Author’s response to reviews

Title: Dynamics of microbiota during mechanical ventilation in aspiration pneumonia

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Author’s response to reviews:

Dear, Mr. Rune Nielsen,

Thank you for carefully reading our manuscript entitled “Dynamics of microbiota during mechanical ventilation in aspiration pneumonia” (PULM-D-19-00238) and for giving insightful comments which have helped us significantly improve our original research. We have read the informative comments very carefully, and have rewritten our manuscript according to those comments. We are hereby sending a revised version of our manuscript.

Our point-by-point responses to the reviewer’s comments are below.

We hope that our responses are satisfactory, and that our manuscript is now acceptable for publication in BMC Pulmonary Medicine. We are looking forward to your favorable consideration.

Sincerely yours,

Ken Otsuji M.D.

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Dear Mr. Gisli G Einarsson (Reviewer #1),

Thank you for carefully reading our manuscript entitled “Dynamics of microbiota during mechanical ventilation in aspiration pneumonia” (PULM-D-19-00238) and for giving insightful comments which have helped us significantly improve our original research. We have read the informative comments very carefully, and have rewritten our manuscript according to those comments. We are hereby sending a revised version of our manuscript.

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Sincerely yours,

Ken Otsuji M.D.

Responses to the Reviewer’s (Reviewer #1) comments:

Gisli G Einarsson (Reviewer #1): General comments

In the following submitted manuscript (PULM-D-19-00238) titled "Dynamics of microbiota during mechanical ventilation in aspiration pneumonia" by Ken Otsuji et al. (2019) the authors describe microbial community composition of the lower and upper respiratory tract in intubated patients diagnosed with aspiration pneumonia, with a further aim of developing treatment strategies against infecting microorganisms. In the study the authors applied 16S rDNA marker-gene cloning methodologies to assess the community composition of the relevant clinical specimens that were collected at predefined time-points during the intubation process. Overall I find this study interesting, especially the longitudinal design and that sampling was both carried out in a paired fashion of the upper and lower airways were possible. However, there are number of limitations to the current manuscript that in my mind would need to be addressed prior to the manuscript further progressing towards publication in BMC Pulmonary Medicine.

Major points:

Comment 1).
In the current manuscript any limitations related to 16S rDNA clonal libraries are missing. For example, how does this methods compare to more recent advantages made through 16S rRNA marker-gene sequencing on the 454-FLX or Illumina MiSeq platforms? Would 16S rDNA
cloning based approaches offer significantly better and more cost-effective ways of assessing microbial community composition than such more modern approaches? I guess that despite potentially allowing better phylogenetic/taxonomic assignment through Sanger sequencing of the longer 16S rRNA gene sequences from the clonal libraries (up-to near full length 16S rRNA gene), biases based on only being able to pick a limited number of clones per sample, the vector may preferentially ligate to one particular sequence type over another, high workload and cost would be a limitation in using the described methodology. This is potentially something that the authors could comment on in discussions.

Response 1).

As you pointed out, using the Sanger sequencing method is one of the limitations of this study. Abundance of nucleotide sequence information is available from NGS (next generation sequencer), and it is commonly used for analysis of human microbiota. On the other hand, there are many kinds of Streptococcus spp. which most NGS cannot classify at the species level in the oral and respiratory tract (Respir Investig. 2018; 56: 432-9). We mentioned the limitations of the Sanger method and sequencing method in a new paragraph at discussion as follows.

Line 285-293 (Discussion)
“Second, the use of the Sanger sequencing method for analysis of the bacterial 16S rRNA gene is another limitation in this study. Recently, NGS (next generation sequencer) is commonly used for analysis of human microbiota (15). An important advantage of NGS is the abundance of available nucleotide sequence information (37). The number of clones identified by the Sanger method in this study is 96 at maximum per sample and significantly fewer than NGS; this is the essence of the limitation. However, there are many kinds of Streptococcus spp. in oral and respiratory tract which most NGS cannot classify at the species level (38) because their sequences of 16S rRNA genes are too similar. The Sanger method used in this study is targeting 550 bp of the gene including three variable regions (V3, V4 and V5), so that it is possible to classify bacteria in more detail.”

Comment 2).
From the current manuscript it is not entirely clear to me how the approaches employed in the current study can infer how best to treat putative bacterial infections in the lower airways of patients diagnosed with aspiration pneumonia. The data shows that there is heterogeneity in community composition, and that this appears to change over time. However, stating that opportunistic pathogens such as Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus etc. are assumed to be resistant to administrated antibiotics is probably an oversimplification. The populations of those clinically relevant taxa may be quite diverse in relations to their responses to treatment, and although many will produce beta-lactamases (incl. ESBL) or other determinants of putative antibiotic resistance there is unlikely to be a single antibiotic treatment approach that fits all members of those taxa. Without testing this in some way, such as through susceptibility testing against a range of antibiotics, we can't speculate or propose what the best treatment option will be. If the authors are eluting towards that we should be using more personalised approaches, rather than the combination of Ampicillin/Sulbactam
which is the most commonly prescribed option here, then that should be highlighted within the manuscript.

Response 2).
As you pointed out, explanation about what kind of treatment approach could be reflected from the results of this study was insufficient. We modified the sentence as follows.

