Online supplement

The dynamics of the pulmonary microbiome during mechanical ventilation in the intensive care unit and the association with occurrence of pneumonia

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Methods

Results

Tables: 2

Figures: 6

Supplemental methods

DNA extraction and amplicon library preparation

Total DNA was extracted from 111 ETA samples using the PowerSoil DNA Isolation kit (Cat. No. 12888-100, MoBio, USA) following the user manual guide. The total DNA amount and purity were evaluated using NanoDrop2000 (ThermoScientific, USA). Bacterial DNA from the samples was quantified by qPCR amplification of the 16S rRNA gene. Primers F-16S-27 (5'-AGAGTTTGATCCTGGCTCA G-3'), R-16S-355 (5'-GCTGCCTCCCGTAGGAGT-3') and probe P-16S-univ-2 (6FAM-CTGGCGGCRKGCYTAACACATGCAAGTCGA-BHQ1) targeting a conserved region of the gene were used. Amplification was carried out in a LightCycler 480 PCR System (Roche Diagnostics, Branford, CT, USA) as followed: 2 min at 50°C for UNG activation; 10 min at 95°C for initial denaturation; 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s.

DNA was diluted to equal concentrations of 100pg/µl and 2 µl was used as template for amplification using primers F-16S-27 and R-16S-355. The reactions were performed using 20 µl (final volume) mixtures containing 10 µl of 10x LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Branford, CT, USA), 2 mM MgCl2, 0.9 µM of each primer, and 0.01U of Uracil N-glycosylase (Fermentas, Thermo Scientific, Waltham, MA, USA). After UNG activation step (50°C for 2 min) and initial denaturation (95°C for 10 min), 30 cycles of amplification consisting of denaturation at 95°C for 15 s, annealing at 50°C for 60 s, and extension at 72°C for 60 s, followed by a final extension step at 72°C for 10 min were carried out.

The resulting amplicons were diluted 1 in 100 in TE and 4 µl of dilution was used as template for addition of barcodes and 454 adapters. The forward primers were tagged at

the 5’ end with a 10 bp barcode specific to each sample. The reactions were performed using 20 µl (final volume) mixtures containing Faststart Taq DNA Polymerase PCR Buffer 1x, 2 µg bovine serum albumin, 2.5 mM $MgCl_2$, 0.8 mM of each deoxynucleoside triphosphate, 0.9 µM of each primer, and 1U of FastTaq DNA polymerase (Roche, Branford, CT, USA). The initial denaturation step was 95°C for 10 min, followed by 10 cycles of denaturation at 95°C for 15 s, annealing at 50°C for 60 s, and extension at 72°C for 60 s, followed by a final extension step at 72°C for 10 min.

The barcoded PCR products were purified using the Agencourt AMPure XP (Beckman Coulter, Woerden, NL) and quantified using the Quant-iT dsDNA Assay Kit on a Qubit fluorometer (Invitrogen). Emulsion PCR was performed according to the protocol (emPCR Method Manual – Lib-A SV jan- 2010) supplied with the GS FLX Titanium XLR 70 Se- quencing kit (Roche Diagnostics, Indianapolis, IN, USA). Two- region 454 sequencing run was performed on a GS FLX Titanium Pico TiterPlate (70 × 75) using a GS FLX Titanium Sequencing Kit XLR70 according to the manufacturer’s instructions (Roche Diagnostics, Indianapolis, IN, USA). Data processing and analysis Data analysis was performed using the software “Quantita- tive Insights into Microbial Ecology” (QIIME 1.8.0). After removal of low quality reads (quality score <25) and chimera (ChimeraSlayer) our data was denoised using the QIIME denoiser program. Then we clustered the sequences into Operational Taxonomical Units (OTU’s) based on 97 % sequence similarity (Uclust). The resulting OTU table was then condensed by removing all OTUs representing less than 0.005 % of the total number of sequences. Uclust classifier was used to assign taxonomy. FastTree was used to construct a phylogenetic tree for downstream analyses.

