

Gut microbiota patterns associated with duration of diarrhea in children under five years of age in Ethiopia



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Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

Impression: This work presents an interesting analysis of potential unique characteristics of the gut microbiomes of Ethiopian children between 0 and 5 years of age. However, the relative sparsity of the outcome of interest (ProPD) in this dataset, and questions regarding some of the methodology employed, warrant considerable revision before consideration for publication.

Summary

The authors present a description of the gut microbiome in 1313 Ethiopian children from 0 – 59 months old with varying states of diarrheal severity, ranging from non-diarrheal controls to children who had experienced diarrhea for 14 days or more at their time of presentation to the participating health centers. This study was prospective but cross-sectional, with an apparent lack of information on disease progression in participants. Using 16S rRNA gene sequencing, the authors describe the relative abundance of the taxa present in these children, grouping them into non-diarrheal controls, acute diarrheal cases, and prolonged diarrheal cases. The authors report age-based differences in gut microbiome composition consistent with previous work. They also claim that presence of diarrhea has pronounced effects on the composition and diversity in their cohort, including differential abundance analysis to highlight differences in alpha and beta diversities between groups, as well as specific taxa enriched in each group. Finally, the authors report on the extent to which relevant clinical metadata explain the variation in gut microbiome composition seen in their cohort groups.

These bacterial compositional analyses are interesting. However, there remain sufficient questions regarding cohort distribution across groups, appropriate confounder control, and other analytical details that must be addressed before further consideration. Detailed critiques and comments are now:

Major comments

1. For the described bacterial compositional analysis, we recommend selecting a minimum threshold for relative abundance for the PCoA and differential abundance

testing. In our experience, a minimum threshold of 0.1% is appropriate for 16S data. We recommend re-analysis of this data with such a minimum threshold in place to filter out taxa that are of very low abundance in the profiled stools. The addition of this filter will likely result in some changes in the differential findings between the groups, especially between the ProPD and AD cases in the section entitled, “Microbial taxa differing between ProPD and AD cases.”

2. The authors report controlling for age, enrollment season, enrollment site, and sex, which are valid and necessary controls. However, it is unclear why the authors only chose these specific variables as controls. It can be inferred based on Table 2 that the authors might have included variables that were significantly different between groups, but we recommend that they explicitly explain how they chose the covariates for their Bray-Curtis dissimilarity comparisons using PERMANOVA and differential abundance testing using DESeq2 in the main text. Furthermore, in Table 2, other factors such as the WAM index and breastfeeding are also significantly associated with gut microbiome composition but are not included as confounders. The authors should both justify their controlling variables and include all possible confounders in all their comparative analyses.

3. This work focuses heavily on differentiating non-diarrheal controls, acute diarrheal cases, and prolonged diarrheal cases. While a sample size of 95 children with prolonged diarrhea is valuable, the fact that this feature of interest composes approximately 7% of the total cohort and 15% of the diarrheal subset should give pause about the conclusions made. We strongly recommend reframing this valuable work as a story focused on non-diarrheal controls versus all diarrheal cases, with a substory at the end exploring the ProPD subset.

4. Should these authors proceed with major comment 3, we recommend performing a sub-sampling of the AD patient samples to generate a balanced one-to-one AD-to-ProPD subset for further analysis.

5. The authors should be realistic about the limitations and interpretations of their findings. The “Discussion” section seems to indicate that they have no data to truly differentiate AD from ProPD, in that they only have the patient or care report on days of diarrhea before collection. Put another way, a child counted as an AD patient with only 13 days of diarrhea at collection may well proceed to subsequent days of diarrhea without the knowledge of these authors. This leads to the concern that this dataset captures the effects of diarrheal duration on the gut microbiome rather than any features truly associated with disease progression. The authors are strongly encouraged to reframe the current work with this significant limitation in mind. As aforementioned, one suggestion would be to restructure

their analysis to a story focused on non-diarrheal controls versus all diarrheal cases, with a substory at the end exploring the ProPD subset.

