Peer Review File

Article information: http://dx.doi.org/10.21037/tp-20-460.

Reviewer A

This paper was described about lower microbiota in with Down syndrome compared to controls with similar symptomatology. It seems to be of interest.

However, to my regret, your study just had negative data that there were no significant differences in presence of pathogens in the lower airways of children with DS and chronic respiratory symptoms, compared to controls, so I am afraid that this paper never had the brand-new findings.

Reply A: This is surely true, however this is what the data has taught us. Despite the more frequent occurrence of respiratory infections and the often more severe disease course in this patient population, the difference cannot be explained by more rare or virulent microorganisms (but rather by the anatomical and immunological abnormalities). In advance, we somewhat had expected this, but it is nice to see that it is confirmed.

Changes in text: none.

Reviewer B

In the present study, The authors retrospectively compared the microbiological data from BAL samples (when available) to a cohort of children with chronic respiratory symptoms but without any other relevant medical history to evaluate the differences in microbiota in the lower airway of patients with DS versus controls. In the end the authors got the conclusion that there were no significant differences in presence of pathogens in the lower airways of children with DS and chronic respiratory symptoms, and antibiotic therapy should not necessarily be adjusted to a more broad spectrum.

In terms of the content of the paper, it has a good conception, but it has no good methodology, rational design and nice illustration. It has not enough merit to be publishable. The details are as follows:

1. Some spelling mistakes: P4L3: “fenotypes” means “phenotypes” ? P5L8: “an anonymized” maight be “and anonymized”?

Reply B1: Thank you for noticing, this is now adjusted in the manuscript.

Changes in the text: The incorrect words are adjusted to ‘phenotypes” and “and”.

2. DESIGN: About controls: The chronic repiratory symptoms are just because of infection? Usually, the children who are prone to be infected or susceptible to the chronic respiratory symptoms are usually because of some other underlying disorders, such as anatomical or immunical or genetic reasons and so on.

Reply B2: The reader indeed will have more background when also reporting the types of symptoms leading up to the examination. This data is available from my previous study, so I will present it shortly here too.

Changes in text: This data was added as the new “table 1”.

3. METHODOLOGY: the paper did not present the specific method to confirm the pathogen? and what about the false negative rate for culture? How to differentiate the colonization and

infection?
Reply B3: The method of pathogen confirmation by cultures was clarified in the "methods" section. A part of this process is definitely operator-dependent (depending on the lab technician on duty), but any doubts are being revisited by additional testing (e.g. with a new culture on blood agar enriched with 'optochine' for detecting pneumococci) or in case of difficult identification a 16s rRNA is performed but this is rarely necessary. The guidelines of the American Society of Microbiology are followed during this process. Limitations of the routine lab must always be taken into account, since there is always a risk of not identifying certain pathogens; also the proportions of commensal bacteria cannot be investigated with this technique.
Since not all patient reports had quantitative measurements of bacteria present and in order to describe the microbial composition as complete as possible, we opted to include all found microorganisms.
Changes in text: In the section "methods", the methods of culturing was added.
4. CONCLUSION: about antibiotic therapy, it is not only related with the types of the pathogens, but also related with the drug resistance, the paper did not present any information about the drug sensitive test.
Reply B4: Indeed it does not report drug sensitivity. Given the more frequent use of antibiotics in the DS population (as reported by other papers), it is definitely worth investigating. I am afraid this will take me too much time to get all of these analyses in before the resubmission deadline. It is however something I should definitely take on in my discussion.
Changes in text: This item was added to limitations and mentioned as an option for future research.

Reviewer C

This study compares ~~upper~~ airway bacteria in children with Down Syndrome vs children with chronic respiratory symptoms. The study concept itself is interesting, given that DS patients do exhibit several immunologic symptoms as well. The researchers are also well-powered to examine these relationships, and this reviewer appreciates the difficulty of obtaining BAL samples from both groups. However, this study requires significant work to become a publishable manuscript.

The use of cultures to identify the presence or absence of microbes may not reflect the entire diversity of microbes present in these samples. If at all possible, 16s rRNA amplicon sequencing and analysis is highly suggested if the interest is in bacterial colonization.
Reply C1: Unfortunately, this technique is not at our disposal, especially not for routine sampling (as was the case here given the retrospective nature of this study).
Changes in text: This limitation was mentioned in the "discussion" section.

Barring the ability to perform amplicon sequencing, much more information is necessary within the methods to describe the culture conditions and experimental protocols used to obtain the culture-based data. Was it a generic culture medium, one designed specifically for respiratory bacteria? The discussion also needs to highlight the limitations of culture-based detection.