Line 272-276 (Discussion)
“Considering the results of this study, antibiotics should be selected on the premise that dynamic changes in microbiota (involved in the reduction of anaerobes in the lower respiratory tract) may occur during mechanical ventilation in intubated patients with aspiration pneumonia. It was suggested that antibiotic treatment for anaerobes in these cases may not be appropriate. Additional pathogenic investigation should be conducted after over 3 h from initiating mechanical ventilation.”

As you mentioned, it was inappropriate expression, we deleted the sentence “These bacteria are assumed to be resistant to the administered antibiotics” in abstract. In addition, we have corrected the content following your suggestion in discussion as follows.

Line 263-268 (Discussion)
“Although antibacterial susceptibilities of causative bacteria—except the isolates—were not evaluated in this study, these bacteria are generally associated with antimicrobial resistance. These bacteria might be major pathogens when initial treatment is ineffective. However, after the administration of antibiotics in this study, the causal relationship between patient background and predominantly detected bacteria was not clear. Since most of these bacteria can be detected by culture method, a personalized approach using the culture method is a practical strategy.”

Minor Points:
Comment 3).
In the manuscript "facultative anaerobes" and "facultative aerobes" are used interchangeably when talking about the same main taxa. The authors should use consistent terminology where appropriate.

Response 3).
Thank you for pointing out. We corrected the term “facultative aerobes” to “facultative anaerobes”.

Comment 4).
Page 5, line 100) stating that "The tracheal aspirate (B) were cultured aerobically at 35C with 5%CO2…". This would be microaerophilic environmental conditions, not aerobic.

Response 4).
Thank you for pointing out. We corrected “aerobically” to “microaerobically”
Comment 5).
Page 12, lines 191-193, stating "Since culture-based methods cannot sufficiently analyse the causative bacteria in aspiration pneumonia..." is not strictly speaking correct as there are number of culture-based approaches that might be useful in this context. In clinical laboratories bacterial cultures attempt to capture a wide range of potentially pathogenic microorganism, including bacteria and fungi. This however can be time consuming and does not allow "rapid" detection of putative infectious agents. Using extended bacterial cultures, i.e. with a number of different bacteriological media and atmospheric conditions (anaerobic atmosphere generally not applied routinely), would allowed the capture of most of the microorganism and furthermore allow for their phenotypic analysis. In this context using 16S rDNA cloning and Sanger sequencing to assess the microbial community composition would not allow for the assessment of functional capabilities of individual members of the community, or the community as a whole, and hence cannot predict the response to antimicrobial therapy with certainty. It would be appreciated if the authors could comment on those limitations when using molecular based profiling.

Response 5).
We modified the sentences following your suggestion.

Line 199-203 (Discussion)
“Recently, DNA sequencing technologies have advanced and have been applied to clinical specimens—although 16S rRNA gene sequencing cannot evaluate certain properties of the detected bacteria (such as drug sensitivity, it is suitable for evaluating bacterial flora and pathogenic bacteria). Because highly diverse microbial communities including anaerobes and indigenous oral bacteria harbor in aspiration pneumonia, 16S rRNA gene sequencing analysis is frequently used to evaluate the causative bacteria [20-22].”

Comment 6).
Page 12, line 196, the use of the word "vicissitude", I would recommend that the authors would use other commonly used words such as "change", "alteration", "modification" etc.

Response 6).
We rephrased the word “vicissitude” to “alteration”.

Dear Mr. Benjamin Wu (Reviewer #2),

Thank you for carefully reading our manuscript entitled “Dynamics of microbiota during mechanical ventilation in aspiration pneumonia” (PULM-D-19-00238) and for giving insightful comments which have helped us significantly improve our original research. We have read the informative comments very carefully, and have rewritten our manuscript according to those comments. We are hereby sending a revised version of our manuscript.

Our point-by-point responses to the reviewer’s comments are below.
We hope that our responses are satisfactory, and that our manuscript is now acceptable for publication in BMC Pulmonary Medicine. We are looking forward to your favorable consideration.

Sincerely yours,

Ken Otsuji M.D.

Responses to the Reviewer’s (Reviewer #2) comments:

Benjamin Wu (Reviewer #2): General comments

Otsuji et al. and authors present an article titled "Dynamics of microbiota during mechanical ventilation in aspiration pneumonia", whereby they demonstrate by using an 16s RNA gene clone library the microbiota dynamics of mechanical ventilation on the lower airway and upper airway of subjects. They show that the act of intubation can have changes to the lung microbiome (via tracheal aspirates), but they also show that there is a depletion of anaerobic bacteria and with the start of IV antibiotics the lower airway microbiome becomes enriched with taxa with an increased potential to be antibiotic resistant.

I have several commendable points to the article: 1) The authors do a very good job with specimen collection and time keeping. If the collection is at the time that authors specify, this is one of the first manuscripts that describe changes to the lung microbiota at certain time points and raises several mechanistic questions to the development of HCAP and VAP. 2) The rigorous clinical data re: reasons for intubation and mechanical ventilation - the authors seem to select their subjects/participants very carefully and captures hopefully a specific group of patients. Finally, 3) The manuscript is overall seems to give specific insight into mechanistically how subjects may develop VAP and I believe that the manuscript has slight overstatements, but overall is coherent and offers a mechanism (e.g., depletion of healthy normal anaerobic microbiota in the airway and possibly a selection pressure of antibiotics on the lower airway microbiota). One important point, but I am unsure where this would go is the trachea width point that the authors bring up - I am not sure what the relevance or the research to back up their statements.

