Article information: https://dx.doi.org/10.21037/tp-23-76

Reviewer A

This manuscript aimed to determine if there were changes in microbiome composition associated with craniosynostosis. There are numerous significant concerns with the methodology that make it difficult to interpret the findings from the study.

First - the participant group that the authors refer to as "healthy controls" were treated in hospital for a head injury. Pediatric head injury, cannot be the control group. This is essentially a study of two distinct brain injury groups, with no control group.

Reply: We greatly appreciate your comments. Previous studies have reported that traumatic brain injury was likely to lead to intestinal dysfunction and changes in gut microbes, but the process is a long-term process of brain-gut regulation (Hanscom M. et al., *J Clin Invest.* 2021. PMID: 34128471). The participants we finally included were almost 10 months' infants with minor and minor head injuries for the first time. Moreover, we try to choose the patients admitted to the same department as the controls. Certainly, we have to admit that this is one of our shortcomings, and future studies should try to select appropriate healthy controls. We also added this content to the Discussion section (see Page 17, lines 351-354). In addition, in order to more precisely express the contents, we have checked the entire manuscript and changed the words "healthy control" into "non-craniosynostosis".

Second- there is no report regarding the age of the children. There are numerous studies demonstrating that the microbiota matures and changes across development. the differences identified by the researchers could have been due to maturation, not the craniosynostosis.

Reply: Thank you very much for your comments. We added some related data in the Table 1 and have modified our text as advised (see Page 10, lines 195-196; Table 1).

Third - All of the statistical analyses for the microbiota requires multiple comparison

corrections (such as a false discovery rate) and this was not done at all.

Reply: Many thanks for your comments and we apologize for our lack of clarity. In this study, unless stated otherwise, statistical analyses of 16S rRNA sequences were carried out using Student's t and Wilcoxon test, when necessary, normalization of the data for comparison with R software. For KEGG pathway enrichment analyses, differential proteins were used to perform enrichment tests of KEGG pathways using two-tailed Fisher's exact test method with a filter value of 0.05 in the FDR (false discovery rate) term filter mode and removal of duplicates. We added this content to the Statistical analysis section (see Page 10, lines 181-185). Thank you again for your advice, and I also hope our reply will answer your questions.

As a minor comment - there are some critical spelling mistakes - for example, fecal samples are incorrectly written as fetal samples

Reply: Many thanks for your comments. We have modified our text as advised and checked the entire manuscript to avoid similar mistakes (see Page 10, line 191).

Reviewer B

This manuscript investigates the potential relationship between craniosynostosis and the composition of the gut microbiome. While mostly descriptive, this study provides important information in human subjects that can be followed by more specific and mechanistic assessments. There are numerous grammatical errors throughout the manuscript that make it very difficult for reading (a clear example of this is the abstract, in which the word "serious" was used instead of "series"). Thus, authors are strongly encouraged to revise the document for a correct use of English grammar. In addition to language imprecisions, other important aspects need to be addressed prior to consider this work for publication.

Major points:

1. It is not clear why subjects with head injury were used as controls and even called "Healthy controls" when that was not the case. This greatly reduces the enthusiasm about the relevance of this study as traumatic brain injury is a well know factor

associated with gut microbiome alterations. This group is far from being a healthy control. In addition, no information about sex or age of the subjects was provided and this is important information for the analyses of microbial populations.

Reply: We greatly appreciate your comments, and we agree with your suggestion. Previous studies have reported that traumatic brain injury was likely to lead to intestinal dysfunction and changes in gut microbes, but the process is a long-term process of brain-gut regulation (Hanscom M. et al., *J Clin Invest.* 2021. PMID: 34128471). The participants we finally included were almost 10 months' infants with minor and minor head injuries for the first time. Moreover, we try to choose the patients admitted to the same department as the controls. Certainly, we have to admit that this is one of our shortcomings, and future studies should try to select appropriate healthy controls. We also added this content to the Discussion section (see Page 17, lines 351-354). Furthermore, in order to more precisely express the contents, we have checked the entire manuscript and changed the words "healthy control" into "non-craniosynostosis". In addition, we added some related data in the Table 1 and have modified our text as advised (see Page 10, lines 195-196).