6. With the substantial cohort imbalance mentioned in major comment 3, analyses that interpret clusters as featuring a “high proportion” of ProPD cases when the cluster in question is more than 70% non-ProPD samples should be removed. However, analyses and interpretation of clusters with clear dominance of diarrheal cases (such as those for AD cases, with 72.6% and 81.3% AD makeup) can remain.

7. Similarly with major comment 6, a cluster that is 57% diarrheal cases and 43% non-diarrheal cases is a balanced cluster, not a diarrheal-dominant cluster, and these authors should interpret it as such. Cluster II, which is composed of 75% diarrheal cases, is more reasonably a diarrhea-dominated cluster.

Minor comments

1. The authors should be wary of over-interpreting statistically significant differences in taxa abundance that may not carry practical or clinical significance, such as the difference in *Eubacterium hardrum* between ProPD (0.23%) and AD (0.45%) patients. Or if they believe such differences are indeed clinically significant, they should describe why, with literature support.

2. Figure 1 is not informative and can be more clearly communicated in text. We recommend removal and replacement with the current Figure 2.

3. We strongly recommend that the authors provide more detail about their study cohort in the main body of the paper. While it is appropriate to leave most methodological details for the “Methods” section, the reader would benefit from substantially more information than currently provided in both the “Introduction” and “Results” sections.

4. We recommend that supplementary Figure 1, which demonstrates the fact that age is an important factor in gut microbiomes despite diarrheal status should be incorporated into the main text Figure 2.

5. Supplementary Figure 4 a and b could replace main text Figure 3 c and d. The appropriately controlled figures should be in the main text, with uncontrolled figures in the supplement if these authors wish to include them.

6. We recommend that the authors replace all panels in Figure 4 with coefficient plots to provide clear interpretation of taxa that are diminished versus enriched in each state.

7. In the section entitled, “Microbial taxa differing between diarrhea cases and non-diarrheal controls,” these authors should include the relative abundances for *Fusobacterium* and *Haemophilus* as they do for *Escherichia*, *Campylobacter*, *Streptococcus*, *Veillonella dispar*, *Prevotella copri*, *Faecalibacterium prausnitzii*, *Dialister succinatiphilus*, and *Bacteroides fragilis*.
8. We recommend aesthetic adjustments to all heatmaps in this work. Firstly, the clustering tree atop each is far too crowded to be informative and should be removed. Secondly, selecting such a bold and dark color for the lowest abundance draws the eye to those rather than the lighter colors of the higher abundance taxa. We strongly recommend changing the color gradient to a true gradient, with the lightest color as the lowest abundance and the darkest color as the highest abundance. Thirdly, we recommend removing the “Age groups (months)” and “Diarrhea status” annotation bars from the top of all heatmaps as they are too small and crowded to assist in interpretation and also do not contribute to later points. Along with this, the key should be adjusted to reflect any implemented changes.
9. In Figure 5, these authors report using Canberra distance for clustering while previous analyses in this work have used Bray-Curtis dissimilarity. The authors should justify their choice.
10. We recommend including Supplementary Figure 8 in the main text for its clear illustration of the enrichment of *Escherichia* spp., *Bifidobacterium*, and *Streptococcus* in the AD-dominated cluster II.
11. We strongly recommend that the authors explicitly state in the main text all models used in DESeq2 and vegan to obtain their results, as inappropriate model choice can skew findings.
12. The authors must correct the numerous typos in Table 2.
13. Rather than Table 2 in its current form, we recommend performing t-tests between diarrheal versus control microbiome profiles to define which variables are statistically significantly different between groups. Further, these authors must be much clearer about how these associations factor into their analysis, similarly to major comment 2.
14. If the authors truly set out to explain ProPD in the collection of this cohort, they must explain why only 7% of their cohort includes their feature of interest, including relevant epidemiological data.

15. Based on the cited previous publication of this cohort, these authors should remark on the presence or absence of cryptosporidiosis in their non-diarrheal, AD, and ProPD subgroups.