Comments 1)-6)
There are several weaknesses, by which are addressed here, but also within the criticisms below. 1) The clonal 16S rRNA library that the authors created and then utilize PCOA and multi-dimensional visualizations to assess may be short-sighted. Using 16S rRNA microbiome via 16S rRNA gene amplification (Illumina MiSeq) and collection may capture more enrichment as microbiota with less presence in the microbiome (less enriched) may be missing from this analysis. This isn't a criticism of the 16S rRNA library clones that the authors have created, it is
pointing out the limitations of their analysis. 2) Small sample size: This is a major criticism which I am not sure if the authors can change, but there is a small n which the authors are making conclusions upon. Most of their discussion is supported by their work, I would just make sure that the small sample size is emphasized in the criticisms/weakness in their discussion. 3) There is a lack of multivariate analysis, there is plenty of clinical data especially with good time keeping, but have the authors controlled for time spent on vent/age/antibiotics/FiO2, length of hospital stay/length of intubation? I have not seen any multivariate analysis - for example, it would be interesting to see if those subjects with Enterococcus in their lower airway started with a small percentage of Enterococcus. Moreover, if the presumption made by the authors is true, there should be analysis performed by performing a regression between time on the ventilator and %anaerobes. 4) The authors should state that they did not obtain any background samples - I would mention that clearly or if they did and were not able to produce clones, I would mention that as well. 5) Finally, the authors should review their figures - supplemental figure s1 may benefit from non-hierarchical heatmaps. In my opinion, the main title figures tell us very little (especially the PCoA) which do not tell us anything at all. 6) One important time point I would like to see is how long the patients were admitted for prior to intubation.

Response 1).
As you pointed out, using the Sanger sequencing method is one of the limitations of this study. An abundance of nucleotide sequence information is available from NGS (next generation sequencer), and it is commonly used for the analysis of human microbiota. On the other hand, there are many kinds of Streptococcus spp, in the oral and respiratory tract, which most NGS cannot classify at the species level (Respir Investig. 2018; 56: 432-9). We mentioned the limitations of the Sanger method and sequencing method in a new paragraph in the discussion section as follows.

Line 285-293 (Discussion)
“Second, the use of the Sanger sequencing method for analysis of the bacterial 16S rRNA gene is another limitation in this study. Recently, NGS (next generation sequencer) is commonly used for analysis of human microbiota (15). An important advantage of NGS is the abundance of available nucleotide sequence information (37). The number of clones identified by the Sanger method in this study is 96 at maximum per sample and significantly fewer than NGS; this is the essence of the limitation. However, there are many kinds of Streptococcus spp. in oral and respiratory tract which most NGS cannot classify at the species level (38) because their sequences of 16S rRNA genes are too similar. The Sanger method used in this study is targeting 550 bp of the gene including three variable regions (V3, V4 and V5), so that it is possible to classify bacteria in more detail.”

Response 2).
We emphasized the small sample size which is the main limitation of this study in the discussion section. We added the following sentences.

Line 282-285 (Discussion)
“There are several limitations in this study. First, the sample size was small, which is one of the major limitations in this study. Because the sample size is small, the dynamics of microbiota
during mechanical ventilation in aspiration pneumonia could not be fully evaluated. It is difficult to say that sufficient information has been obtained for treatment strategy from this study.”

Response 3).
As you suggested, we obtained other clinical data such as vasopressin use, hospital stay, mechanical ventilation days, ICU stay, mortality, and adjusted for multivariate analysis. However, they did not change the result. Relationship between mechanical ventilation time and proportion of anaerobe was evaluated using incident rate ratios estimated by a multilevel Poisson regression model. We modified the description following your suggestion.

Line 183-196 (Result)
“There was no significant difference in the proportion of anaerobes between saliva (A) to (B) (IRR: 0.97, 95% CI 0.85-1.12, p=0.76). On the other hand, the proportion of anaerobes were significantly lowered between tracheal aspirate (A) to (B) (IRR: 0.34, 95% CI 0.26-0.45, p&lt;0.001). Fig. 3 shows the changes in proportion of anaerobes in tracheal aspirate from (A) to (B) during mechanical ventilation (individual cases). As a result of multivariate analysis using clinical factors such as FiO2, tracheal diameter, vasopressor use, ICU stay, mechanical ventilation days, hospital stay and mortality, there was no significant relationship between the proportion of anaerobes and these clinical factors. However, the extent of decrease in anaerobes was fully dependent on the time difference between the sampling of tracheal aspirate (A) and (B). There was no significant decrease of anaerobes in the tracheal aspirate (B) collected within 2 h after collecting tracheal aspirate (A) (IRR: 1.23, 95% CI 0.73-2.05, p=0.43); significant decrease in anaerobes occurred in tracheal aspirate (B) collected 3 to 10 h after collecting tracheal aspirate (A) (IRR: 0.22, 95% CI 0.13-0.38, p&lt;0.001) and collected 11 h or longer after collecting tracheal aspirate (A) (IRR: 0.23, 95% CI 0.15-0.35, p&lt;0.001). It is noteworthy that most anaerobes were not detected in tracheal aspirate (B) which was collected 11 h or longer after collecting (A) (Fig. 3).”

Response 4).
Although we analyzed the samples with control (PBS used for dilution), we did not obtain background samples. We described about this limitation in discussion as follows.

