Author’s response to reviews

Title: Characterization of gut microbiota in children with pulmonary tuberculosis

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Author’s response to reviews:

Reply letter

Dear editor:

Thanks for your attention on our manuscript and suppose many perfect advices to our paper. We have revised our paper according to your comments.

Review 1:

1. The study group age ranges from 0.2 to 15.5 years for PTB patients and from 0.6 to 16.0 years for healthy controls. The WHO uses an age threshold of <15 years in its epidemiological analysis of childhood TB. In terms of WHO treatment guidelines, a child refers to the 0-10-year age group, within which infant refers to the <1 year age, and adolescent refers to the 10-18-year age group. The wide range ages used in this study for both groups across which a relatively small number of subjects (n=18) is spread creates the possibility of over- or under-representation of an age subset within one or both of the subject groups in the microbiome analysis. More precise information on the individual ages contained in the two subject groups, and a more refined analysis of age group subsets, is required. The latter may necessitate a larger patient cohort to be sampled in order to provide reliable data on any differences in microbiota structure or content.
Response

Thanks for your comments and we think this is a perfect advice.

Actually, at first we wanted to divide the patients into several groups according to their age. However, only 18 patients were enrolled in our study and we have divided them into three groups (less than 1-year-old, 1 to 10 years old and older than 10 years old) according to your comments. But there were only three patients who were less than 1-year-old and six patients who were older than 10 years old in our study. So if we divided the patients into three groups and analyzed the gut microbiota of these groups, we thought it would cause bias because of the small sample size. Our study is the first one to explore the characterizations of gut microbiota in children with PTB. And the next step, we will complete a large scale research and enroll more children with PTB and divided them according to their age to explore the characterizations of gut microbiota in each subgroup. So in the limitation of our paper, we added that that: “Finally, a large-scale, multi-center study would be required to validate this initial characterization of the gut microbiota of pediatric TB patients before and after treatment” on page 19 line 5 to 8.

2. The manuscript overstates its findings in places. For example,

"Our finding implicated the critical role of the gut microbiota and gut-lung axis in the development of PTB and provides a theoretical basis for the treatment of PTB through gut microbiota intervention." No experimental data are provided in this study to support the critical role of the gut-lung axis in the development of PTB, or the use of gut microbiota intervention in PTB treatment.

Response

Yes, we agree with your comments. So in the revision, we have deleted this sentence in the conclusion part.

3. The manuscript refers to the study by Namasivayam et al (Longitudinal profiling reveals a persistent intestinal dysbiosis triggered by conventional anti-tuberculosis therapy. Microbiome 2017, 5(1):71) i.e. "In addition, Namasivayam et al found that compared with healthy controls, the diversity of gut microbiota in a murine model of TB presented a slight but significant reduction in the 12 weeks after It is important to refer to the specific findings by Namasivayam et al i.e.
"To determine the changes in the intestinal microbiota due to Mtb infection in this particular experimental setting, we first performed a longitudinal comparison of the microbiota of the mice from the untreated naïve and TB groups. When data from all of the time points were pooled, we did not observe a statistically significant change in diversity resulting from Mtb infection as assessed by Chao1 and Shannon indices (Additional file 2: Figure S2a) which measure the total number of operational taxonomic units (OTUs) and, in case of the Shannon index, the richness, abundance, and evenness of the OTU distribution. However, a slight but significant decrease in diversity was evident at W12 of infection (Fig. 1a). We then used the phylogeny based UniFrac method to compare the bacterial communities in the naïve versus TB animals (Fig. 1b). Although unweighted UniFrac analyses, which cluster the data based on presence or absence of OTUs, clustered the naïve and TB samples separately (p < 0.001), the clustering driven by Mtb infection was not statistically significant based on weighted UniFrac distances (p = 0.203) that also take into account the relative abundance of the OTUs. We next compared the composition of the microbiome to identify bacterial taxa that differ between the two groups. In agreement with Winglee et al. [54], we observed trends of differential abundance in members of the order Clostridiales of phylum Firmicutes and certain members of phyla Bacteroidetes and Tenericutes between the two groups (Fig. 1c, Additional file 3: Figure S3). Nevertheless, none of these differences, except genus Alkaliphilus that was increased in naïve mice, remained significant over the entire duration of the experiment. Together, these findings involving our specific infection and animal housing conditions and one inbred host genetic background, while distinct in detail from the previously published data, confirm that Mtb exposure by itself causes only minor changes in the composition of the murine intestinal flora."