2. The way authors refer to the craniosynostosis (CS) group is not consistent, some times it is called "Case group" or "Group B". Authors should aim for consistency.

Reply: Many thanks for your comments. We have modified our text as advised (see Page 10, line 197; Page 11, line 200-201).

3. While rarefaction curves were presented, there was no indication as to whether differences in sequence number were present between groups. This is important because if the sequences differed, subsampling had to be done to avoid artificial effects due to this phenomenon.

Reply: Many thanks for your comments. Rarefaction curve refers to the random selection of a certain number of individuals from the sample, the statistics of the number of species represented by these individuals, and the number of individuals and species to build. When the curve tends to be flat, it indicates a reasonable amount of sequencing

data, and more data will only generate a few new OTUs. As was shown in the Figure 2A, the results showed that the number of sequencing data was reasonable, and the included sample size was sufficient to reflect the species composition of the community. Thank you again for your advice. In addition, I also hope our reply will answer your questions.

4. Results from table 1 do not include any statistics. This is very important for making conclusive remarks.

Reply: Thank you a lot for your valuable advice, and we agree with your suggestion. We added some related data in the Table 1 and have modified our text as advised (see Page 10, lines 195-196; lines 198-199).

5. The Results section is written very vaguely, describing alterations or changes rather than specifying the directionality of the effects (e.g., increased or decreased).

Reply: Thank you very much for your advice. We have modified our text as advised (see Page 10, lines 198-199; Page 12, lines 233-241).

6. Figure 1A and 1B are lacking a description of the statistical analyses employed to compare the two groups, if any.

Reply: We greatly appreciate your suggestions. Unfortunately, there were not *P* value for Figure 1A and 1B. We only observed the composition in the different groups. But we can find the dominant bacteria through Figure 3 and Figure 4.

7. How can authors explain the lack of differences between the two groups for beta-diversity?

Reply: Thank you a lot for your valuable advice. Figure 3 showed the major trends in data change of two groups based on the principal coordinate analysis (PCoA) and Nonmetric Multidimensional scaling (NMDS) methods. Then, we used the OIME2 software to test the difference of two groups, the detailed parameters were as follows:

P- permutations = 999, P-methods = “permanova”，“anosim”，“permdisp”. The results there was no statistical difference in PERMANOVA test (All corrected *P* values >0.05) (see Page 12, lines 230-232).

8. The discussion of LEfSe analyses is based on species of bacteria that were not specifically found in the study (e.g., L reuteri, E fecalis, etc). Most of the authors findings were at the level of order. Thus, their discussion of these bacterial species is artificial and irrelevant for the present studies.

Reply: Thank you a lot for your valuable advice, and we agree with your suggestion. We have made the suggested change in the Results section (see Page 15, lines 308).

9. The first paragraph of the discussion focuses on reduced OUT number and bacterial taxa in the CS group, but no statistics were provided. Discussion on the differences in alpha diversity indexes (why some are reduced whereas others are increased) is missing.

Reply: Thank you for your comments, and we agree with your suggestion. In this study, we found that the CS group have significantly lower levels of Family, Genus, and Species than non-craniosynostosis group. And we have modified our text as advised (see Page 10, lines 198-199; Table 1).