Line 293-302 (Discussion)
“Third, background samples were not obtained during sequence analysis in this study. When using sequencing techniques to assess microbiota in low biomass environments, care should be taken regarding the potential contamination of background (39). In this study, the DNA extract from only PBS (without specimen) was used as the control. No obvious band (with 2% agarose gel electrophoresis analysis) was detected from the control sample after the PCR (30 cycles) and the cloning-sequencing analyses were performed with only PCR positive samples. Since significant amplification was not observed from the control samples and most bacterial phylotypes detected in this study are commonly found in the upper and lower respiratory tract, the contamination of the background was regarded small and insignificant. However, we cannot deny that background contamination may have influenced the results of the analysis in some way.”
Response 5).
We created unsupervised hierarchical clustering map and replaced the figure following your suggestion (Fig S2-1, S2-2). As a result of unsupervised hierarchical clustering, the relationship between the detected phylotypes and the timing of recruitment to the study or the timing of specimen collection was not clear. Since the PCA result revealed that tendency of reduction of anaerobes in tracheal aspirate, we believe the result of PCA is worth to describe.

Response 6).
We described the days from admission to recruitment with other parameters you suggested in corrected Table 1.

We will be referring to page numbers on bottom of the page and the leftmost line numbers.

Abstract:

Comment 7).
Line 23 "On the other hand, …" It's not clear what the authors are referring to "(A) to (B)". I am sure that the context is provided in the article, but unfortunately it is not clear from just reading the abstract. I would recommend that the authors re-write for clarity and allowing for readers to read the abstract as a stand-alone abstract.

Response 7).
We rephrased the sentence following your suggestion.

Line 30-34 (Abstract)
“Principal component analysis based on the composition of genus revealed that although the changes of microbiota in the saliva from (A) to (B) were not clear, the composition of anaerobes in the tracheal aspirate (B) was lower than (A). In fact, the reduction of anaerobes, not in the saliva but in the tracheal aspirate from (A) to (B), was confirmed by incident rate ratios estimated by a multilevel Poisson regression model (p<0.001).”

Comment 8).
Line 31 "There was a significant trend …" What exactly is the trend that the authors are implying?

Response 8).
We corrected the sentence to clarify as follows.

Line 34-35 (Abstract)
“The extent of decrease in anaerobes was fully dependent on the time difference between the sampling of tracheal aspirate (A) and (B)—in particular, over 3h of mechanical ventilation.”
Comment 9).
Line 31 "It is suggested that …" What or how exactly is it suggested that there is a loss of anaerobic bacteria from the lower respiratory tract? I think that the authors should speak with slightly more specificity in this abstract.

Response 9).
We rephrased the sentence for more specificity as follows.

Line 32-37(Abstract)
“In fact, the reduction of anaerobes, not in the saliva but in the tracheal aspirate from (A) to (B), was confirmed by incident rate ratios estimated by a multilevel Poisson regression model (p<0.001). The extent of decrease in anaerobes was fully dependent on the time difference between the sampling of tracheal aspirate (A) and (B)—in particular, over 3h of mechanical ventilation. It is suggested that the alterations of microbiota (involving the reduction of anaerobes in the lower respiratory tract) occurred during mechanical ventilation before administration of antibiotics.”

Comment 10).
Line 46 "These bacteria are assumed …" I believe that this is an overstatement and should be removed from the abstract.

Response 10).
We removed the sentence following your suggestion.

Background:

Comment 11).
Page 3, Line 12 "Several studies indicate that aspiration pneumonia …" While I do not have issue with the sentence preceding this sentence, I have issue with this sentence. I believe that aspiration pneumonia should be considered a distinct entity. I believe that the authors mean to indicate that aspiration/microaspiration is a risk factor/involved in CAP and HCAP. The authors should not conflate aspiration pneumonia and aspiration. I would clarify this sentence as either aspiration or aspiration pneumonia. If the authors did mean aspiration pneumonia, I would recommend that the authors clarify if aspiration pneumonia may actually be misidentified as CAP or HCAP. OR that aspiration is a risk or present in CAP or HCAP (this is what I believe the authors meant, but I am asking the authors to clarify this statement).

Response 11).
Thank you for pointing this out. We meant aspiration/microaspiration is involved in CAP and HCAP. We corrected the sentence as follows.

Line 49-51 (Background)
“Several studies indicate that aspiration is involved in 5 to 20 percent of community acquired pneumonia [2-4] and 15 to 75 percent of healthcare-associated pneumonia [5-7].”
Comment 12).
Page 3, Line 23 "Culture methods are used for the surveying …" The anaerobic bacteria are fastidious. The tracheal aspirate that is with oral bacteria may be deleterious as well.

Response 12).
We rephrased the sentence following your suggestion as follows.

Line 51-53 (Background)
“As for causative agents, anaerobic bacteria and facultative anaerobic bacteria such as Staphylococcus aureus, Klebsiella pneumoniae and Escherichia coli were detected from patients with aspiration pneumonia [1,8-10]. Culture methods are used to identify the causative agents [11].”

Comment 13).
Page 3, Line 37 "In some cases, antibiotic treatment …" I am having difficulty making the logical leap between aspiration pneumonia and multi-drug resistant organisms. I believe that I understand that the authors want to connect the two, but is there evidence that oral carriage of antibiotic resistant markers are of concern? As far as I know, nasal carriage of MRSA increases risk of bacteremia, but it is unclear how oral bacteria/flora is connected to drug-resistance. This seems arbitrarily connected to the research focus of the article, and I caution leaving it in as it is misleading to the reader. (as far as I know, 16s rRNA gene marker identification cannot identify bacterial resistance to antibiotics per se, but can possibly aid in the recognition of bacteria that MAY have increased resistance).