Reviewer #2:

Remarks to the Author:

The manuscript analyzed the gut microbiota over 3.000 Ethiopian children under five years of age, comparing the gut microbiota found in feces collected from healthy children and children with prolonged, persistent and combined diarrhea. Despite this study, is one of the largest studies in this area, the article is limited to a superficial description of the microbiota findings, with very simple analyzes and without correlating the findings with the current published data. The poor discussion of the results is striking. It is suggested to perform an in-depth analysis of the data and focus on the impact of the findings found in the context of diarrhea in children and the projections that this study may have on the management of diarrheal infections in LMIC.

One of the worrying aspects of the data analysis is the information provided regarding the use of antibiotics before obtaining the samples. The % of the samples obtained from children in which an antimicrobial was used is indicated where around 10% antimicrobials were used, without indicating the type of antimicrobial and the duration of treatment. Although this variable was not associated with changes in the intestinal microbiota, there is sufficient evidence to support that the use of antimicrobials has a significant effect on the composition of the intestinal microbiota.

Response to the comments of the reviewers

We would like to thank the reviewers for their thoughtful comments, which has helped us to improve the manuscript. The comments of the reviewers are stated in black font, while our responses are in blue font and italics.

Reviewer #1 (Remarks to the Author):

Impression: This work presents an interesting analysis of potential unique characteristics of the gut microbiomes of Ethiopian children between 0 and 5 years of age. However, the relative sparsity of the outcome of interest (ProPD) in this dataset, and questions regarding some of the methodology employed, warrant considerable revision before consideration for publication.

Summary

The authors present a description of the gut microbiome in 1313 Ethiopian children from 0 – 59 months old with varying states of diarrheal severity, ranging from non-diarrheal controls to children who had experienced diarrhea for 14 days or more at their time of presentation to the participating health centers. This study was prospective but cross-sectional, with an apparent lack of information on disease progression in participants. Using 16S rRNA gene sequencing, the authors describe the relative abundance of the taxa present in these children, grouping them into non-diarrheal controls, acute diarrheal cases, and prolonged diarrheal cases. The authors report age-based differences in gut microbiome composition consistent with previous work. They also claim that presence of diarrhea has pronounced effects on the composition and diversity in their cohort, including differential abundance analysis to highlight differences in alpha and beta diversities between groups, as well as specific taxa enriched in each group. Finally, the authors report on the extent to which relevant clinical metadata explain the variation in gut microbiome composition seen in their cohort groups.

These bacterial compositional analyses are interesting. However, there remain sufficient questions regarding cohort distribution across groups, appropriate confounder control, and other analytical details that must be addressed before further consideration. Detailed critiques and comments are now:

Major comments

1. For the described bacterial compositional analysis, we recommend selecting a minimum threshold for relative abundance for the PCoA and differential abundance testing. In our experience, a minimum threshold of 0.1% is appropriate for 16S data. We recommend re-analysis of this data with such a minimum threshold in place to filter out taxa that are of very low abundance in the profiled stools. The addition of this filter will likely result in some changes in the differential findings between the groups, especially between the ProPD and AD cases in the section entitled, “Microbial taxa differing between ProPD and AD cases.”

We appreciate the comment of the reviewer. We have re-analysed our data using the suggested minimum threshold of 0.1% relative abundance in all differential abundance tests as well as the beta-diversity based analysis (Figures and Tables have been updated accordingly). Introduction of this threshold does not change our

findings and conclusions. The introduction of a minimum abundance threshold is now mentioned in the Materials and Methods section, see page 17.

2. The authors report controlling for age, enrollment season, enrollment site, and sex, which are valid and necessary controls. However, it is unclear why the authors only chose these specific variables as controls. It can be inferred based on Table 2 that the authors might have included variables that were significantly different between groups, but we recommend that they explicitly explain how they chose the covariates for their Bray-Curtis dissimilarity comparisons using PERMANOVA and differential abundance testing using DESeq2 in the main text. Furthermore, in Table 2, other factors such as the WAM index and breastfeeding are also significantly associated with gut microbiome composition but are not included as confounders. The authors should both justify their controlling variables and include all possible confounders in all their comparative analyses.

