Author's response to reviews

Title: Gut microbiota in children with type 1 diabetes differs from healthy children: a case-control study.

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Author's response to reviews: see over
The Editor,

*BMC Medicine*

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Ref: Gut microbiota in children with type 1 diabetes differs from healthy children: a case-control study (MS: 1845900297788147).

Dear Editor,

Thank you for the opportunity to resubmit a revised version of our manuscript with the appropriate changes in accordance with the Reviewers’ suggestions. We set out below our responses to the comments of the reviewers, together with the action taken.

Yours sincerely,

Dr. María Isabel Queipo-Ortuño
Responses to the reviewers’ comments for MS: 1845900297788147

Reviewer 1 general comment

Murri and colleagues present a new analysis of the microbiome in T1D. The use a strong control group and good analytical approaches. However, I think that some of the interpretations of their data are stronger than the data warrant and more attention should be paid to technical controls to allow the reader to better assess the data. Therefore, I suggest the following major compulsory revisions:

Response: We really appreciate the general comment of reviewer 1. In the text we have now added some comments relating to the reviewer’s suggestions.

-Comment 1: In the discussions, the authors propose that the microbial diversity is higher in the healthy children. It is not possible to conclude this from the presented data. Because of the sensitivity of the DGGE techniques, low frequency bacteria are not identified. Thus, it remains possible that those bacteria that were not detected by this analysis due to their low abundance could be much more diverse in children with T1D than controls (that is, children with T1D could have a large number of low abundance organisms whereas healthy children may have fewer).

Response: In accordance with the reviewer’s logical commentary, we have now eliminated this sentence in the discussion section of the revised manuscript.

-Comment 2: The qPCR data should be presented with more detail. The use of a standard curve is laudable and a great way to do this analysis. However, for appropriate use of the standard curve, the authors need to report the efficiency of the qPCR with all primer pairs on their samples. If the efficiency is substantially greater than 110% or less than 90%, then the data may not be reliable. The sample may have PCR altering conditions that are not present in the standard curve.

Response: As it has been indicated by the reviewer, the amplification efficiency of the qPCR using all bacterial primer pairs has been added in the method section under "Microbial quantification by real-time qPCR" (Page 11, lines 8-12). Moreover, we have now added that to monitor for the possible presence of qPCR inhibitor in the study samples, each subject’s extracted DNA was subjected to a human β-Globin PCR (Page 10, lines 15-17).

-Comment 3: Negative controls are not reported. The authors should determine and state whether any product is detected by PCR on a non-template control. Similarly, in the absence of template, is any product(s) detected by DGGE?

Response: We are sorry this omission. We have always included negative controls containing all the elements of the reaction mixture except template DNA in all the PCR tests and no product was ever detected. With respect to the second question, non-template controls were also included in all DGGE analysis and no bands were ever
detected. This data have been added in the page 8 (lines 15-16), page 9 (line 6) and page 11 (lines 6-7) in the method section of the revised manuscript.

-Comment 4: The authors also indicate a correlation between glucose control and the microbiome. The data would be made much stronger if they were able to show analysis on the same subject at a time when glucose control was different. Since glucose control is generally dynamic within an individual, this may be possible and would substantially strengthen the presentation and interpretation of the collected data.

Response: We agree with the reviewer's observation. But, unfortunately we do not have in our database the glucose profiles of seven points from several days of the patients involved in the study. So, to overcome this limitation we have considered in the correlation analysis the long-term blood glucose messenger HbA1c, which is like a marker for average blood glucose levels in the last three months and it is a more correct evaluation of blood glucose. The correlation analysis between the HbA1c levels and the microbiome were shown in the result section of the manuscript under "Relation between gut microbiota composition in children with type 1 diabetes and glycemic level" (Page 14, lines 16-25 and page 15, lines 1-3).

I also hope the authors may consider the following discretionary revisions:

-Comment 1: Did the authors control for other therapies that may alter the microbiome such as therapy with proton pump inhibitors or corticosteroid bursts?

Response: We thank the reviewer for this observation. But none of the study subjects were subjected to therapy with proton pump inhibitors or corticosteroid bursts.

-Comment 2: The authors should consider including additional data relevant to the role of the microbiome in T1D from mouse studies such as the nature paper by Li Wen and colleagues or the several excellent recent papers on the role of SFB-Th17 interactions in mice. Do we have a grasp yet on the related mechanisms/interactions in humans? Does this study advance this field?

