Author’s response to reviews

Title: Apical Periodontitis: preliminary assessment of microbiota by 16S rRNA high throughput amplicon target sequencing.

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Author’s response to reviews:

Please find here the point by point response letter to the reviewers.

OHEA-D-17-00463  Reviewer reports: Yuichiro Kikuchi (Reviewer 1): Reviewer’s report:
This manuscript reports on the comparison of the microbial composition of the periapical granulomas and radicular cysts. This manuscript has shown some interesting results about the microbial differences in periapical granulomas and radicular cysts, which can help us to understand the mechanism of apical periodontitis and also can be helpful for the therapy. The experimental procedures seem to be sound. However, some changes are necessary to make this paper more clear and complete. We would like to thank very much Prof. Kikuchi for appreciating and helping us improving our work. The revised manuscript was modified according to the Reviewer’s kind suggestions.
Major Compulsory Revisions  It is difficult to understand the Figure 1 at a glance. I think it is not possible to simply compare PGs and RCs. I ask the authors to create another table showing the ratio of bacterial species detected in each PGs and RCs sample. According to the reviewer, a new table (Table 3) reporting the ratio of the OTUs was added.

Minor Essential Revisions  1. There are no page numbers making it difficult for the reviewers to make specific comments based on page numbers.

We apologize for this inconvenience and added line numbers and page numbers accordingly.

2. (Title) I've never seen the expression of "16S high throughput amplicon target sequencing". For example; Change "16S high throughput amplicon target sequencing" to "16S rRNA high throughput amplicon target sequencing".

The title was changed according to the reviewer’s comment.

3. (Abstract, line6-7) Change "space." to "space.". (period: red→black)

The manuscript was amended following the reviewer’s indications.


The manuscript was corrected following the reviewer’s indications.

5. (Background, line28-29)

Change "Aps" to "APs".

The manuscript was amended following the reviewer’s indications.

6. (Background, line36-37) Is "Ricucci & Siqueira [14]" correct? I think "Ricucci & Siqueira [13, 14]" is correct.

The manuscript was corrected following the reviewer’s kind suggestion.
7. (Materials and methods, study design and patients, line18-22) Change "the Ethics committee of the Dental School" to "the Ethics committee of the Dental School, University of Turin". And what is the committee's reference number?

The manuscript was amended following the reviewer’s indications.

8. (Results, first paragraph, line45-52) Delete "Apical periodontitis consisted of------------------ while RCs by its presence." These sentences were not results.

We agree and amended the text accordingly.

9. (Results, second paragraph, line1) It is to be noted that all patients were older than 18 years and were in good health conditions as per inclusion criteria [Table 1].16S rRNA gene sequencing. A total of 163,832 raw reads were obtained after the sequencing. What is "16S rRNA gene sequencing"?

We added this as a new sub-paragraph entitled “Microbial diversity”.

10. (Results, second paragraph, line12-13) Change "102 OTUs" to "202 OTUs".

We apologize for the typo and corrected the text accordingly.

11. (Results, third paragraph, line15-19) Alpha-diversity indices (Table 1) showed no difference on the level of complexity (P > 0.05) of RCs samples compared to PGs. Is "Table 1" correct?

We apologize for the typo, it is indeed table 2.

12. (Results, six paragraph, line5-6) Is "Fig. 4" correct?

We apologize for the typo, we meant Fig. 3.
13. (References) Please correct the notation of all author's name and page numbers as shown below. (ex) BMC Oral Health example reference style: Article within a journal Smith JJ. The world of science. Am J Sci. 1999;36:234-5.

References were corrected following the aforementioned notation.

14. (Table 1) Sample _code " RC_2" is used twice. Please correct this. Sample _code " RC_4"
Change "13,5" to "13.5".

The text has been amended, as suggested.

15. (Figure 1) The resolution of the figure is quite low, so I don't read the letters. Replace this figure with still higher magnification photos. And, there is no description about the difference of the red box and the blue box in figure legends.

We added a figure with higher resolution, along with the required information in its figure legend.

16. (Figure 2 legend) Change "granulom" to "granuloma".

The text was amended, as suggested.

