Responses to Reviewers

Minor revisions, if not explained, are applied to mostly for shorter manuscript and for minor modification along with revisions explained in this document. This document includes our responses to reviewers' comments:

* The reviewers comments are in italic font. Our responses are in regular font and embedded in dotted borders.

Reviewer1 (Tommi Vatanen)

Basic reporting

Authors present a cohort of preterm babies followed by longitudinal stool sampling at NICU. Small subset of babies developed NEC (n=4) or LOS (n=3) which are then compared to healthy controls.

Response:
We acknowledge the limited sample size in our cohort. To our knowledge, this is the first study profiling NEC and LOS patients among Asian population. So our data serves as a starting point for further studies in relevant fields.

The presentation of the data, bioinformatic and statistical methods used and English language are currently inadequate for publishing the manuscript.

Response:
We reanalyzed our data utilized Zero-Inflated Beta Random Effect model and other appropriate statistical methods including two way RM ANOVA. So we came up with more solid and reliable results and the data illustration has been improved greatly. We rewrote the whole manuscript and got editing help from our two of Jiayi Liu's previous lab mates at City of Hope National Medical Center with full professional proficiency in English. So we believe that our manuscript is now readable and understandable.

The introduction is jumping between different topics and many citations are old and appeared in low impact journals (e.g. Matamoros et al.). There is abudance of recent work in infant gut microbiome which should be cited to introduce readers to the field. The importance of sentence on lines 97-100 is unclear. I suggest starting with couple of sentences on post-birth gut microbiome, briefly defining the conditions in focus (NEC and LOS), describing previous gut microbiome work on these diseases and leading up to the motivations and goals of the current study.

Response:
We sincerely appreciate your suggestion and rewrote the introduction part. In detail:

1. We started with introducing microbiota and its role in human health (line 48-54).
2. Then we briefly reviewed how temporal microbiota in children would lead to (line 55-61), followed by the microbiota characteristics in preterm infants(line 62-66).
3. We described previous works focusing on NEC and LOS (line 67-75 and line 76-84, respectively).
4. Lastly, it came up with our motivations and goals of our study(line 85-95).

Lines 326-328: Authors are overselling their study

Response:
In our new manuscript, we have delete this part.
Yes, we did oversell our story in the original description. Thank you so much for pointing this out. We apologize for overselling the story thus conveying misleading informations.

Experimental design

Authors use an outdated method for processing 16S rRNA gene sequecing data. Independent comparisons operating with mock community data have shown that uparse is prone to false positive OTUs (see e.g. PMID: 27822515). Authors should process the 16s amplicon data using an up-to-date method such as DADA2 (PMID: 27214047) or deblur (PMID: 28289731)

Response:
We sincerely appreciate your providing us with two updated DADA2 and deblur, which represents the state-of-the-art in the field of 16s data processin. For us, these two pipelines are completely new to our team so it still need our time and efforts to optimize before we could get solid, reliable and reproducible results for publishing.
Previous study showed that DADA2 and uparse are both capable of discriminating samples by treatment, leading to similar biological conclusions (PMID: 28903732).
Taken together, we keep using our previous pipelines. However, in the near future we will surely use DADA2 as our new pipeline to publish. We apologize for not using the pipelines mentioned in your comments.

Validity of the findings

Figure 2 legend is missing and any information on statistical test used is missing even though p-values are reported on panel B. Panel B is also mostly unreadable since the relative abundance of most taxa is low and the bars are not scaled accordingly. In Methods, explain the statistical methods used and how do they account for longitudinality in the data.

Response:
We have reanalyzed our data to show their longitudinal nature. (Discussed below)

Authors compare bacterial OTU counts between groups but this is not feasible since the groups differ by size and lacks any statistical assessment. I suggest measure bacterial richness (number of OTUs) or other measures of alpha-diversity per each stool sample and conducting statistical testing between groups.