Reply C2: The techniques are now clarified in the “methods” section and the limitations in the “discussion” section.
Changes in text: Extra paragraph in the “methods” section and also a list of limitations in the “discussion” section.

Several modifications need to be made to Table 1 and Table 2 as well. First, for Table 1, this reviewer is unsure of the utility of stratifying cultured bacterial by typical vs atypical. Instead, this reviewer requests that tests in Table 2 be carried out at a culture-specific level (ie, Presence/Absence of Moraxella catarrhalis, Streptococcus pneumoniae, etc). Please consider that there are strain-level differences in the pathogenicity of microbes, and thus grouping them may give inconclusive results. For instance, Staphylococcus aureus is typically considered pathogenic while Staphylococcus epidermidis is considered protective. Thus examining relationships for each encountered microorganism is vital to sufficient interpretation of this data.
Reply C3: I have adjusted all of the tables. The new “table 2” contains a list of all detected microbes, with the number of patients per subgroup. The most encountered bacteria (with > 10 are now also compared by Chi square test in “table 3”. The others not given the very small numbers per microorganism.
Changes in text: The tables (now called 2 and 3 since I added a “table 1” with patient characteristics) contain more detailed info per microorganism. This was also shortly described in the main text at the end of the “results” section (“We also compared the most frequently reported bacteria…”).

If available, the authors should consider analyzing these bacteria using a quantitative metric such as CFU/ml. The methods state that “all positive cultures (even with small numbers of colony forming units per ml) were taken into account”. The bacterial load may also be discriminatory, and those with several thousand CFUs may be distinct from those with one or two.
Reply C4: For some patients, this is available but not for all of them. Discriminating would therefore also mean a loss of information (or patients), so the authors opted to describe all found bacteria and viruses (the latter also being reported by the lab as present or absent and not quantitatively). Nevertheless, a good suggestion in case of further studies.
Changes in text: none.

Refrain from describing the comparison group as controls. Also, describing all possible reasons for endoscopic evaluation should be provided somewhere in the paper. If the finding is still null after considering the comments above, discussion as to whether these respiratory conditions may also influence bacterial colonization is certainly warranted. The conclusion should reflect this as well.
Reply C5: The reader indeed will have more background when also reporting the types of symptoms leading up to the examination. This data is available from my previous study, so I will present it shortly here too. When fitting, the “control group” was described as the children without underlying conditions.
Changes in text: Reasons for endoscopic evaluation were added in the new “table 1”. Throught the text, the “control group” was described as the children without underlying conditions when

fitting.

Some kind of visualization of this data would be greatly appreciated. This reviewer can see several possibilities, including a heatmap.
Reply C6: The new "table 2" now contains the numbers of patients with the microorganisms detected; I have made both a heat map and bar chart below this table.
Changes in text: See "table 2" for 2 different visualizations.

All bacteria names must be italicized.
Reply C7: Thank you for this correction.
Changes in text: Throughout the text and in the different tables, bacteria names were italicized (except when for example 'Streptococci' was used).

Finally, this reviewer believes that the abstract must be modified to clarify that data is being derived from culture-based techniques. As written, it suggests microbiota composition analysis via amplicon sequencing.
Reply C8: True, this was clarified.
Changes in text: In the abstract (in both the "methods" and "results" section), this was mentioned.

Reviewer D

In the current manuscript, De Lausnay et al. present the results of a study focused on exploring differences in microbial composition between subjects with and without Down syndrome (DS). Previously it has been reported that individuals with that pathology are prone to developing several comorbidities. Among them, respiratory symptoms appear to be the most common. To address the origin of that high prevalence, authors compared the microbiological composition between DS individuals and controls without DS. No differences were observed between groups.

In general, the analyses have been performed correctly and the manuscript reads well. However, some flaws should be addressed to improve its quality prior publication.

