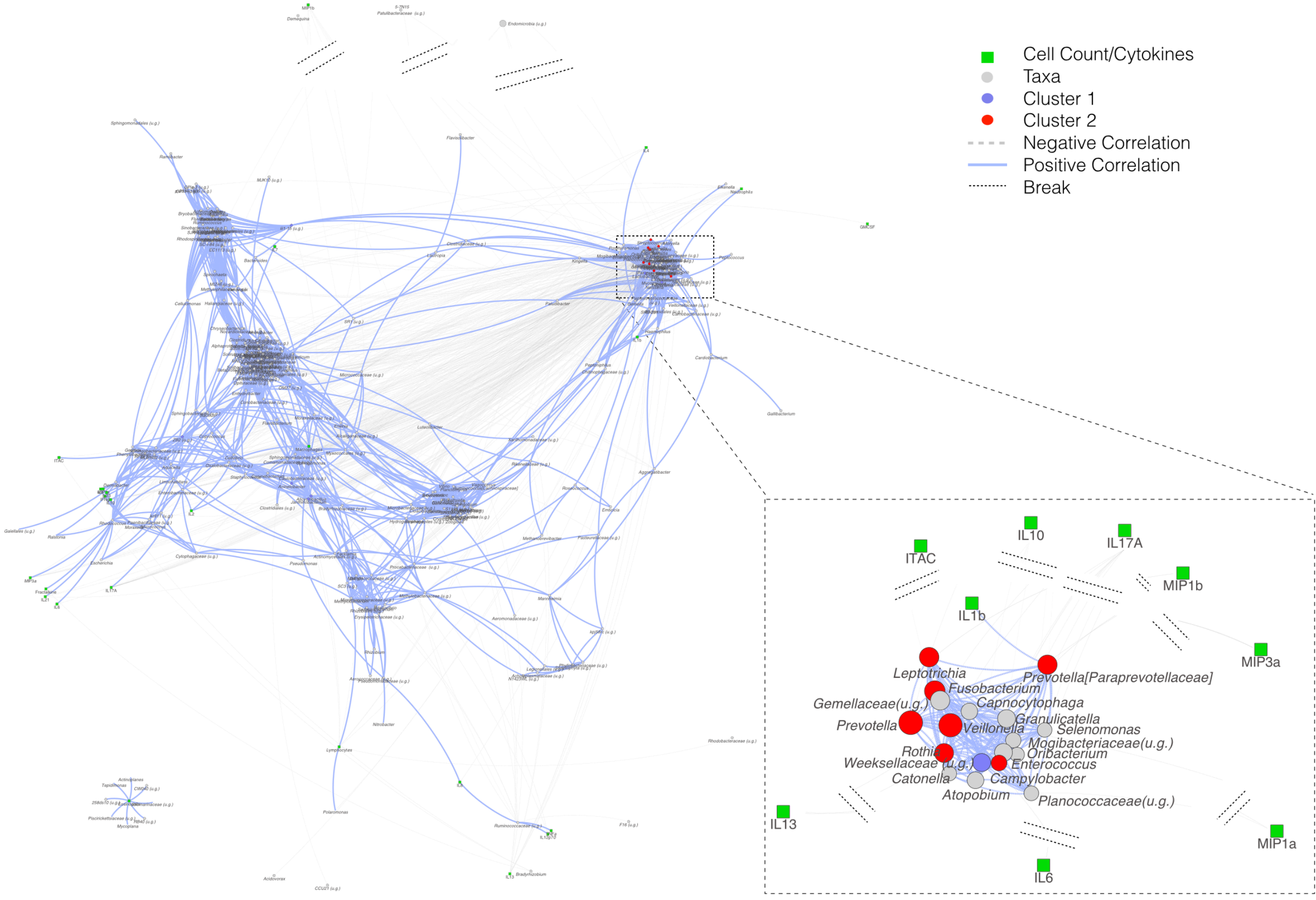
Cluster 2

C.



Supplementary Figure S10: Associations between taxa and inflammatory biomarkers in the lower airways for NTM- samples. Correlations seen with taxa identified as oral commensals and Cluster 2 (from DMM) are not seen with NTM- samples (in comparison to Figure 5)

Supplementary Figure 10



Supplementary Table S1: Oral and induced sputum samples.

	Oral Wash	Sputum	Total
Baseline	106	106	212
2-4 Months	15	13	28
5-7 Months	8	9	17
8-10 Months	4	3	7
11-13 Months	5	3	8
14-16 Months	2	4	6
17-19 Months	6	4	10
20-22 Months	3	3	6
23-24 Months	1	2	3
Total	150	147	297

Supplementary Table S2: Demographic and pulmonary function data of the bronchoscopy cohort