The Namasivayam et al study found very limited changes in M. tuberculosis infected mice versus naïve controls and most were not significant. The main findings of the Namasivayam et al study were that administration of anti-TB drugs isoniazid (H), rifampin (R), and pyrazinamide (Z) was significantly associated with an immediate decrease in the Shannon and Chao1 microbial diversity indices in the first two weeks of therapy. They reported changes in the abundance of specific components such as members of the order Clostridiales which were maintained for at least 3 months after cessation of HRZ treatment.

Unfortunately, a major drawback of the submitted manuscript by Zhu and colleagues is that no data are provided on samples obtained for gut microbiota analysis following commencement of TB treatment (the Zhu et al., manuscript states that "none of the enrolled participants including the PTB group and healthy controls had received probiotics, prebiotics or antibiotics within one month before admission" and that "Stool samples were collected from all participants on the day of their admission"). The provision of additional data post commencement of TB treatment would have been insightful with regard to any effect of TB drugs on the gut microbiome of children.
Response

Thanks for your comments, and in Namasivayam et al study, they reported that a slight but significant decrease in diversity was evident at W12 of infection (Fig. 1a). this result was constant with our results. So we cite this reference when we discuss the diversity of the gut microbiota on page 13 line 21 to 22 and page 14 line 1.

In fact, six PTB patients in our study completed the follow-up after receiving one-month anti-tuberculosis treatment. We collected the stool samples from them and explored the difference of the gut microbiota in patients before and after anti-tuberculosis treatment. Because of considering the length of our paper, so we didn’t present these contents. According to your comments, we have added these contents in our revision (in the abstract, method, results and discussion parts) (on page 12 and page 17 line 15 to line 22 and page 18 line 1 to line 15). What’s more, in the discussion part, we also cite the study of Namasivayam et al, because our study got a similar result that after one-month anti-tuberculosis treatment, the richness of gut microbiota decreased. However, the relative abundance of gut microbiota between the patients upon admission and after one-month of treatment presented no significant difference in our study, which was in contrast to the results of Namasivayam et. al. Namasivayam et. al. reported that the relative abundance of the gut microbiota in TB mice before and after anti-tuberculosis treatment were significantly different. We have also discussed the reasons about this difference in the discussion part (on page 18 line 2 to 15)

4. The manuscript requires thorough proof reading as it contains multiple typographical errors e.g. "Tuberculosis (TB) is one of the most common infectious disease in the word"

"caused by mycobacterium tuberculosis" - should be Mycobacterium tuberculosis

"interventions needs to be addressed"

"the critical role of the gut microbiota in pulmonary infectious disease has also been increasing recognized"

There is an overuse of the term "What's more" i.e.

"What's more, the PTB patients presented a significant reduction of beneficial bacteria"

"What's more, the growing epidemics of drug-resistant TB is a continuing threat"

"What's more, in the present study none of the enrolled participants"

"What's more, a prospective exploration performed by Luo et al"
"What's more, Sabino et al reported that the microbiota of patients with primary sclerosing cholangitis" 
"What's more, SCFAs can regulate the proliferation of colonic epithelial cells and enhance the permeability of the intestinal mucosa"

Overuse of "great" and "many" e.g. "A great amount of evidence suggests that the gut microbiota exerts many beneficial effects on humans through the involvement of many significant physiological processes"

Response

Thanks for your comments and because English is not our mother language, so we have invited an English speaker to polish the language of our manuscript.

Reviewer 2

P4 L4: Please use the latest WHO report and insert its reference.

Response

We have used the latest WHO report and inserted the reference on page 4 line 9 to 14

P4 L7-9: Please use the latest WHO report.

Response

We have corrected the contents with the latest WHO report. The contents were in yellow on page 4 line 9 to 14

P6 L1-12: Explanation of TB diagnosis with such detailed isn't necessary and relative.

Response

Thanks for your comments

Because the diagnosis of tuberculosis includes confirmed tuberculosis and probable tuberculosis in our study. So we think we need to state the diagnostic criteria clearly.

P10 L13-17: Reporting of species isn't according to standard structures.

Response

We used the standard structure of the species on page 11 line 19 to 22.

P12 L8-12: Comparison of results with an organism in vagina isn't interesting.
Response

Thanks for your comments

We agree with you that comparison of results with an organism in vagina isn't interesting. So in the revision, we deleted these contents on page 15 line 1.

P14 L13: Bifidobacteriaceae is a family not phylum.

Response

We have corrected this in the revision on page 17 line 8.

P14 L20: Any indicated limitations aren't acceptable.

Response

Thanks for your comments and the limitation of our paper include three parts. The first one is about healthy controls. Because the healthy controls in our study didn’t complete chest x-ray to exclude PTB, so we think we should explain this. The second one is about the cross-sectional research design. Because this design cannot get the causal relationship, so we think we should explain this. The third is about gut microbiota after receiving anti-tuberculosis treatment. Because only six patients have completed the follow-up, so we think that the relative small sample size should be mentioned in this part. So in the revision, we have revised our limitation.