We accept the reviewer comment about controlling all confounders and repeated all DESeq2 and PERMANOVA analysis using these settings. We selected the confounding variables based on their significant contribution on the gut microbiome in our db-RDA analysis (Table 2). We furthermore also control for confounders not found to be significant in our analysis (Table 2) but known from other studies to influence the gut microbiome composition. For example, we controlled for sex, enrollment season and site. We expected seasonal nutritional shifts associated with Ethiopian dietary habits, where most families consume vegetables during the wet season and mainly cereals during dry season and enrolment site in which the children enrolled from Jimma town have different environmental exposures than children enrolled in Serbo town, a more rural catchment area. We now explained the selection of confounding variables in the main text page 18.

3. This work focuses heavily on differentiating non-diarrheal controls, acute diarrheal cases, and prolonged diarrheal cases. While a sample size of 95 children with prolonged diarrhea is valuable, the fact that this feature of interest composes approximately 7% of the total cohort and 15% of the diarrheal subset should give pause about the conclusions made. We strongly recommend reframing this valuable work as a story focused on non-diarrheal controls versus all diarrheal cases, with a substory at the end exploring the ProPD subset.

4. Should these authors proceed with major comment 3, we recommend performing a sub-sampling of the AD patient samples to generate a balanced one-to-one AD-to-ProPD subset for further analysis.

We acknowledge this valid suggestion of the reviewer, but also argue, that our findings regarding the characteristics of the prolonged diarrheal cases are important. Despite only representing 15% of the diarrhea cases enrolled in the study, the ProPD cases nevertheless mounts up to 95 cases due to the large size of the cohort. However, we have now reframed the manuscript based on the reviewer comments and focus more on a diarrhea vs. non-diarrhea case comparison. The findings from analysing differences between acute diarrhea (AD) and prolonged or persistent

diarrhea (ProPD) is now included in the manuscript as a substory after the main one focusing on diarrhea vs. non-diarrhea cases. We explained this in the Introduction page 4, and the Results section on pages 6-9, and in the Discussion pages 10-14.

The reviewer's suggestion of doing a balanced one-to-one AD-to-ProPD analysis has also been implemented in the manuscript (page 9/ Supplementary Fig. 7).

However, one can also argue, that doing a one-to-one analysis between AD and ProPD leads to "loss" of good and valid gut microbiome information from 459 AD cases and reduces the total power of the analysis. Therefore, we decided to also keep the analysis of all 554 AD cases vs. the 95 ProPD cases (see page 9). Having both analysis in the manuscript allows the reader to better judge the findings.

5. The authors should be realistic about the limitations and interpretations of their findings. The "Discussion" section seems to indicate that they have no data to truly differentiate AD from ProPD, in that they only have the patient or care report on days of diarrhea before collection. Put another way, a child counted as an AD patient with only 13 days of diarrhea at collection may well proceed to subsequent days of diarrhea without the knowledge of these authors. This leads to the concern that this dataset captures the effects of diarrheal duration on the gut microbiome rather than any features truly associated with disease progression. The authors are strongly encouraged to reframe the current work with this significant limitation in mind. As aforementioned, one suggestion would be to restructure their analysis to a story focused on non-diarrheal controls versus all diarrheal cases, with a substory at the end exploring the ProPD subset.

The reviewer is raising a valid point. Obviously, while most AD cases fortunately recover without developing into ProPD, some AD cases will naturally turn into ProPD cases. This limitation of the study is now discussed in more detail on page 14.

6. With the substantial cohort imbalance mentioned in major comment 3, analyses that interpret clusters as featuring a "high proportion" of ProPD cases when the cluster in question is more than 70% non-ProPD samples should be removed. However, analyses and interpretation of clusters with clear dominance of diarrheal cases (such as those for AD cases, with 72.6% and 81.3% AD makeup) can remain.