Response 13).
We apologize for causing a misunderstanding due to insufficient explanation. We don’t want to connect aspiration pneumonia and multi-drug resistant organisms. We consider that inappropriate administration of antibiotics may emerge drug resistant pathogens. We rephrased this part as follows.

Line 57-62 (Background)
“Therefore, it is difficult to evaluate pathogens of aspiration pneumonia comprehensively via culture-dependent methods. Difficulty in identification of causative bacteria may lead to inappropriate administration of antibiotics and the emergence of drug resistant pathogens. The proportion of nosocomial infections caused by multi-drug resistant pathogens is increasing, resulting in an increased length of hospital stay, mortality, and cost [14]. The emergence of multi-drug resistant pathogens is an urgent social problem, and proper use of antibiotics is necessary.”

Comment 14).
Page 3, Line 55 "In cases of aspiration pneumonia, …" Please provide a hypothesis statement for the readers. I am not sure what the authors want to focus on, however I believe that without a hypothesis statement, the research article runs the risk of being descriptive.

Response 14).
We provided hypothesis statement following your suggestion.
In particular, high concentrations of oxygen may be administered during mechanical ventilation, and we have found no reports regarding the dynamics of anaerobes in the lower respiratory tract during mechanical ventilation. We therefore hypothesized that microbiota in patients with aspiration pneumonia—especially with anaerobes in the lower respiratory tract—change dynamically during mechanical ventilation and administration of antibiotics."

Comment 15).
Page 4, Line 6, "To develop a treatment strategy for patients with aspiration pneumonia, …" Not sure what this study would add to the already well-developed treatment strategy that the authors are discussion. I would expand upon this idea.

Response 15).
We rephrased the sentence following your suggestion.

To confirm our hypothesis, we evaluated the changes in microbiota of oral cavities and lower respiratory tracts in cases of mechanically ventilated patients with aspiration pneumonia using the clone library analysis based on highly accurate 16S rRNA gene sequencing."

Methods

Comment 16).
Page 4, Line 38 "Inclusion criteria" Was there an inclusion of how long a patient can be admitted prior to recruitment in the study? Was there conclusive diagnosis of aspiration? How was the diagnosis of aspiration pneumonia made?

Response 16).
There were no inclusion criteria for admission to recruitment period in this study.

Aspiration pneumonia was diagnosed by medical history of aspiration (considerations were background information such as medical history inclusive of status of comatose) and diagnosed with pneumonia according to IDSA/ATS guidelines.

Comment 17).
Sample handing is missing from the methods section. Issues such as 1) were samples spun down? 2) were samples evaluated for cross contamination? 3) Why were samples stored at 4C and left no more than 7 days? (wouldn't the authors keep them colder at -20C)? 4) was the volume of samples recovered? Moreover, regarding lower airway samples, did the authors use the cell-free or cell-associated bacteria component? (please see Dickson 2014 Microbiome "Cell-associated bacteria in the human lung microbiome").

Response 17-1).
The samples were vortexed before analysis. We rephrased the sentence as follows.
The collected samples of saliva and tracheal aspirate were vortexed and split for the total bacterial cell count and bacterial DNA extraction.

Our analysis was performed with great care to avoid contamination. For example, to reduce the potential effects of oral care, the samples were collected 2 h before (or after) the scheduled oral care. Also, cuff pressure of the endotracheal tube was aptly monitored to prevent saliva leakage.

In this study, we evaluated not only microbiota but also bacterial number of the samples. To avoid effects on bacterial counting due to repeat freezing and thawing the sample, we stored the samples at 4°C until analysis. We added about this limitation in discussion as follows.

“The final limitation to be discussed regarding this study is that samples for DNA analysis were stored at 4°C until time of analysis. In this study, not only the evaluations of the microbiota but also the number of bacteria contained in the specimen were analyzed. In order to avoid changes of the bacterial count result by repeated freezing and thawing of the samples, the samples were stored not at -20°C or -80°C but at 4°C until analysis. We cannot deny the possibility that this method may have affected on the results in some way. Although, as a result in this study, the microbiota of the saliva had hardly changed from (A) to (B), the anaerobic bacteria count significantly decreased in the tracheal aspirate. Therefore, storing the sample at 4°C seems to have had little (or no) influence on the results.”

We did not use cell-free samples for analysis. In order to evaluate the association between microbiota of oral cavity and lower respiratory tract, an analysis using whole sample other than cell-free sample may be appropriate.

The authors do not describe any background control sampling, while the oral/saliva microbiome is likely very abundant, it is also good practice to amplify samples for control including: sampling fluid, sampling tools. For example, what we tend to do is take sampling fluid (e.g., sterile saline) on the day of the procedure for control. Others have collected a sterile flush through instruments such as bronchoscope/endoscopy tools in order to understand the possibility of collected bacterial DNA on instruments and to ensure that attribution from the background microbiome can be accounted for in the samples.

Technical controls can include the 16S rRNA of the elution buffer by which the bacterial DNA was collected. I would like to see this data from the authors (for a citation please see Salter et al. BMC Biology 2014).
As we described this in Response 4). Although we analyzed the samples with control (PBS used for dilution), we did not obtain background samples. We described this limitation in discussion as follows.