17. (Figure 3) The resolution of the figure is quite low, so I don't read the letters. Replace this figure with still higher magnification photos. As required, we added a figure with higher resolution. Yasuo Takeuchi (Reviewer 2): This is a preliminary study examined the microbiota of periapical granulomas (PGs) and radicular cysts (RCs). The samples of periapical lesions were collected from 5 PGs and 5 RCs, and the bacterial presence was examined by pyrosequencing of the 16S rRNA genes. In the present study, facultative anaerobes were mainly found in both PGs and RCs, while PCoA plots with a weighted UniFrac distance matrix showed the difference of microbiota between PGs and RCs. In addition, abundance of several minor OTUs differed among these two groups. They concluded that characteristics of microbiota differed between PGs and RCs. Main criticism of this study is very small sample size. It is fact that there are limited studies performed comprehensive analysis of microbiota associated with apical periodontitis.
This reviewer also understands that studies of this nature using the sequencing approach are usually plagued by a small sample size. However, each five sample is not enough for an evidence based evaluation / comparison of microbiota among two groups.

We would like to thank very much Prof. Takeuchi for reading our work. The revised manuscript was modified according to the Reviewer’s suggestions. Although we agree with the reviewer that one of the limitations of this study is its small sample size, we observed strong signs of association. Indeed, we found a specific signature of the microbiota and, to our knowledge, very few studies with this approach have been dedicated to characterize the microbiota in both PGs and RCs underlying the originality of the study.

Specific comments are as follows; - English style should be refine throughout the paper. It should get some English proofreading from native speakers. Correct the spelling of bacterial names (ex; Staphilococcus warneri → Staphylococcus warneri).

We apologize for the mistake and corrected it. To refine the English style the paper was proofread by a mother tongue teacher.

- P3, Abstract / P5, Paragraph 2: What is the mean of the abbreviation "OPT"?

OPT means OrthoPanTomography, which is a synonym of panoramic radiograph.

- P3, Abstract / P5, Paragraph 2: State more clearly where and how clinical samples were collected in the study. The authors mentioned that "The main cause of apical periodontitis is the bacterial colonization of the root canal space (in Abstract)". If so, it seems reasonable to collect the sample from the root canal space.

As stated, clinical samples were harvested during the surgical procedure. Attention was paid to handle the periapical lesions in a sterile way so as not to contaminate them. Immediately, the samples were fixed in 4% formalin. The manuscript was changed to underscore this concept. We did not collect samples from the root canal space, as it was not the purpose of our study.

- Materials and Methods: All of the statistical methods used in the study should be written in this section, not in the Results.
The text was changed according to the reviewer’s suggestion.

- Results: Although the authors showed the relative abundance of bacterial species, it is difficult to understand the whole picture of microbiota in PGs and RCs. This reviewer recommends to show the data of microbiota at phylum and/or genus level.

According to the reviewer, table 3 reporting the ratio of the OTUs was added.

- P8, Paragraph 1: The richness of the samples varied from 22 to 202 OTUs, didn't them?

We corrected this typo in the revised manuscript.

- P8, Paragraph 2: Alpha-diversity indices were showed in Table 2, weren't them?

The correct one is indeed table 2. We thank the reviewer for picking up this typo.

- P8, Paragraph 3: The words "ADONIS" and "ANOSIM" are recommended to write in all capital letters.

The text was changed according to the reviewer’s suggestion.

- Discussion: The authors mentioned that some facultative anaerobes such as Lactococcus lactis, Propionibacterium acnes, Staphylococcus warneri, Acinetobacter johnsonii and Gemellales dominated in periapical lesions. I'm not sure but these are unfamiliar as usual bacteria in oral cavity, and it is something of a surprise for me. The authors cited Ref No. 24 and mentioned that "these species are all normal commensals of the human oral cavity and were isolated in radicular cyst (Page 10, Paragraph 2)". However, it was the case report and was also not mentioned the characteristics and/or prevalence of these bacteria. I cannot get over the possibility that the samples were contaminated. The authors should collect more periapical samples and confirm the prevalence of these bacteria using PCR or other bacterial identificational methods. Otherwise, if you collected the saliva from the same subjects, you can examine the presence of these bacteria for checking the contamination of saliva. How do you think that these bacteria are associated with the pathogenicity of periapical lesions? Add the discussion about this topic.
The main objective of this study was to assess the microbiota of PGs compared with RCs samples, which -we think- is its novelty. We believe that readers will be interested in and benefit from the outcome of this study. As pointed out, we based our study on a few samples (only 5 per group) due to the difficulties encountered in finding more specimens. We did not collect saliva samples in order to compare the results because this was not the aim of the present research, although it is for sure an interesting point. Probably, from those results we will try, in the future, to go more deeply by using shotgun metagenomic approaches. We would like to avoid excessive speculations on the discussion about the pathogenicity of the taxa we found in our datasets even because we did not performed classical plate count or classical microbiological analysis on those samples. In addition, please consider that, for instance, the presence of Staphylococci in human periodontal disease was demonstrated by Rams et al. (Rams TE, Feik D, Slots J. Staphylococci in human periodontal diseases Oral Microbiol Immunol. 1990;5(1):29-32) and Scalas and colleagues (Scalas D, Roana J, Boffano P, Mandras N, Gallesio C, Amasio M, Banche G, Allizond V, Cuffini AM. Bacteriological findings in radicular cyst and keratocystic odontogenic tumor fluids from asymptomatic patients. Arch Oral Biol. 2013;58(11):1578-83). Gemellales were isolated within RCs by Tek (Tek M, Metin M, Sener I, Bereket C, Tokac M, Kazancioglu HO, et al. The predominant bacteria isolated from radicular cysts. Head Face Med. 2013;9:25).