Details on statistical analyses

Statistical analysis was performed in R (version 3.2.3) via the R-studio interface and topological analysis was performed in Cytoscape (version 3.3.0) (23). The relative abundance table ranked by order and a correlation matrix for the abundances of the selected taxonomic orders were imported into R and used for the downstream analysis. Following R libraries were used: *plyr* and *reshape2* (data manipulation) *ade4* (principal component analysis), *lme4* (linear mixed effect model), *vegan* (biodiversity index), *ggplot2* (data visualization). All sequenced samples were used to study the influence of duration of mechanical ventilation and the administration of antibiotics in a linear mixed effect model using the following formula "*lmer*(*diversity ~ duration of mechanical ventilation + antibiotic exposure (1|patient identification number))"* with the *lme4* package in R.

Supplemental results

The DNA concentration of the samples was measured by qPCR as described in the Material and Methods section. All samples showing a qPCR Cp value at least 3 Cp inferior to the negative control (corresponding to approximately 13000 16S rRNA gene copies per µl) were kept for further analysis.

The total output for the sequencing of 120 samples and a negative control (sterile water) were 1307829 sequenced. After quality trimming, removal of duplicates, human genome sequences and chimeras a total of 643392 sequences (49.2%) remained for analysis. The sequence count per sample ranged from 157 to 11769 with a median of 5433, a mean of 5361 and a standard deviation of 2183.9. Seven samples had a sequence count more than 2 standard deviations inferior to the mean (sequence count:

157 to 448) and were thus not used for following analyses. Consequently, the number of sequences per sample was normalized to 2315 for alpha-diversity analyses, corresponding to the lowest sequence count in the remaining samples.

Using a 97% similarity cutoff, a total of 17150 OTUs were found across all samples. 10265 OTUs were represented by a single sequence. The number of OTUs ranged from 250 to 903 per sample (median = 499). 369 OTUs regrouped 90% of the sequences, and only 21 OTUs regrouped 50% of the sequences.

The taxonomic analysis assigned the sequences to 99 family-level bacterial taxa, belonging to 19 different phyla. In order to assess the relevance of the chosen sequencing depth, rarefaction plots were generated at different ranges (11 equal steps ranging from 10 to the median sequence count per sample) for all samples using 3 different diversity measures: number of observed species, chao1, phylogenetic distance (the minimum total length of all the phylogenetic branches required to span all the sample’s taxa on the phylogenetic tree).

Table S1: Antibiotic therapy per group

	Colonization 315 days	Control 415 days	HAP/CAP 130 days	VAP 1206 days
3rd GENERATION CEFALOSP.	0	0	0	0.7
Aminoglucoydes. other	0	0	0	0
AMINOGLYCOSIDES	0	0	0	0
Amoxyciline+Clavulanate	1.6	0.2	8.5	1.9
Amphotericin B	0	0	0	0
Amphotericin B lipid formulation	0	0	0	0
Ampicillin	0.6	0	0	0
Amykacine	0	0	0	1.7
Antifungal. other	0	0	0	0
Antimicobacteria antibiotics	0	0	0	0
Antiviral agents	0	0	0	0
Aztreonam	0	0	0	0
Azytromicin	3.8	0	0	0
B-lactamic. other	0	0	0	0
Benzylpenicillin	0	0	0	0
CARBAPENEMS	0	0	0	0.4
Caspofungine	0	0	0	0
Cefazoline	0	0	0	0
Cefepime/Cefpirome	0	1	0.8	0.7
Cefotaxima	1.6	0	0	0
Ceftazidime	0.6	0	0	0
Ceftriaxone	3.8	0	0	0.3
Cefuroxime	0	0	0	0
Cephalosporin. other	0	0	0	0
Ciprofloxacine	0	0	0	0.1
Clindamicina	0	1.2	0	0.7
Cloxacilline	0	5.1	0	1.2
Cotrimoxazole	0	0	0	0.1
Daptomicina	0	0	0	0
Ertapenem	0	0	0	0
Erytromicin	0	0	0	0
Fluconazole	0	0	0	0.6
Gentamycine	0	0	0	0
Levofloxacine	0	1.2	0	0.8
Linezolyd	1.6	0	0	2
Macrolides. other	0	5.1	0	0
Meropenem/Imipenem	10.5	6.5	0	2.7

Metronidazole	0	0	0	0
Penicillin. other	0	0	0	0
PIPER./TAZOB.	1.3	1.7	0	1.7
QUINOLONES	0	0	0	0
Quinolones. other	0	0	0	0
Tamiflu	0	0	0	0
Teicoplanin	0	0	0	0
Temocillin	0	0	0	0
Tigeciclina	0	0	0	0
Tobramycine	0	0	0	0.2
Vancomycin	5.4	1.4	0	0.5

Percentage of the days that the group was exposed to a certain antibiotic therapy.