Response: In accordance with the reviewer's suggestion, we have now added in the introduction section of the revised manuscript (Page 5, lines 15-22) these important data about the role of the intestinal microbiota on the immune response homeostasis in T1D described in the papers of Wen et al. and Ivanov et al. At present, we have not analyzed in our patients the mechanisms of autoimmunity protection and promotion by bacterial colonization proposed by these authors in animal models. Nevertheless, the translation of this finding in animal models to human opens new important opportunities to intervene in the progression and prevention of T1D. Actually, our study is in the threshold of understanding whose specific microbes are responsible of the appearance of T1D as well as the function they exert in the environment.

-Comment 3: Could the authors discuss how these data relate to the concept and definition of enterotypes as forwarded in other studies?
Response: We appreciate this comment. We have now discussed our data related to the concept and definition of enterotypes in the discussion section of the revised manuscript (Page 16, lines 5-12).

Reviewer 2 general comment

This is an interesting manuscript that corroborates previous observations in animal studies on microbial groups negatively correlated with diabetes type 1. A positive and well-thought aspect of this study is that the diet of the patients was carefully screened.

Response: We really appreciate the general comment of reviewer 2. In the text we have now added some comments relating to the reviewer's suggestions.

Specific comments

Comment 1: Page 6. When the authors said that no probiotics or prebiotics were consumed, does it also include yogurt?

Response: Now in page 7, when we said that no probiotics or prebiotics were consumed, we also included the yogurt consumption. The study subjects were forbidden to consume yogurt during the three months prior to start of the study.

Comment 2: Page 11. The authors state that they have similar patterns in consumption of rice…although the diabetic children had a fast carbohydrate restriction”… BUT rice is classified as a high glycemic carbohydrate.

Response: Diabetic children had no restriction in rice consumption. When we say that the diabetic children had a fast carbohydrate restriction we refer to the consumption of foods made with white flour and refined sugar. To avoid this confusion we have now added this information in the revised manuscript (Page 12, lines 8-9).

-Comment 3: Page 9. Change to the past tense the description of the methodology under “Sequencing of selected bands from DGGE gels”

Response: In accordance with the reviewer's suggestion, we have changed to the past tense the description of the methodology under “Sequencing of selected bands from DGGE gels” (Page 9, lines 16-24).

-Comment 4: Page 10. The concentration of bacteria was calculated using standard curves. “Standard curves were created using serial tenfold dilutions of DNA from pure cultures, corresponding to $10^1–10^{10}$ copies/gram of feces”. However, the different genera analyzed have different copy number of the 16S rRNA gene. Have the results been normalized for that?

Response: We thank the reviewer for this observation. We are sorry not having added this information before. As the reviewer indicates the copy number of the 16S rRNA gene present in the different genera of bacteria are different and for this reason the standard curves have been normalized to the copy number of the 16S rRNA gene for
each species. Moreover, for the species whose copy number of 16S rRNA gene was not published, it was calculated by averaging the gene numbers of the closest bacterial taxa from the ribosomal RNA database rrnDB (http://ribosome.mmg.msu.edu/rrndb/index.php). We have now added this information in the methods section, in the "Microbial quantification by real-time qPCR" of the revised manuscript (Page 11, lines 2-6).

**Comment 5:** Page 12, end of the page. In this section the authors evaluated the abundance of some specific genera of bacteria NOT the “Differences in the size of the bacterial population” Please revise this statement and conclusions based on this.

**Response:** We thank the reviewer for this observation. We have now revised and changed this statement, (page 13, line 22) and conclusion based on this observation in the revised manuscript.

**Comment 6:** Figure 1. I couldn’t see it (it was really small). Check for formatting issues.

**Response:** We are so sorry about the problem that the reviewer has had with the image size. We have now improved the image formatting in the figure 1 to avoid this problem.

**Comment 7:** Page 14-19. The discussion should be summarized. It is too long and too repetitive. Recently (2011) Giongo et al. (ISME J) reported a study on children with diabetes type I where microbiota sequencing was performed. This manuscript would benefit of a discussion of similarities and differences observed between the two studies.

**Response:** We thank the reviewer for this observation. We have now summarized and restructured the discussion section of this manuscript using for this purpose the reference suggested by the reviewer (page 15-19). However, we would like to indicate that new data have been added to the discussion because they have been required by the reviewer 1.