Line 293-302 (Discussion)
“Third, background samples were not obtained during sequence analysis in this study. When using sequencing techniques to assess microbiota in low biomass environments, care should be taken regarding the potential contamination of background (39). In this study, the DNA extract from only PBS (without specimen) was used as the control. No obvious band (with 2% agarose gel electrophoresis analysis) was detected from the control sample after the PCR (30 cycles) and the cloning-sequencing analyses were performed with only PCR positive samples. Since significant amplification was not observed from the control samples and most bacterial phylotypes detected in this study are commonly found in the upper and lower respiratory tract, the contamination of the background was regarded small and insignificant. However, we cannot deny that background contamination may have influenced the results of the analysis in some way.”

Results
Comment 19).
Page 7, Line 27 "The remaining 22 subjects ..." One important time point that the authors did not collect, but is of interest is the time of admission to recruitment into the study. There is some evidence that admission to the hospital system can impact upper airway microbiome. I would recommend that the authors list the time of admission into the hospital as another in their table. I would also include medications, especially immune modulating medications. I would include it both for possible biases introduced on delay into recruitment, but also to do analysis to see if anyone's baseline microbiome is impacted by early vs. late recruitment into the study and/or early/late recruitment due to respiratory failure.

Response 19).
We corrected the Table 1 following your suggestion. To evaluate microbiome between early vs late recruitment into the study, we added this information in unsupervised hierarchical clustering map (Fig S1). We described about this in results as follows.

Line 155-158 (Results)
“The results of unsupervised hierarchical clustering of genus for samples of saliva and tracheal aspirates are shown in Additional file 4 (Figure S2-1, S2-2). As a result of unsupervised hierarchical clustering, the relationship between the detected phylotypes and the timing of recruitment to the study or the timing of specimen collection was not clear.”

Comment 20).
Figure 1 I would line up the # of cases and at each time point in the flow/time course figure. The section provided by the authors is slightly difficult to understand and should be re-written for improved clarity.
Response 20). We changed the figure following your suggestion.

Comment 21). Page 8, table 1 Characteristics of the subjects. Some other information I would think important for the authors: that would make the study more interesting:

1) Mortality and mortality related to pneumonia/infection
2) Length of stay in ICU, mechanical ventilation, and hospital
3) Medications: Vasopressors, antibiotics utilized per patient, culture data (e.g., positive culture, identification of organism)
4) Co-morbidities
5) Is the primary diagnosis the diagnosis that resulted in respiratory failure? This is not clear from the table

I would work on expanding some more information for the table, I believe the requested data are clinically relevant and would be of interest for other readers.

Response 21). We changed the table following your suggestion.

Comment 22). Page 9, Line 12 "The composition of clones assigned to each genus …" I am not sure why the table is split between the orange/white groupings on the left. I would like to see a non-hierarchical evaluation (e.g., using a distance matrix of the authors choice) of these taxa including grouping by both all samples and by time point (please see heat maps in Segal et al. 2016 Nature Microbiology).

1) We would like to know if samples are more similar to patients (they will cluster closer w/ the patients) or if clinical events such as intubation will make patients more similar to each other (collapse of taxa etc.)
2) We would also like to know of co-occurring bacteria in the tracheal/oral flora travel together, for example in lung microbiome it is recognized that Streptococcus, Prevotella, and Veillonella can be found in the lower airway.
3) There may be limitations to the heatmaps, as the authors use a clone library and not a true microbiome matrix, but I would like to see unsupervised analysis to understand the data.

Response 22). As described in Figure legend S1 (Appendix S1) we defined as follows. “Obligate anaerobes are indicated as orange background (Species of Actinomyces, Campylobacter, and Treponema detected in this study were obligate anaerobes). Aerobes and facultative anaerobes are indicated as white background.” We created heat maps following your suggestion (Fig S2). We added the sentences in results as follows.

Line 155-158 (Results) “The results of unsupervised hierarchical clustering of genus for samples of saliva and tracheal aspirates are shown in Additional file 4 (Figure S2-1, S2-2). As a result of unsupervised hierarchical clustering, the relationship between the detected phylotypes and the timing of recruitment to the study or the timing of specimen collection was not clear.”
Comment 23).
Page 10, Table 2 - this table is a slight convoluted, I would bold the data where the culture results matched the dominant phylotype at point B. Although, this doesn't say much, especially when the culture data is effective at clinically diagnosing a potential pathogen. What is interesting is when the data is mismatched, there should be a distinction made (possibly re-organizing the table).

Response 23).
We re-organized Table 2 following your suggestion. We rephrased (Result) and added (Discussion) the sentences as follows.

Line 164-165 (Result)
“Although the culture method detected predominant phylotypes of the tracheal aspirate (B) in many [85.7% (18/21)] of the subjects, it failed to detect any in the remaining cases (case 9, 14 and 17).”

Line 252-258 (Discussion)
“In this study, the culture method detected predominant phylotypes of the tracheal aspirate (B) in many of the cases (Table 2). However, the culture method could not detect predominant phylotypes in three of the cases (case 9, 14 and 17). In case 9, since Lactobacillus is a gram positive rod, it is possible that Lactobacillus was detected in culture but could not be identified in the clinical laboratory of the hospital. In case 17, Granulicatella is difficult to culture because of their pyridoxal dependence and slow growth characteristics (35), so it may not have been detected by the culture method. It is not clear why no growth was detected in case 14.”