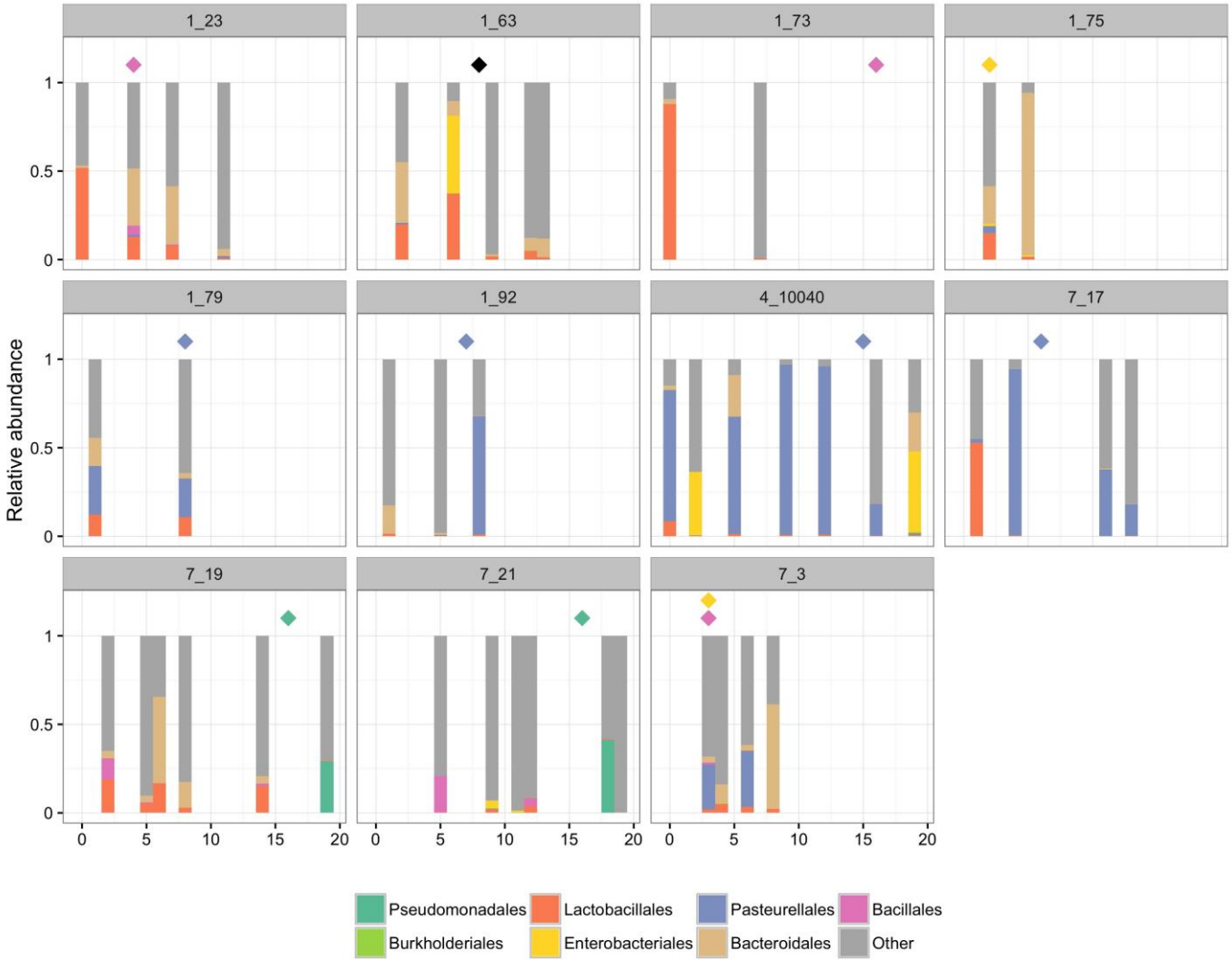
Table S2: Relative abundance of cultured pathogens in metagenomic analysis.