The reviewer has a valid point and we have updated the figures accordingly and made the necessary adjustments of the manuscript. See page 8 (Figure 7)

7. Similarly with major comment 6, a cluster that is 57% diarrheal cases and 43% non-diarrheal cases is a balanced cluster, not a diarrheal-dominant cluster, and these authors should interpret it as such. Cluster II, which is composed of 75% diarrheal cases, is more reasonably a diarrhea-dominated cluster.

Again, the reviewer has a valid point and we have thus updated the figure (Figure 5) accordingly and made the necessary adjustments of the manuscript. See page 7.

Minor comments

1. The authors should be wary of over-interpreting statistically significant differences in taxa abundance that may not carry practical or clinical significance, such as the difference in *Eubacterium hadrum* between ProPD (0.23%) and AD (0.45%) patients. Or if they believe such differences are indeed clinically significant, they should describe why, with literature support.

*The reviewer has valid argument, but we will also argue that the reduced relative abundance of *Eubacterium hadrum* (synonym *Anaerostipes hadrum*) from 0.49% relative abundance in AD cases and 0.52% in healthy controls to 0.27% in ProPD cases and might still have health implications for the individual child, as this bacterium is an efficient butyrate producer that contains > 45 butyrate-associated genes including 9 butyryl- coenzyme A (CoA) dehydrogenase-linked genes and thus may play major role in butyrate production (Ravi K. et.al 2015, <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4384487/>).*

2. Figure 1 is not informative and can be more clearly communicated in text. We recommend removal and replacement with the current Figure 2.

Figure 1 has been removed and the recruitment is described in the main manuscript in more detail instead. See page 4-5.

3. We strongly recommend that the authors provide more detail about their study cohort in the main body of the paper. While it is appropriate to leave most methodological details for the “Methods” section, the reader would benefit from substantially more information than currently provided in both the “Introduction” and “Results” sections.

We have added more information about our cohort characteristics in the Introduction and Result sections. See Introduction page 3 and 4 and Result page 5.

4. We recommend that supplementary Figure 1, which demonstrates the fact that age is an important factor in gut microbiomes despite diarrheal status should be incorporated into the main text Figure 2.

Updated as suggested. Supplementary Figure 1 has been added to the main manuscript as Figure 2.

5. Supplementary Figure 4 a and b could replace main text Figure 3 c and d. The appropriately controlled figures should be in the main text, with uncontrolled figures in the supplement if these authors wish to include them.

The manuscript has been updated as suggested. See Figure 3.

6. We recommend that the authors replace all panels in Figure 4 with coefficient plots to provide clear interpretation of taxa that are diminished versus enriched in each state.

Figure 4 have been updated along the lines suggested by the reviewer. However, to make the visualization easier to read, we prefer Raboplot (<https://github.com/jstokholm/rabuplot>) which is commonly used for microbiome data visualization using boxplots.

7. In the section entitled, “Microbial taxa differing between diarrhea cases and non-diarrheal controls,” these authors should include the relative abundances for *Fusobacterium* and *Haemophilus* as they do for *Escherichia*, *Campylobacter*, *Streptococcus*, *Veillonella dispar*, *Prevotella copri*, *Faecalibacterium prausnitzii*, *Dialister succinatiphilus*, and *Bacteroides fragilis*.

*The manuscript has been updated as suggested. However, *Fusobacterium* is no longer mentioned as it is no longer significant after the updated analysis. See page 7.*

8. We recommend aesthetic adjustments to all heatmaps in this work. Firstly, the clustering tree atop each is far too crowded to be informative and should be removed. Secondly, selecting such a bold and dark color for the lowest abundance draws the eye to those rather than the lighter colors of the higher abundance taxa. We strongly recommend changing the color gradient to a true gradient, with the lightest color as the lowest abundance and the darkest color as the highest abundance. Thirdly, we recommend removing the “Age groups (months)” and “Diarrhea status” annotation bars from the top of all heatmaps as they are too small and crowded to assist in interpretation and also do not contribute to later points. Along with this, the key should be adjusted to reflect any implemented changes.