Comment 24).
Page 11, Line 1
I would discuss the PCoA plot before speaking about the individual components - move this section behind the discussion of the plot. Also, the PCOA plot (figure 2) likely is very non-descriptive as the authors excluded lower abundant phylotypes/clones - these may have been interesting to add back as they may make the distinctions between the saliva and trachea more apparent. Based on supplemental figure 1 of the clones, it is clear that the PCOA plot was likely not going to find any differences between the saliva and the tracheal aspirates - the grouping looks too similar.

One recommendation is to do a Procrustes analysis of the individuals - it is clear at time C that individuals start to resemble each other, but it would be interesting to see if there is any distinction between individual and their samples. Some predictions:
1) Subjects that change the greatest on Procrustes may have significant culture findings 
2) Subjects that change the least on Procrustes may be the most "protected" (best clinical outcomes), etc.

Response 24).
Since the PCA result revealed that tendency of reduction of anaerobes in tracheal aspirate, we believe the result of PCA is worth description. Procrustes analysis is beyond our understanding and we could not perform the analysis. Because the number of cases in our study is small, the
Comment 25).
Page 11, Line 23 "Dynamics of microbiota..." Could the authors define which bacteria are anaerobes? I reviewed the methods section in the paper and the appendix, but neither has the information, I would write it into the methods for reference and mention it again here.

Response 25).
We described the obligate anaerobes, aerobes and facultative anaerobes in figure legend of Fig S1 (Appendix S1). We added the sentence regarding this (in method) as you suggested.

Line 75-78 (Appendix S1)
“Obligate anaerobes are indicated as orange background (Species of Actinomyces, Campylobacter, and Treponema detected in this study were obligate anaerobes). Aerobes and facultative anaerobes are indicated as white background.”

Line 131-133 (Method)
“Regarding the obligate anaerobes, aerobes, and facultative anaerobes followed the description of Bergey’s Manual of Systematics of Archaea and Bacteria [28].”

Comment 26).
Page 11 Line 57 "It is noteworthy that most anaerobes .." Some data presented in abstract form has shown that hyperoxia can change the lower microbiome. Which has relevance to subjects who receive mechanical ventilation - did the authors do a regression on the %percentage of anaerobes and the FiO2% of the subjects? (Prediction higher FiO2 lower anaerobe percentage), also the authors can also do a regression on time on vent and percentage of anaerobes while controlling for FiO2%.


Response 26).
As we described this content in Response 3), relationship between mechanical ventilation time and proportion of anaerobe was evaluated using incident rate ratios estimated by a multilevel Poisson regression model. It is described at Line 183-196 (Result) as follows.

“There was no significant difference in the proportion of anaerobes between saliva (A) to (B) (IRR: 0.97, 95% CI 0.85-1.12, p=0.76). On the other hand, the proportion of anaerobes were significantly lowered between tracheal aspirate (A) to (B) (IRR: 0.34, 95% CI 0.26-0.45, p&lt;0.001). Fig. 3 shows the changes in proportion of anaerobes in tracheal aspirate from (A) to (B) during mechanical ventilation (individual cases). As a result of multivariate analysis using clinical factors such as FiO2, tracheal diameter, vasopressor use, ICU stay, mechanical ventilation days, hospital stay and mortality, there was no significant relationship between the proportion of anaerobes and these clinical factors. However, the extent of decrease in anaerobes was fully dependent on the time difference between the sampling of tracheal aspirate (A) and
There was no significant decrease of anaerobes in the tracheal aspirate (B) collected within 2 h after collecting tracheal aspirate (A) (IRR: 1.23, 95% CI 0.73-2.05, p=0.43); significant decrease in anaerobes occurred in tracheal aspirate (B) collected 3 to 10 h after collecting tracheal aspirate (A) (IRR: 0.22, 95% CI 0.13-0.38, p<0.001) and collected 11 h or longer after collecting tracheal aspirate (A) (IRR: 0.23, 95% CI 0.15-0.35, p<0.001). It is noteworthy that most anaerobes were not detected in tracheal aspirate (B) which was collected 11 h or longer after collecting (A) (Fig. 3).”

Discussion:

Comment 27).
Page 12 Line 14, I do believe there has been studies that examined the lung microbiome / tracheal aspirates of patient who are mechanically ventilated, the authors should be commended on the work they did regarding the timing of their samples. The study is novel as it longitudinally assessed the changes to the microbiome. The changes to the microbiome however still needs to be fully assessed - one simple assessment that the authors have not performed was individual changes (e.g., persistence of similar microbiota signals throughout all time points or changes). The authors should also do multivariate analysis with various clinical data which they collected, but did not use this data in their analysis.

Response 27).
Thank you for the suggestion. We described the studies that evaluated bacterial flora in the lower respiratory tract in mechanically ventilated patients as follows.

Line 204-217 (Discussion)
“The dynamics of the bacterial flora in the lower respiratory tract in mechanically ventilated patients are evaluated as follows [29-31]. Berdal and colleagues [29] evaluated tracheal suction samples from 48 h after intubation to every 72 h thereafter in 74 mechanically ventilated patients. They found the microbiota in the upper and lower airways of mechanically ventilated patients to be closely correlated and relatively stable over a period of 72 h. Kelly and colleagues [30] evaluated oropharyngeal and endotracheal secretions sampled within 24 h of intubation, and every 48–72 h thereafter, from 15 mechanically ventilated patients with respiratory failure which were compared with samples obtained from a healthy control group. They found that critically ill subjects had lower initial diversity in upper and lower respiratory tract microbiota compared to those of the healthy control and the diversity further diminished over time on the ventilator. Zakharkina and colleagues [31] evaluated endotracheal aspirate samples collected at intensive care unit admission and then subsequently twice a week from 35 mechanically ventilated patients for a non-infectious cause of respiratory failure. They found that mechanical ventilation, but not antibiotic administration, was associated with changes in the respiratory microbiome and that dysbiosis of microbial flora in the respiratory tract was most profound in patients whom developed ventilator associated pneumonia (VAP).”