Pathogen	Order	Relative abundance
Acinetobacter baumanii	Pseudomonadales	0.0019
Acinetobacter baumanii	Pseudomonadales	0.4117
Acinetobacter baumanii	Pseudomonadales	0.0003
E. Coli	Enterobacteriales	0.1170
E. Coli	Enterobacteriales	0.0003
E. Coli	Enterobacteriales	0
Enterobacteriae	Enterobacteriales	0
Gram negative rod	Enterobacteriales	0.0146
Haem influenzae	Pasteurellales	0.6796
Haem influenzae	Pasteurellales	0.2170
Haem influenzae	Pasteurellales	0.6696
Haem influenzae	Pasteurellales	0.1795
Haem influenzae	Pasteurellales	0.9386
Kleb pneumoniae	Enterobacteriales	0.2906
Moraxella catheralis	Pseudomonadales	0.0009
Pseudomonas aeruginosa	Pseudomonadales	0.0004
Staph aureus	Bacillales	0.0510
Staph aureus	Bacillales	0.3739
Staph aureus	Bacillales	0.0002
Staph aureus	Bacillales	0.0007
Staph aureus	Bacillales	0.0114
Staph aureus	Bacillales	0
Staph aureus	Bacillales	0
Staph aureus	Bacillales	0.0018
Staph aureus	Bacillales	0.2109
Staph aureus	Bacillales	0.0937

Figure S1: Per patient display of administrated antibiotics.

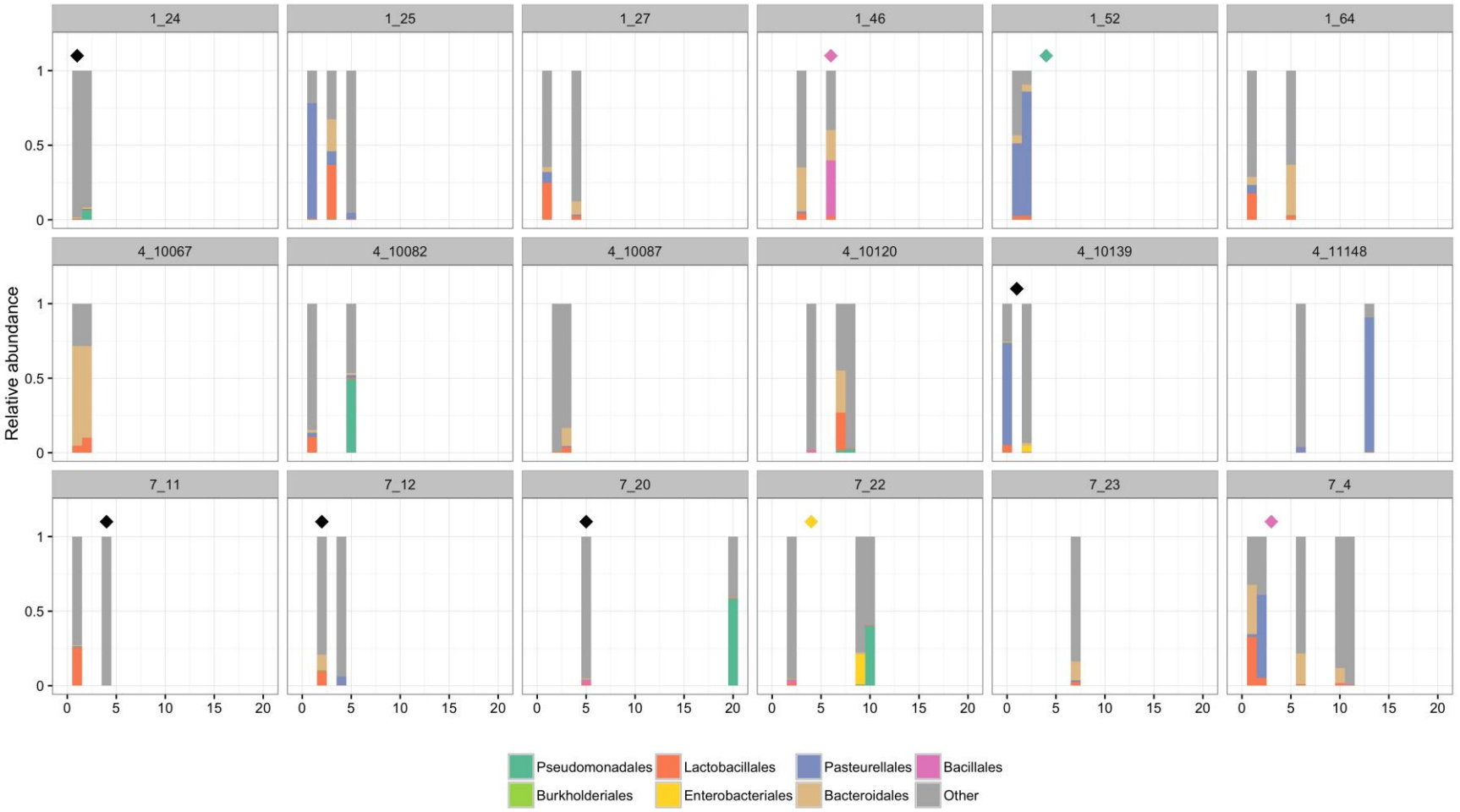
Per patient summary of antibiotic administration. Every dot displays an antibiotic given on a certain day.