The visual appearance of the relevant figures has been updated as suggested by the reviewer with a more visually appealing use of colours, i.e. gradient blue for low abundance and red for high abundance. Given the age distribution of the cohort, we have decided to not remove the “Age group” annotation bar. As the reviewer understands “age” is the most significant covariate that influence the gut microbiome in this cohort, and we thus strongly believe the results of this study should be interpreted having in mind the effect age on the gut microbiota of this cohort.

9. In Figure 5, these authors report using Canberra distance for clustering while previous analyses in this work have used Bray-Curtis dissimilarity. The authors should justify their choice.

We base the analysis on hierarchical clustering of taxa found by differential abundance analysis to differ between two given groups, where Canberra distance-based clustering is a common approach and applied this in Figure 5 and in all subsequent heatmaps. We could also have chosen e.g. Euclidean distance metrics or Manhattan and did indeed also initially test this, but the obtained results were overall similar to the Canberra based results, and we decided to stick with this.

10. We recommend including Supplementary Figure 8 in the main text for its clear illustration of the enrichment of *Escherichia* spp., *Bifidobacterium*, and *Streptococcus* in the AD-dominated cluster II.

*Figure 7 has been updated as suggested. For consistency, we choose include all the DESeq2 differential abundance analysis results in the main Figures and show all sPLS-DA results as **Supplementary**.*

11. We strongly recommend that the authors explicitly state in the main text all models used in DESeq2 and vegan to obtain their results, as inappropriate model choice can skew findings.

The requested information was already stated in the original manuscript. However, we have slightly updated the text to make it easier to find. See page 17 and 18.

12. The authors must correct the numerous typos in Table 2.

Table 2 has been carefully revised as suggested by the reviewer.

13. Rather than Table 2 in its current form, we recommend performing t-tests between diarrheal versus control microbiome profiles to define which variables are statistically significantly different between groups. Further, these authors must be much clearer about how these associations factor into their analysis, similarly to major comment 2.

We appreciate the reviewer recommendation to explore our data with different statistical tests such as the t-test. But we believe distance based Redundancy analysis(db-RDA) is the common method of statistical analysis to identify covariates that influence the gut microbiome as seen here ([Jiyeon Si .et.al 2022, https://www.nature.com/articles/s43587-022-00286-w](https://www.nature.com/articles/s43587-022-00286-w)). This is due to different factors such as the nature of zero inflated microbiome compositional data and the non-normal distribution of microbiome data and also the need for multiple comparison of microbiome taxa.

14. If the authors truly set out to explain ProPD in the collection of this cohort, they must explain why only 7% of their cohort includes their feature of interest, including relevant epidemiological data.

We have responded to a similar comment above (comments 3 and 4). First and foremost, we were limited due the natural low prevalence of ProPD cases (around 15%) but due to the size of the cohort we still nevertheless end up on a decent 95 ProPD cases. Of note, it took two years of enrollment to reach this number. As explained in the Methods part the data and sample material utilized in this study were obtained in a study focusing on diagnostic accuracy of a diagnostic test, and not powered or directed towards infants with ProPD. We would like to add, that we believe this study on the gut microbiome of ProPD is the first on its kind and can be regarded as an exploratory study to design a large-scale multicenter study to robustly determine the role of the gut microiome in children with ProPD across different enrollment sites etc.

15. Based on the cited previous publication of this cohort, these authors should remark on the presence or absence of cryptosporidiosis in their non-diarrheal, AD, and ProPD subgroups.

The manuscript has been updated as suggested. See page 5.