VARIABLES	All Patients	NTM Status (-)	NTM Status (+)	p value
N	20	12	8	
Age	63.8 (12.9)	60.3 (14.0)	69.0 (8.8)	0.46
No. Female (%)	19 (95)	11 (92)	8 (100)	0.33
No. Caucasian (%)	15 (75)	8 (42)	7 (88)	0.56
BMI	22.6 (4.8)	23.7 (5.4)	21.2 (3.6)	0.31
Packs Per Day	1.2 (0.7)	1.1 (0.7)	1.2 (1.1)	0.93
No. Years Smoking	22.1 (12.2)	21.2 (14.3)	25.0 (0.0)	0.73
Lung Physiology*				
FVC (% predicted)	95.5 (10.1)	97.7 (12.3)	93.7 (8.2)	0.50
FEV_1 (% predicted)	92.3 (10.7)	91.7 (13.5)	92.9 (8.7)	0.85
FEV_1/FVC (%)	76.2 (7.8)	74.8 (8.7)	77.4 (7.3)	0.57
RV (% predicted)	116.0 (25.4)	106.0 (22.4)	131.0 (24.5)	0.13
TLC (% predicted)	105.9 (9.0)	101.5 (7.5)	111.2 (8.2)	0.07
DLCO (% predicted)	98.1 (23.5)	105.6 (22.8)	88.8 (24.0)	0.32

* Available in 13 subjects

Supplementary Table S3: *Ex Vivo* (TLR4 stimulated) cytokine production of BAL cells in the 20 patients from the bronchoscopy cohort

	NTM- (n=12)			NTM+ (n=8)		
	Involved	Non-Involved	P value	Involved	Non-Involved	P value
Ex Vivo Cytokine Production (fold change)						
GM-CSF	12.922 [0-47.6]	89.799 [47.6-123.4]**	ns	8.284 [4.3-9.3]	33.105 [22.7-71.2]**	*0.05*
IFNγ	0.966 [0-1.8]	4.647 [3.3-15.2]**	ns	0.06 [0-0.5]	1.158 [1.1-1.3]**	*0.05*
MIP1β	4.192 [-0.1-4.7]	16.468 [12-74.6]	ns	5.231 [4.1-7.7]	13.354 [11.4-28.7]	ns
IL-23	0.145 [0.1-0.8]	1.948 [1.8-3.8]	ns	1.344 [1.2-1.4]	2.018 [1.7-2.0]	ns
MIP1α	3.134 [0-3.4]	0.2 [0.1-2.2]	ns	6.885 [3.5-7.2]	22.03 [11.4-23.0]	ns
IL-8	0 [0-0.8]	-0.052 [-0.1-0]	ns	16.841 [12.2-81.2]	122.885 [60.9-292.8]	ns
IL-5	0.147 [0-2.8]	4.2 [2.4-4.9]	ns	4.531 [2.3-6.4]	10.982 [9.5-12.7]	ns
MIP3α	3.017 [0.1-12.2]	24.74 [12.5-36.9]	ns	8.414 [6.3-21.3]	41.411 [29.1-51.9]	ns
IL-4	0.29 [0-0.4]	0.832 [0.6-1.1]	ns	0.908 [0.8-1]	0.849 [0.8-1.4]	ns
IL-6	32.907 [0-34.5]	1165.817 [589.7-1380.4]	ns	890.798 [458.9-1171.9]	866.575 [602.9-1109.1]	ns
IL-21	3.141 [-0.2-4.1]	3.719 [1.9-4.3]	ns	2.156 [1.7-2.9]	3.885 [3.6-5.6]	ns
TNFα	9.132 [0.2-10.7]	16.773 [8.4-26]	ns	15.494 [10.9-25.2]	36.424 [27.4-50.8]	ns
Fractalkine	1.982 [-0.1-2.5]	2.127 [1.2-2.8]	ns	1.364 [1.1-2.2]	2.024 [2-2.5]	ns
IL-1β	14.293 [0.1-19.1]	106.462 [54.4-252.6]	ns	11.535 [8.7-15.3]	34.318 [18.9-74.8]	ns
IL-10	9.747 [0.1-18.9]	58.508 [30.7-148.5]	ns	7.229 [5-38.4]	27.244 [16.7-41.5]	ns
IL-2	0.559 [0.5-0.7]	0.376 [0.3-0.5]	ns	-0.036 [-0.2-1.1]	0.031 [0--0.5]	ns
IL-7	3.258 [-0.1-5]	4.475 [2.3-5.6]	ns	4.231 [2.9-5.5]	4.221 [3.7-5.3]	ns
IL-13	1.087 [0.2-1.8]	1.225 [0.8-2]	ns	1.392 [1.1-1.6]	1.921 [1.7-2.2]	ns
IL-12 p70	2.765 [1.9-3.3]	6.118 [3-35.6]	ns	6.462 [4.8-9.3]	7.283 [5.7-7.6]	ns
IL-17A	0.526 [0-0.9]	0.557 [0.5-2.2]	ns	0.723 [0.6-1.3]	1.213 [0.8-1.3]	ns
ITAC	0.224 [0.1-0.4]	0.111 [0.1-1.5]	ns	-0.14 [-0.2-0.2]	0.756 [0.5-0.9]	ns

Data represented as Median [IQR]. p-value based on Mann Whitney. *Comparing involved sites by NTM status. **Comparing non-involved sites by NTM status