As we mentioned in Response 24), because the number of case in this study is small, the analysis of individual changes was limited to the evaluation of anaerobic bacteria which actually reduced during mechanical ventilation.
We collected clinical data and performed multivariate analysis following your suggestion. However, maybe this is due to small sample size as well, as described at Response 3), they did not change the result. We described about this in Result as follows.

Line 187-191 (Result)
“As a result of multivariate analysis using clinical factors such as FiO2, tracheal diameter, vasopressor use, ICU stay, mechanical ventilation days, hospital stay and mortality, there was no significant relationship between proportion of anaerobes and these clinical factors. However, the extent of decrease in anaerobes was fully dependent on the time difference between the sampling of tracheal aspirate (A) and (B).”

Comment 28).
Page 12 Line 60, "This phenomenon supports…” I am not sure how the presumption that anaerobes are major pathogens, they may be major players in the lung microbiota during intubation and disappear when the subjects get intubated. Is it plausible that the other way of thinking about this event is that with the disruption of a normal lung microbiome/microbiota, the lung becomes more vulnerable to lower airway infections? It is difficult to interpret the data unless there are mechanistic experiments, so the authors must entertain all assumptions.

Response 28).
We rephrased the expression following your suggestion.

Line 233-237 (Discussion)
“Although further investigation is necessary to explain this phenomenon clearly, several assumptions can be made. There is a possibility that the normal lung microbiome was disrupted by mechanical ventilation, or that those fluctuations may be caused by oxygen concentration increase via mechanical ventilation (because obligate anaerobes cannot survive in the presence of oxygen).”

Comment 29).
Page 13, Line 20 "so it was possible that the cuff of the endotracheal tube was not adequately 218 adhered to the trachea.” I would hope that ventilatory technicians are checking cuff pressure and checking for cuff leak. Unless there's another proposal that possibly positional changes impacted the way that the cuff isolated the tracheal. Moreover, it is also well described that intubation does not stop aspiration.

Response 29).
In this case, the cuff pressure was checked as appropriate as in the other cases. Unlike other cases, many anaerobic bacteria were detected in the tracheal aspirate after mechanical ventilation. We examined the patient background as a possible to explain this phenomenon. Because trachea diameter of this case was especially larger than other cases, we considered it was possible that more saliva might sag in the lower respiratory tract than other cases. Since the number of cases is limited and this phenomenon is just an estimate, we expect this to trigger future research.
Comment 30).
Page 13, Line 43 "After the administration of antibiotics, Enterobacter asbriae, E. cloacae, Corynebacterium propinquum, C. acoolens, Pseudomonas aeruginosa, Klebsiella pneumoniae…"
I find this fascinating. There's been some studies that have found that antibiotic application has reduced metrics of microbiomes (decreased alpha diversity), but the authors show that the application of antibiotics increase the taxa which are associated with antibiotic resistant infections, thus this is one of the most interesting conclusions of their study and should be emphasized. One important comparator which is missing are those subjects who are intubated but not given antibiotics - do they go on to develop changes in their lung microbiome? It is not the focus of their study, but would be interesting for them to emphasize (as a potential next step to study).

Response 30).
Thank you for the suggestion. We rephrased the sentences as follows.

Line 262-271 (Discussion)
“It is noteworthy that these pathogenic bacteria were detected as predominant 48-72 h after the administration of antibiotics. Although antibacterial susceptibilities of causative bacteria—except the isolates—were not evaluated in this study, these bacteria are generally associated with antimicrobial resistance. These bacteria might be major pathogens when initial treatment is ineffective. However, after the administration of antibiotics in this study, the causal relationship between patient background and predominantly detected bacteria was not clear. Since most of these bacteria can be detected by culture method, a personalized approach using the culture method is a practical strategy. There were only two cases (13 and 17) where patients were intubated but did not receive antibiotics. Large scale analysis of the dynamics of bacterial flora of such cases may provide interesting results, and are expected to be in future research.”

Comment 31).
Page 14, Line 1 - the authors should have a comprehensive accounting of the strengths and weaknesses of their study - if background samples are unavailable then they should say so.

Response 31).
As we mentioned in Response 4), we pointed to this limitation (in discussion) following your suggestion.

Line 293-302 (Discussion)
“Third, background samples were not obtained during sequence analysis in this study. When using sequencing techniques to assess microbiota in low biomass environments, care should be taken regarding the potential contamination of background (39). In this study, the DNA extract from only PBS (without specimen) was used as the control. No obvious band (with 2% agarose gel electrophoresis analysis) was detected from the control sample after the PCR (30 cycles) and the cloning-sequencing analyses were performed with only PCR positive samples. Since significant amplification was not observed from the control samples and most bacterial phylotypes detected in this study are commonly found in the upper and lower respiratory tract, the contamination of the background was regarded small and insignificant. However, we cannot
deny that background contamination may have influenced the results of the analysis in some way.”