Reviewer #2 (Remarks to the Author):

The manuscript analyzed the gut microbiota over 3,000 Ethiopian children under five years of age, comparing the gut microbiota found in feces collected from healthy children and children with prolonged, persistent and combined diarrhea. Despite this study, is one of the largest studies in this area, the article is limited to a superficial description of the microbiota findings, with very simple analyzes and without correlating the findings with the current published data. The poor discussion of the results is striking. It is suggested to perform an in-depth analysis of the data and focus on the impact of the findings found in the context of diarrhea in children and the projections that this study may have on the management of diarrheal infections in LMIC.

One of the worrying aspects of the data analysis is the information provided regarding the use of antibiotics before obtaining the samples. The % of the samples obtained from children in which an antimicrobial was used is indicated where around 10% antimicrobials were used, without indicating the type of antimicrobial and the duration of treatment. Although this variable was not associated with changes in the intestinal microbiota, there is sufficient evidence to support that the use of antimicrobials has a significant effect on the composition of the intestinal microbiota.

The reviewer raises several valid points. We have as indicated above expanded the analysis e.g. by including a one-to-one comparison of AD vs. ProPD cases (See Result section pages 9). Furthermore, we have changed especially the Discussion section to better place our findings in the context of previous studies aiming to elucidate links between gut microbiome and diarrhea in LMIC settings. See Discussion pages 10-14.

Regarding antibiotics use in our cohort, we have now stated the used types of antibiotics on Result section page 5. Importantly, data on antibiotics use was collected from the caretaker responsible for the child and information on the duration of antibiotics treatment was not available. Antibiotics consumed due to diarrhea and upper respiratory infections are bought at local drug stores by the caretaker and they often stop treatment when symptoms improve. While these data are not optimal, they unfortunately reflect reality in Ethiopia. Of note, the fact that antibiotics use was not associated with a clear gut microbiome pattern may reflect that only a minor fraction of the children received antibiotics, that several different antibiotic types were used and possibly also widespread antibiotic resistance in Ethiopia which potentially limits the influence of antibiotics on gut microbial structure across the cohort. Similar observations have been made in another cohort in another LMIC, namely Vietnam (<https://www.nature.com/articles/s41598-022-24488-9>). We have updated the Discussion to better reflect this.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Major critiques

- The authors switch from non-constricted PCoAs (Figures 1 and 2) to a constricted PCoA (Figure 3) without clear justification or explanation. The authors should perform consistent analyses or (recommended) perform both analyses, reporting one in main text and one in a supplementary figure, and providing in-text justification for the PCoA visualization method chosen.
- The conclusion stated in the figure legend title for Figure 9 does not match Figure 9. As there is no clustering of ProPD cases, this figure does not demonstrate a distinction between AD and ProPD. Please rephrase this figure legend to reflect the evidence or replace this figure with one similar to Figure 6.

Minor critiques

- Lines 135-142: These authors should explicitly state the metrics and tests used to compare these GMs.
- Lines 147–158: As the authors report in the methods section, they should report in-text here the controlled variables in their DESeq2 analysis.
- Line 153: An enrichment of 1.5% versus 1.2% is an extremely modest enrichment to report, despite its statistical significance. Please qualify the interpretation of this enrichment and discuss its potential clinical significance (or lack thereof)
- Lines 171 – 175: For consistency with the rest of the work, these authors should report the relative abundance values for the taxa in question in-text.
- Line 192: Typo, the authors omitted a “%”.
- Line 195: Typo, the authors omitted a “%”.
- Line 246: Typo, “e.g.” is unnecessary in this sentence.
- Line 299: Typo, “e.g.” is unnecessary in this sentence.
- Figure 2 legend: Typo, the authors missed an “a” for bolding.

Reviewer #2 (Remarks to the Author):

The revised manuscript addresses all comments made and meets the requirements for publication.

Replies to referees:

We thank the reviewers for their comments and for taking the time to read the manuscript.

We took all of them into account in the revised version of the manuscript and corrected any typos or sentences that required rephrasing.