The others

There are many repetitive words in the text, Such as "what's more".

Response

We have polished our English by an English speaker.

The novelty of the text should explain with more details, too.

Response

We have added more details in the first paragraph of discussion to explain the novelty of the present study. (on page 13 line 3 to12)

The method section is extensively specified to samples and diagnosis of TB and explanation of molecular approaches is limited.

Response:
Thanks for your comments

Because the diagnosis of tuberculosis includes confirmed tuberculosis and probable tuberculosis in our study. So we think we need to state the diagnostic criteria clearly.

About molecular approaches, do you mean these parts: DNA extraction, Amplification and sequencing of 16S rRNA encoding gene and Bioinformatic analysis?

Because the genomic DNA was extracted from each sample using the E.Z.N.A. ®Stool DNA Kit, so we think it doesn’t need explain too much. In the Amplification and sequencing of 16S rRNA encoding gene part, we added the corresponding hypervariable region in the 16S rRNA that is isolated using the primers we specified (338F, 806R) on page 7 line 21 to line 22. what’s more, we have explained more details about the alpha diversity and beta diversity on page 8 line 12 to 20

I can't find any ethical committee in your paper

Response

Actually we have, it was on page 7 line 10 to 11

Generally, writing of the text is so poor and should rewrite, clearly.

Response

Thanks for your comments and because English is not our mother language, so we have invited an English speaker to polish the language of our manuscript.

Reviewer 3:

Abstract :

1. Please include the age range of the participants in your abstract text or reference the participants as "pediatric patients with PTB" instead of only "PTB patients."

Response : we have added theses contents in our abstract on page 3 line 5, 7, 12, 19.

2. Please note misspelled F. prausnitzii (pg 3, line 14). Also, please use proper scientific nomenclature for referencing bacteria at the genus (and species) level (see further comments).

Response: we have corrected these contents in our revision on page 3 line 16. And we used the proper scientific nomenclature for referencing bacteria at the species levels on page 11 line 19 to 21.
Background:

1. Please use proper scientific nomenclature when referencing bacteria (pg 4, line 1): Mycobacterium tuberculosis (genus is capitalized)

Response: Thanks for your comments and we have corrected these contents on page 4 line 8

2. Please consider including a reference for your statement, "...pediatric populations who are deemed as a high-risk group" (pg 5, line 10-11)

Response: Thanks for your comments and we are very sorry, in fact, in the WHO guidelines on tuberculosis infection prevention and control, it is said that: TB can affect everyone, but specific population groups have a higher risk of acquiring TB infection and progressing to disease once infected; these groups include people living with HIV infection, health workers and others in settings with a high risk of transmission of M. tuberculosis[1]. So in the revision, we changed this contents into: especially in pediatric groups which represent a clinically important population with increased susceptibility to TB[2]. (on page 5 line 14 to 15)

Reference:


Methods:

1. Please use proper scientific nomenclature when referencing bacteria (pg 6, line 4): Mycobacterium tuberculosis (genus is capitalized).

Response: we have corrected this in the revision on page 6 line 10 to 11.

2. Please use the proper format for referencing the Bacillus Calmette-Guérin vaccine (capitalize, use hyphen).

Response: thanks for your comments, we have corrected this contents on page 7 line 1.

3. Please include the corresponding hypervariable region in the 16S rRNA that is isolated using the primers you specified (338F, 806R).

Response: we have added these contents about 338F,806R on page 7 line 21 to 22 and page 8 line 1.
4. In your abstract you specified that the Illumina HiSeq platform was used. In the methods in the paper this is referenced as Illumina MiSeq. Please check which instrument was used.

Response: we used the Illumina HiSeq platform. So in the method part, we have corrected this on page 8 line 2.

5. Please include which version of QIIME you used. Also, please cite this using the correct reference paper (see the QIIME website for how to cite their materials)

response: Thanks for your comments and we have added the version of the QIIME and cite the relative reference in the revision paper according to your advice on page 8 line 6.

6. Please note which version of the Greengenes reference database was used.

Response: we have added the version of the greengenes reference database in the revision on page 8 line 10.

7. Please fix misspellings:

"microbota" (pg 4, line 22) to microbiota

"Bata" (pg 8, line 1) (pg 7, line 1,7) to beta

response: thanks for your comments and we have corrected these content on page 5 line 4 and page 8 line 18.

Results

1. Please correct "weight Unifrac" (it is called "weighted UniFrac") and cite accordingly. (pg 8, line 2) (pg 9, line 15). This is correctly referenced in your figure legend 1.