Major comments
1. Title: It should specify that both Down Syndrome patients and controls suffer from respiratory symptoms.
Reply D1: The title was altered.
Changes in text: The title was altered to "…compared to controls with similar respiratory symptomatology"
2. Abstract and main text: Consider adding a sentence in which you explain the motives behind studying microbial composition differences between DS patients and controls.
Reply D2:
Changes in text: In the abstract the following was added: "Children with DS… generally have a more severe and prolonged disease course in case of infection. … We aim to compare

microbiota… to see if we can explain the difference in disease course." In the main text, at the end of the introduction: "…the aim of this study is to evaluate if the difference in prevalence, severity and duration of respiratory tract infections can be (partially) explained by comparing results from BAL fluid cultures…"
3. Page 2 line 17: This reviewer suggests rephrasing the sentence "Mostly present…". It is not correct to affirm that if the information is based only on culture tests for specific pathogens. Suggestion: "Among the microorganisms tested, the most frequently reported were H. influenzae, M. catarrhalis, Streptococci and Staphylococci."
Reply D3: Thank you for this suggestion.
Changes in text: The sentence was adjusted as proposed ("Among the microorganisms tested, the most frequently reported were…").
4. A table showing clinical data of the subjects is encouraged.
Reply D4: The requested table was added.
Changes in text: See newly added "table 1".
5. Add a table depicting all the tested pathogens/ commensals. Another option is to mention it throughout the text.
Reply D5: All encountered microorganisms are listed in "table 2", with specific numbers of patients in whom these were detected. Our cultures detect practically all relevant aerobic and anaerobic bacteria; mycobacteria and viruses when requested.
Changes in text: "See table 2".
6. Include a table summarizing the microorganisms observed in each group (DS and controls).
Reply D6: The tables were all adjusted, see the new "table 2" with quantification of patients in whom the different microorganisms were detected.
Changes in text: "See table 2".
7. Sample size limitation could be disguising some promising results. Is in authors minds to keep including individuals in this study/ cohort?
Reply D7: Absolutely! However, this last year we were forced to dramatically reduce the number of (non-essential or less urgent) procedures due to the COVID-19 pandemic, so not many DS patients had a bronchoscopy performed (in the years before, this were about 5 to 10 DS patients annually).
Changes in text: none.
8. With regards to statistical analyses, Fisher's exact test is preferred when at least one of the categories in the contingency table has an expected value less than 5. Please take this into consideration. Furthermore, the use of logistic regressions is encouraged to take into account possible confounders such as age or biological sex.
Microbial category ~ DS + Age + Sex + other confounders
Reply D8: A Fisher exact test was indeed used when one or more cells had an expected value <5. Logistic regression showed no statistical interference of age or sex.
Changes in text: This was also mentioned at the end of the "methods" section between brackets.
9. Analyzing microorganisms in categories assesses differences in the community as a whole but analyzing the presence/absence of each microorganism separately could reveal interesting results as well.
Reply D9: As mentioned above, the specific numbers of patients per microorganism are

mentioned in “table 2”, however it contains very small numbers for most. I added a statistical comparison in “table 3” for the most detected bacteria (i.e. H. influenzae, M. catarrhalis, S. pneumonia an S. aureus).
Changes in text: “See table 3” and in the main text, the last 2 sentences of the “results” section.
10. Replication in an independent cohort would give robustness to the results found. Is there a cohort with similar characteristics that you know of?
Reply D10: I believe it would be rather interesting to compare the microbial composition of children with cerebral palsy and chronic airway disease (given we expect less anatomical anomalies, more functional problems such as chronic pulmonary aspiration). Or CF patients (the latter is now being organized in our university). Also the microbial composition in younger versus older DS children (e.g. toddlers and adolescents with respiratory symptomatology).
Changes in text: none.
11. The discussion would be strengthened by adding a section discussing the study limitations and strengths.
Reply D11: This is very true, I added some sentences about the lack of information about drug resistance, the small number of most specific pathogens, and the technique used.
Changes in text: In the “discussion” section, lines 9-15 were added.
Suggestions for future studies/ publications
1. In collation with previous comments, currently there are more precise techniques to characterize microbiological communities (bacteria, fungi and viruses) based on DNA information, often call microbiome analyses. It would add an extra layer of information if those methods could be applied to this research. For bacterial communities, 16S rRNA sequencing is the most extensively used.
Reply D12: This advice was given by other reviewers too and sounds very fitting. However , this technique is not at our disposal, especially not for routine sampling (as was the case here given the retrospective nature of this study). But useful comment for future research!
Changes in text: This limitation was mentioned in the “discussion”.
2. Another interesting analysis would be the comparative between DS patients with and without respiratory symptoms to assess potential differences in lower airways microbial composition as one of the reasons causing respiratory symptoms.
Reply D13: This indeed would be very interesting, but I believe it to be unethical to do this kind of invasive sampling in children without clinical indication for a bronchoscopy. Less invasive sampling may not give the same specificity.
Changes in text: none.