There is one point that are not able to answer regarding the impact of SCO-RCG on opacities made by reviewer 1 on page 13. As no full tables are available for SCO-RCG, we cannot compute the calibration of a solar model and quantify this precisely. It is clear from experience with OPLIB and OPAS opacities that an impact can be expected, but a quantitative estimate would only be possible if we could compute a full evolutionary sequence.

Response to the comments of the reviewers

We would like to thank the reviewers for the final revision our manuscript. The comments of the reviewers are stated in black font, while our responses are in blue font and italics.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Major critiques

- The authors switch from non-constricted PCoAs (Figures 1 and 2) to a constricted PCoA (Figure 3) without clear justification or explanation. The authors should perform consistent analyses or (recommended) perform both analyses, reporting one in main text and one in a supplementary figure, and providing in-text justification for the PCoA visualization method chosen.

We appreciate the reviewer's suggestion, comment regarding the need for consistent constrained analysis in Figures 1 and 2. Figures 1 and 2 aims to illustrate the gut microbiome development across the entire cohort (Figure 1) as well as separately within cases (diarrhea) and controls (Figure 2). We performed constrained analysis in Figure 3 to control for any confounding variables that might affect the visualization of the diarrhea versus control comparison, but also included the unconstrained analysis as Supplementary Figure 2. We believe that applying constrained analysis to the age categories in Figures 1 and 2 will not change the interpretation of our results, but have made sure to clearly indicate in the text the possible confounders we control for in the constrained analysis (Figure 3).

- The conclusion stated in the figure legend title for Figure 9 does not match Figure 9. As there is no clustering of ProPD cases, this figure does not demonstrate a distinction between AD and ProPD. Please rephrase this figure legend to reflect the evidence or replace this figure with one similar to Figure 6.

The reviewer raises a valid point. We have rephrased the legend of Figure 9 accordingly. See lines 812-820 on page 37.

Minor critiques

- Lines 135-142: These authors should explicitly state the metrics and tests used to compare these GMs.

Corrected as suggested. We now mention the metric and tests used for comparison between diarrhea cases vs. non-diarrhea controls, AD cases vs. ProPD cases, and non-diarrhea controls compared with either AD or ProPD. Pairwise permutation multivariate analysis of variance (PERMANOVA) and distance-based Redundancy Analysis (db-RDA) based on Bray-Curtis dissimilarity metrics were used for these analysis (see lines 137-146 on pages 5 and 6).

- Lines 147–158: As the authors report in the methods section, they should report in-text here the controlled variables in their DESeq2 analysis.

We now mention the variables controlled including age group, enrolment season, enrolment site, sex, WAM index, current breastfeeding, and diarrhea in the previous month (see lines 149 and 150 page 7).

- Line 153: An enrichment of 1.5% versus 1.2% is an extremely modest enrichment to report, despite its statistical significance. Please qualify the interpretation of this enrichment and discuss its potential clinical significance (or lack thereof)

*We now discuss the proliferation of *H. parainfluenzae* in diarrhea cases and its role in causing acute gastroenteritis in the discussion part (see lines 270-272 on page 12).*

- Lines 171 – 175: For consistency with the rest of the work, these authors should report the relative abundance values for the taxa in question in-text.

Relative abundances of taxa are now added accordingly (see lines 177-179 and lines 187-188 on page 8).

- Line 192: Typo, the authors omitted a “%”.

“%” is added accordingly (see line 198 on page 9).

- Line 195: Typo, the authors omitted a “%”.

“%” is added accordingly (see line 201 on page 9).

- Line 246: Typo, “e.g.” is unnecessary in this sentence.

“e.g.” is deleted accordingly.

- Line 299: Typo, “e.g.” is unnecessary in this sentence.

“e.g.” is deleted accordingly.

- Figure 2 legend: Typo, the authors missed an “a” for bolding.

“a” is now in bold (see line 735 page 34).

Reviewer #2 (Remarks to the Author):

The revised manuscript addresses all comments made and meets the requirements for publication.

Thank you for your constructive feedback.