In this study, we assessed the alpha diversity based on seven indexes. Chao1 and Observed species indices were used to characterize richness, while Shannon and Simpson indices were used to characterize diversity. In addition, Michaelis Menten fit was used to fit the rarefaction curve of observed OTUs, Pielou's evenness index was for evenness, and Good's coverage index for coverage. Among these indexes, the distributions of the Chao1, Good's coverage, Michaelis Menten fit and Observed species was different between the two groups. Except for Good's coverage index, the values of other three indexes were lower in the CS group than non-craniosynostosis group, which indicated that the abundance of species in the CS group than non-craniosynostosis group. However, another clinical study showed a decreasing trend of alpha diversity in patients with osteoporosis compared with the normal bone mineral density (BMD) control, which is inverse to our results. These discrepancies might

potentially be due to the different numbers of included patients, population information (age, gender compositions), living habits including diet and exercise, and medicine use including antibiotics and anti-osteoporosis drugs in these clinical studies. We have modified our text as advised (see Page 14, line 277-284).

10. It is not clear why authors mentioned in the discussion (2nd paragraph) that their findings on the Firmicutes to Bacteroidetes ratio was consistent with the literature when they did not find any changes in Firmicutes.

Reply: Thank you for your comments and we apologize for our lack of clarity. Previous studies have found that the ratio of Firmicutes/Bacteroidetes was negatively correlated with the loss of bone. Our study found that Bacteroidetes was significantly enriched in the non-craniosynostosis group, while Firmicutes (Phylum) had no difference between the two groups. That is say, the ratio of Firmicutes/Bacteroidetes was negatively correlated with the non-craniosynostosis group. At the same time, bone development in the non-craniosynostosis group was lower than that in the CS group, and bone content was also lower. In short, Previous studies thought that the ratio of Firmicutes/Bacteroidetes was negatively correlated with the loss of bone. Our study believed that the ratio of Firmicutes/Bacteroidetes was negatively correlated with the non-craniosynostosis group. Thus, we thought the results were similar. Thank you again for your comments. In addition, I also hope our reply will answer your questions.

11. The results displayed in Supplementary Fig 2 are lacking statistical analyses.

Reply: Thank you for your comments. There were not *P* values for Supplementary Fig 2. We only observed the composition in the different groups. But we can find the dominant bacteria through Fig 3 and Fig 4.

Minor points:

1. In the abstract, the total of 93 children with CS and 71 children with head injury should be removed as it is misleading as only 30 subjects of each condition were sampled in the end.

Reply: Many thanks for your comments. We have modified our text as advised (see Page 3, line 50).

2. Introduction, second paragraph: Non-syndromic CS is mentioned but never defined. Please specify.

Reply: Thank you a lot for your valuable advice. We have modified our text as advised (see Page 6, lines 103-105).

3. Labels in figures are too small to be read, please consider increasing their font size. In addition, use the same labels for CS and controls as in the main text to label figures to keep consistency.

Reply: Thank you for your comments, and we agree with your suggestion. We have made the suggested change in the figures.

4. Acronyms like BMD are abruptly introduced without any previous spelling.

Reply: Many thanks for your comments. We have modified our text as advised (see Page 6, line 108) and checked the entire manuscript to avoid similar mistakes.

5. Pielou's evenness, and Shannon indices are missing in the methods but are included in the results.

Reply: Many thanks for your comments. We have modified our text as advised (see Page 9, lines 176-177).

6. Good's coverage is described under the section for statistical analyses as an alpha diversity index when it is not. Please correct.

Reply: We greatly appreciate your suggestions. Good's coverage index was generally used to assess the alpha diversity, representing coverage (Good, 1953. https://doi.org/10.1093/biomet/40.3-4.237). In this study, we used the OIME2 software to calculate the value, the detailed parameters were as follows: p-steps 10 --p-min-depth 10 --p-iterations 10. Then, we used Wilcoxon test the difference of two groups.

7. Second paragraph of the Statistical analyses section ends with LEfSe results. Please consider moving these two sentences down to join the 3rd paragraph of this same section, in which more results on LEfSe are provided.

Reply: Many thanks for your comments. We have modified our text as advised and re-expanded the statistical analysis section (see Page 10, lines 174-188).