Figure S2: Microbiome dynamics during mechanical ventilation in patients that developed VAP.



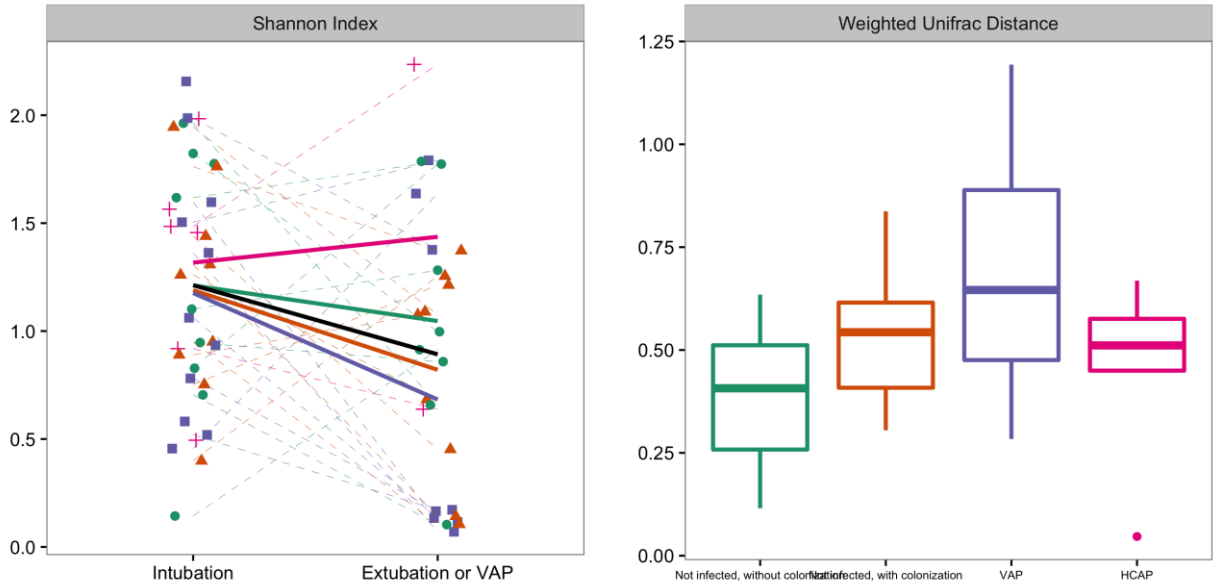
OTU dynamics during mechanical ventilation. The dots above the bars indicate culture results, for indication colours see legend.

Figure S3: Microbiome dynamics during mechanical ventilation in patients that didn't develop VAP.