- P10, Paragraph 3: Although the authors mentioned the anaerobic taxa were abundant in RCs samples, there were no information about it in Results. Again, the sentence "Aerobe and facultative anaerobic bacteria growth was seen in 10.8% of the cases." should be written in Results. The sentence was removed from the discussion session.

- P11, Paragraph 2: Show the proportions of aerobic and anaerobic bacteria to whole microbiota in Results.

We added this information in the results session as well as in the table 3.

- Figure 1: What is the mean of descriptions (Numbers) at "Region" and "Canal Root therapy" part?
We apologize for not being sufficiently explanatory. In Table 1, the numbers refer to the WHO classification of teeth and therefore indicate the tooth/teeth adjacent to the region. Table 1 was modified accordingly.

Kiyonobu Honma (Reviewer 3): General comment  The authors attempted to reveal diversity of microbiome at two different kind of apical periodontitis, periapical granulomas and radicular cysts by pyro-sequence analysis. The authors found similar kind of anaerobe and facultative anaerobe with culture based study. I think the aim of this study is important to expand understanding about microbiome environment at apical periodontitis. However this manuscript needs improvement for data presentation to recommend for the publication. We would like to thank very much Prof. Honma for appreciating and helping us improving our work. The revised manuscript was modified according to the Reviewer’s kind suggestions.

Specific comment  Please add page number to the manuscript.

We apologize for the inconvenience and amended the text as suggested. Please introduce all abbreviation if it is shown first time in the manuscript. We apologize for the inconvenience and amended the text as suggested. * Materials and Methods, Line 11 please introduce about "OPT".

OPT means OrthoPanTomography, which is a synonym of panoramic radiograph.

* DNA analysis by pyrosequencing  How did the authors prepare samples for DNA extraction? Please add more detail about DNA extraction procedure. We modified and improved the text as suggested. Please describe about ANOSIM and ADONIS analysis what the authors mentioned at results section. If the authors can present ANOSIM and ADONIS data as table, it make the manuscript better. We added this information in “Materials and Methods” section, however we did not add a table since the results of the scripts generated only the P value of the statistical test.

* Results, I think make a table of the species that was found at each group with OTUs data to
summarize the data is helpful. According to the reviewer, table 3, which reports on the ratio of the OTUs, was added in the revised manuscript.

* Page?, L9-10, "0.2% in at least two samples. [Fig.1]” Please mention sample names.

Here we meant that the OTU table was filtered by a minimum of the relative abundance of 0.2% in a minimum of two samples.

* Page?, L1-2 "species-level taxonomic assignment and significant correlations at FDR <0.05 (Fig.4). * What is the "FDR" standing for? Can I understand "Fig.4" as "Fig 3"? We apologize for our typo. The correct figure is Fig. 3. FDR meant False Discover Rate. We corrected accordingly the revised manuscript.

* Discussion, What is the authors idea about PGs samples shows less diversity than RCs sample? The topic is very interesting, but, unfortunately, controversial. From our data -partially supported by literature-, the presence of the epithelium may be seemingly associated with the selection of a more complex microbiota. To establish a possible cause-effect relationship a series of complex experiments are needed, exceeding the purposes and limits of this research. Thus, in absence of objective results, every hypothesis being speculative, we preferred not to address the matter.

* Fig 1 I think the authors can make a table to summarize the data and the figure with plot data should be placed as a supplement data. The figure is including too many plots and each plot is too small to see the data. According to the reviewer, table 3 reporting the ratio of the OTUs was added in the revised manuscript.

* Fig. 3 The figure also hard to understand. Please shows as a table. Although we appreciate the kind remark aimed at improving the manuscript, we believe that a table showing the R and P values together would be even more difficult to read than this figure. Fig.3 is now available in high resolution.