Minor comments
Abstract:
Line 9: consider replacing “microbiota” with “microbial composition”. Reply D14: done.
Line 12: define BAL acronym. Reply D15: done.
Line 17: Consider replacing “compared in both groups” with “compared between both groups”. Reply D16: done.
Line 18:
Consider revising the taxonomic nomenclature. Species names should be written in italics,

while substantives referring to groups of organisms such as Streptococci are not italicized. Please, make this comment extensive to page 4 lines 6, 12, 15, and 16, page 6 line 14, 15.
Also consider typing H. as Haemophilus to be coherent with the other genera or the other way around. Reply D17: done, thank you for this clarification.
Line 20: Consider rephrasing this sentence. Suggestion: No significant differences in lower airways microbial composition of children with DS and chronic respiratory symptoms were found when compared to controls presenting similar symptomatology. Reply D18: done.
Line 23: Consider adding a keyword related to respiratory symptoms. Reply D19: done.
Introduction:
Page 3 line 2: Please provide a reference. Reply D20: done.
Page 3 line 5: Remove "s" in the word "makes" of the following sentence "These features makes them more vulnerable…". Reply D21: done.
Page 3 line 7:
Consider replacing "vulnerable for" with "vulnerable to". Reply D22: done.
Consider rephrasing this sentence. Suggestion: "Children with DS are more vulnerable to infections possibly due to, among other factors, an altered immune status (3; 4)". Reply D23: done.
Page 3 line 11: Consider adding a comma in the following sentence "In a previous study (8), we focused on the anatomy of the lower airways"," and reviewed endoscopic…" Reply D24: I divided it into two separate sentences.
Page 3 line 17: Consider specifying that respiratory microbiota is the one not studied to date. There are a few articles analyzing digestive as well as oral microbiota in Down Syndrome patients. Reply D25: a sentence was added.
Page 3 line 19: Consider rephrasing this sentence. Suggestion: "Nowadays it is widely accepted that the lower airways harbor a complex and diverse microbiota that differs substantially from the upper airways, which in turn represents different sub-niches such as nasal or oral cavity (12)". Reply D26: Very nicely phrased, thank you for the adjustment.

Page 3 line 21: Please provide a reference for the challenging sampling of the lower airways. Reply D27: done.
Page 3 line 24: Change "10.000" to "10,000". Reply D28: done.
Page 4 line 1: Please provide a reference for the bacterial challenges mentioned. Reply D29: done.
Page 4 line 4: Consider removing the comma in the following sentence "Studies in children with chronic cough and diagnosis of protracted bacterial bronchitis"," have shown…". Reply D30: done.
Page 4 line 5: Change "BAL (bronchoalveolar lavage) for "bronchoalveolar lavage (BAL)" so the acronym follows the definition. Reply D31: done.
Page 4 line 7: Do all the references (13,14,15) refer to all the genera? If that is not the case, this reviewer suggests placing each reference where it is more appropriate. Reply D32: Yes they do, so I believe it fitting to let the references as they were.
Page 4 line 10: Consider removing "exist that". Reply D33: done.
Page 4 line 11: Consider replacing "4" with "four". Reply D34: done.
Page 4 line 14: Consider replacing "3" with "three". Reply D35: done.

Page 4 line 21: Consider rephrasing this sentence. Suggestion: “Therefore, the aim of this study was to compare the lower airways microbiota between a cohort of children with DS from XXX and a group of controls with similar respiratory symptoms but without significant medical history”. Reply D36: This phrase was already altered to clarify why the microbiota was compared between the two groups.

Also, consider clarifying the name of the institution or adding a reference if it has been described previously. Are the controls part of a larger cohort? Reply D37: The name of our institution is now mentioned. The controls are selected from log of all pediatric bronchoscopies performed, where I excluded all children with underlying disorders (such as cystic fibrosis, cerebral palsy, bronchopulmonary dysplasia, etcetera). This was described in my first paper (reference 8).

Page 4 line 23: Please, include an explanation about the kind of treatment it is referred to in this sentence or to what type of patients this sentence applies. Reply D38: The follow was added: “e.g. for choosing empirical antibiotic therapy”.

Page 4 line 24-25: Consider changing “MDAR (Materials Design Analysis Reporting)” for “Materials Design Analysis Reporting (MDAR)”, so that the definition precedes the acronym. Reply D39: done.

Methods

Page 5 line: Consider using a passive voice: “Information gathered in databases from our previous study was considered. Retrospective chart review of all endoscopic procedures (flexible bronchoscopy and flexible/ rigid laryngoscopy, all under general anesthesia with spontaneous breathing) was performed in pediatric patients with DS from April 2011 until June 2019.” Reply D40: done + the following sentence was also put in a passive voice.