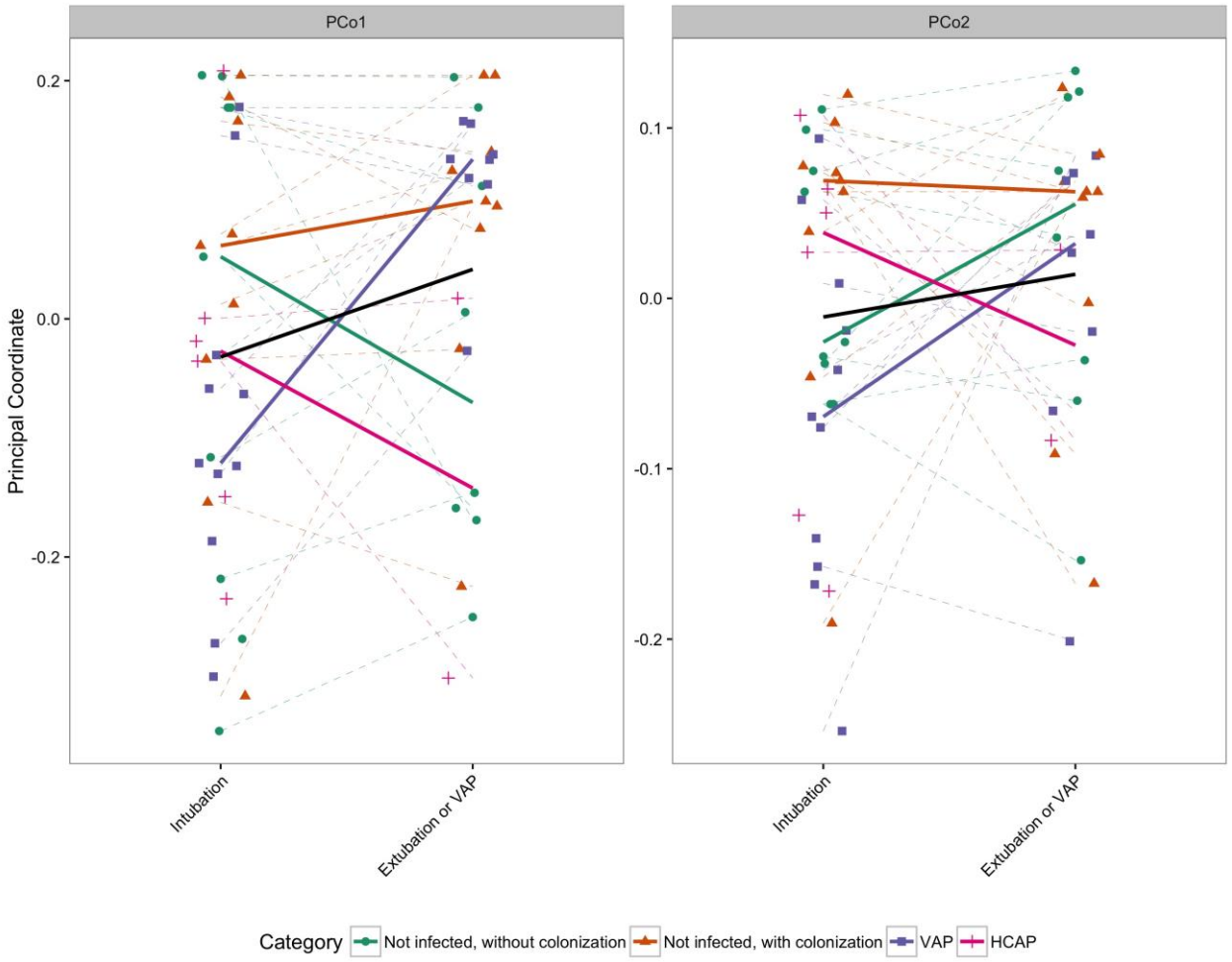
OTU dynamics during mechanical ventilation. The dots above the bars indicate culture results, for indication colours see legend.

Figure S4: Changes in diversity.