Response: thanks for your comments and we have corrected this on page 8 line 17

2. Page 11, line 8 - why is "aerosolized Mycobacterium tuberculosis" capitalized?

Response: we are very sorry for this mistake. In revision, we have corrected this on page 13 line 20.

3. Consider also presenting the relative abundance of phyla in each group, as this measure is commonly reported in microbiome papers and would enable comparison with other research.

Response: thanks for your comments and we have added the relative abundance of phyla in each group in the revision on page 10 line 20 to 22 and page 11 line 1 to 4.

4. The paper mentions the Shannon Index but did not report associations with the Shannon Index, only Simpson index. Please consider at least mentioning that no associations with the Shannon Index were observed if that is the case.
Response: thanks for your comments and we have mentioned this contents on page 10 line 6 to line 7.

Discussion

1. Page 11, line 15: Please consider elaborating on the differences between Luo et al’s study and yours (e.g., age, sex ratio, etc).

Response: thanks for your comments and we have discussed the difference between the two studies on page 14 line 5 to 11.

2. Please correct misspelling - pg 12, line 5, "Prevolla"

response: we have corrected this word on page 14 line 20.

3. Please use proper scientific nomenclature when referencing bacteria (pg 13, line 19) - species names are not capitalized. Consider rephrasing (note underlined edits as well) to: "In the present study, F. ruminococcaceae and F. prausnitzii were significantly lower in the PTB group compared to healthy controls. Previous studies have described that F. ruminococcaceae and F. prausnitzii may exert...

Response: Thanks for your comments and we have corrected the species names on page 11 line 19 to 21. what’s more we have corrected the contents according to your advice on page 16 line 13 to 18.

4. Please note, Bifidobacteriaceae is not a phylum, it is a family (pg 14, line 12-13).

Response: we have corrected this on page 17 line 8.

5. Please consider discussing that many pathogens are strain-specific. For example, though Enterococcus was increased in PTB patients, many harmless commensal bacterial species/strains are contained in this genus (namely, E. faecalis and E. faecium) and resolution to the species or strain level presents a future direction or limitation of the study.

Response: thanks for your comments and we have discussed this on page 16 line 6 to line 10 according to your advice.

6. You build a case for Prevotella as a pro-inflammatory, immune-regulating genus of bacteria, which is accurate, but species of Prevotella have also been found in populations consuming more carbohydrates, namely fiber. Do you have data on the dietary intakes of the cases and controls? This may also be contributing to the differences observed.

Response: thanks for your comments and we also think this is a perfect idea. However, we didn’t collect the dietary intakes of the subjects enrolled in our study and maybe when we design the nest research about the gut microbiota, we will collect this. And thanks for your comments again.
7. Please consider giving examples of microbiota interventions in your conclusion (pg 15, line 16).

Response: thanks for your comments and some reviewers said that we overstated our findings in conclusion. And there were no microbiota interventions for PTB treatment, so in the revision, we have deleted this contents: Our finding implicated the critical role of the gut microbiota and gut-lung axis in the development of PTB and provides a theoretical basis for the treatment of PTB through gut microbiota intervention.

8. On an analysis level, some additional ideas: Are there other clinical characteristics of the participants that you may wish to analyze with parameters of the gut microbiota? (For example, signs or self-reported symptoms, etc.). Though sample size may present a challenge, it would be interesting to determine if there are any differences in gut microbiome metrics by sex, age/age group, or weight within cases and controls, separately. Is there information on the duration or specific treatment that PTB cases were given (or if no treatment given then please state)?

Response: thanks for your advice and because of the limitation of the small sample size, so we didn’t analyze the relationship between the clinic characterizations and gut microbiota. But we will explore this in further with a large-scale study. What ‘more, we have analyzed that whether there were any differences in gut microbiome metrics by sex, age/age group, or weight within cases and controls separately. What a pity, there were no significant difference, we speculated that it may be related to the small sample size.

In fact, six PTB children in our study completed the follow-up after receiving one-month anti-tuberculosis treatment. We collected the stool samples from them and explored the difference of the gut microbiota in patients before and after anti-tuberculosis treatment. Because of considering the length of our paper, so we didn’t present these contents. According to your comments, we have added these contents in our revision (in the abstract, method, results and discussion parts). (on page 12 and page 17 line 15 to line 22 and page 18 line 1 to line 15)

What’s more, we have revised the name and brand of the Stool DNA Kit. We are very sorry for this mistake, and we use this kit: E.Z.N.A. ®Stool DNA Kit (D4015, Omega, Inc., USA). So we have revised. On page 7 line 18 to 19.