If this cohort of patients has been described previously a reference should be included. Reply D41: done.

Please, state the institution where those procedures were performed. Reply D42: done.

Page 5 line 9: Consider rephrasing this sentence. Suggestion “For each subject, availability of BAL sample obtained during the endoscopic procedure for its further microbiological investigation was checked in their medical records”. Reply D43: done.

Page 5 line 10: Consider removing the following sentence “Of course, this is only possible if a bronchoscopy was performed”. Reply D44: done.

Page 5 line 14: Please, state how the samples were categorized and what criteria were used for that purpose. Reply D45: I find it more logical the way it is described more detailed in the “results” section. This way it is not repeated unnecessarily.

Page 5 line 15: Replace “are” with “were”. Reply D46: This sentence was altered because of your following comment.

After simulating the analyses, this reviewer guesses the comparatives are between being included in one category against the rest. Please, extend the methodology about the chi square test, it is not clear what are you comparing and what are the p-values reporting. Reply D47: This sentence was altered to clarify what is being compared: (“We used a Mann-Whitney U test to compare ages between the two cohorts and Chi square test (or Fisher exact test when appropriate) to compare the two cohorts in terms of sex distribution, reason for endoscopic evaluation and microbial composition.”

Results:

Page 5 line 20: Does 2.89 refer to the mean or median age? Reply D48: mean age, this is now mentioned more clearly on page 6, line 6-7. This mean age is now specifically from the 182 patients who had a bronchoscopy with BAL instead of the 215 patients from the original study. ("The groups are matched in terms of age and sex, with an overall mean age at time of bronchoscopy of 3.4 years and the majority being male (63.7%).")
Page 5 line 23:
State clearly if the data displayed in this sentence corresponds to controls alone or the overall group. Reply D49: cfr previous reply, the overall group.
Please state the comparative behind the p-values. Reply 50: This information (separate mean ages, sexes, p-values) can be found in detail in "table 1".
Page 5 line 24:
Please, consider moving up the sentence "Reasons for endoscopic…" after "Our control group consists of 150 children with respiratory symptoms that warranted endoscopic evaluation, but without additional underlying conditions". Reply D50: done.
Please, state if this sentence refers to the overall group or case/control group. Reply D51: done, these can be found in detail in "table 1".
Page 6 line 3: Consider moving this sentence to the Material and Method section for a better understanding of the methodology followed. Extend this comment to line 5, 6, and 7. Reply D52: I find it more clearly this way, so I can refer the reader to the tables immediately.
Discussion
Page 6 line 11: Rewrite this sentence as "Even though children with DS are more prone to infections (often originating in the respiratory tract) than children not affected by DS due to several predisposing factors (4; 5; 10), this…" Reply D53: done.
Page 6 line 13: Consider being explicit about the controls having respiratory symptoms.
Page 6 line 14: Correct "catarrhalis" for "Moraxella catarrhalis" Reply D54: done.
Page 6 line 16: "When treating…". Cultures are not always effective to assess microbiological composition since a great range of microorganisms are not yet culturable. Bearing that in mind, this reviewer considers that statement ambitious. Reply D55: This statement is now followed by the limitations and that these conclusions must be approached with caution.
Conclusion
Page 6 line 23: If this reviewer is not mistaken, in any moment the hypothesis was that differences were not to be found. Reply D56: "As suspected" was deleted.
Page 6 line 24: Consider rewriting that conclusion. You can not conclude the differences in infectious burden are due to immunological and abnormalities because they have not been analyzed in this manuscript. Reply D57: This is true, I have rewritten this as follows: "…we conclude that the higher infectious burden in children with DS is not caused by a different microbial composition, but could perhaps be better explained by the immunological and anatomical abnormalities, these topics too deserve more clarification."
Page 7 line 2: Consider revising the term pathogen throughout the manuscript. Here it looks like you are referring to microorganism as a general term not just to the infection-causing ones. Reply D58: This is also true, thank you for pointing that out.
Table 1:
This reviewer suggests ordering taxonomical names alphabetically. Reply D59: done.
Table 2:

Categories did not sum up the total in Down group, my guess is that the category “combination” is missing one individual. Reply D60: I have noticed this when remaking my tables, so this was adjusted.

Please clarify how you classify microorganisms as commensal. In table 1 no “commensal” category is depicted. Reply D61: This is determined by the lab techinician on duty, who states if the detected colonies are relevant or not. Microorganisms such as viridans Streptococci and several Staphylococcus species are often determined as commensals, unless when they grow manifold and purely.