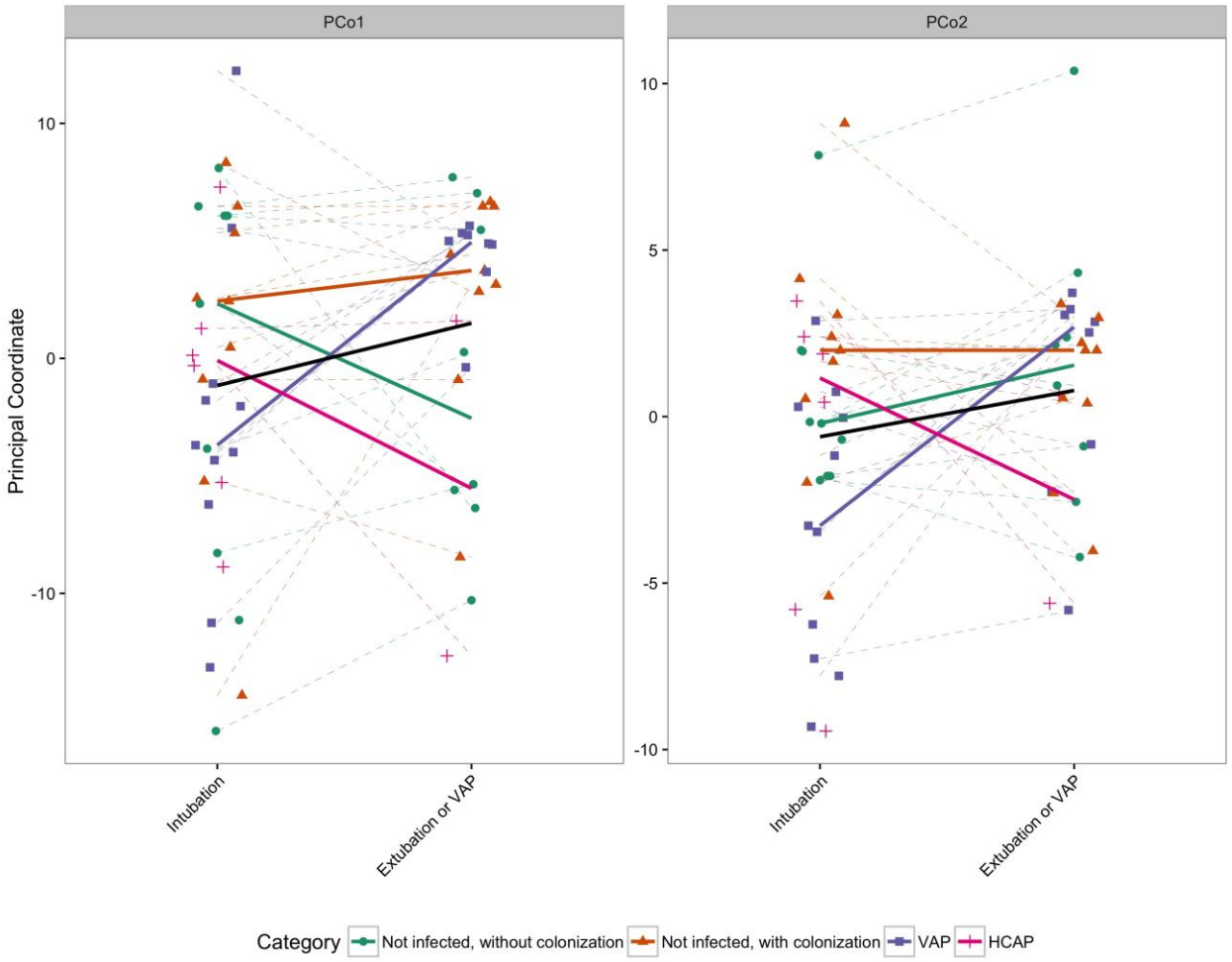
Left graph: Changes in diversity between two time points; sample just taken after initiation of mechanical ventilation and before extubation or at the development of VAP, whatever came first. Shannon diversity is a measure of alpha diversity. Right graph: Weighted Unifrac distance between these two samples, represented per group. The trend lines per patient group are visualized in dark green for non-infected patients without colonization, in red for non-infected patients with colonization, in purple for patients that developed VAP and in pink for patients that developed pneumonia within the first 48 hours of mechanical ventilation.

Figure S5: Changes in beta diversity; Bray-Curtis distance summarized by principal coordinates.



Changes in diversity between two time points; sample just taken after initiation of mechanical ventilation and before extubation or at the development of VAP, whatever came first. Bray-Curtis distance is a measure of beta diversity, which is summarized into two principal coordinates. The trend lines per patient group are visualized in dark green for non-infected patients without colonization, in red for non-infected patients with colonization, in purple for patients that developed VAP and in pink for patients that developed pneumonia within the first 48 hours of mechanical ventilation.

Figure S6: Changes in beta diversity; Manhattan distance summarized by principal coordinates.



Changes in diversity between two time points; sample just taken after initiation of mechanical ventilation and before extubation or at the development of VAP, whatever came first. Manhattan distance is a measure of beta diversity, which is summarized into two principal coordinates. The trend lines per patient group are visualized in dark green for non-infected patients without colonization, in red for non-infected patients with colonization, in purple for patients that developed VAP and in pink for patients that developed pneumonia within the first 48 hours of mechanical ventilation.