Response:
Thank you so much for the suggestions. We discarded the comparisons in merely OTU counts. Instead, we firstly reset the analysis time intervals as: early post-partum, early pre-onset, late pre-onset, early disease, middle disease, late disease, and post disease. (Because the disease onset time for each patient is not on the same day of life, it is not wise to analyze on a weekly scale.)(Detailed description is in line 183- 196). Then we calculate sobs and shannon diversity of each group in each intervals, followed by statistical tests.

Longitudinal nature of the data (repeated measures from same subjects) needs to be taken in to account in selecting the statistical test used. Similarly, comparing mean relative abundances between groups is not adequate since it doesn't account for repeated measures. I suggest using mixed effects linear models or something similar to conduct statistical testing for relative abundances.

Response:
Yes, we agree that mean taxa% is far from adequate analysis.

In our new analysis, we use the Zero-Inflated Beta Random Effect model to illustrated the longitudinal nature of our data, in details:

1. we splitted the whole sampling span into 7 time intervals: the analysis time interval as: early post-partum, early pre-onset, late pre-onset, early disease, middle disease, late disease, and post disease(Detailed description is in line 183- 196).
2. .
3. we set “Treat” (i.e. NEC, LOS, control) ,“Time” (as mentioned above) and “subjects” (24 patients identified by 24 unique IDs) as covariates and applied into the model.
4. we got the p value of each OTU and assign the OTUs to the genus.

From the model we detected OTUs with significant differences which matched to Bacillus and *Solibacillus.* Back to our genus trend (Fig 6), we found out the greatest difference in these two genus occurred at the early postpartum interval. Thus we speculate the initial microbiota compositions may contribute to the later health outcomes in preterm infants.

For your perusal, the source code for our modeling is available in our GitHub repository.

Besides, we also did mixed effects linear mode and got similar results (source code data were shown along with the ZIBR model at the GitHub repository). So we illustrated the former results. Please let us know if we should present the other model or both.

For diversity indices, we also utilized appropriate statistical tests (including two way RM ANOVA) and reported all significant results.
For relative abundance, we used alluvial plot to illustrate the trend of abundance changes over time and compare the abundance within each analysis interval to show their longitudinal natures (Fig 6).

Comments for the author

BioProjecgt PRJNA470548 was not found in NCBI SRA.

Response:
As for our raw data, we've uploaded to a public platform at figshare and its doi is 10.6084/m9.figshare.7205102. And the data at SRA(SRX4056077) has been set "private until paper is published" at SRA, so it is not public for now and our apologies for the inconveniences. .

Reviewer2 (William Schierding)

Basic reporting

A small scale study of NEC and LOS in preterm infants, showing the difference in basic metagenomes between controls and NEC/LOS. Clear and unambiguous, professional English used throughout. Sufficient field background/context provided. Professional article structure, figs, tables.

Response:
We appreciate your comments!

However, some novel data is first presented only in the discussion section.

Response:
We have moved the data from discussion to results section.

Raw data (link to SRA) has been shared privately. However, a link to the SRA does not appear to be with the paper text. There needs to be a mention of the SRA ID in the text. Apologies if I missed it.

Response:
As for our raw data, we've uploaded to a public platform at figshare and its doi is 10.6084/m9.figshare.7205102. And the data at SRA(SRX4056077) has been set "private until paper is published" at SRA, so it is not public for now and our apologies for the inconveniences. .

Experimental design

Very well done experimental design and research question. Methods sufficiently described. Sequencing depth (data generation) and analysis pipeline is appropriate to achieve aims of analysis.

Response:
Thank you so much for valuing our works!

However, results reveal that the design was underpowered for full 16S analysis across the two treatment and one control group. Thus, few significant results. Appropriately powered to generate a hypothesis, seems appropriate for a small journal such as PeerJ.

Response:
We have enriched our analysis from the following perspective:
We compare the longitudinal diversity indices using two way RM ANOVA.

With use the Zero-Inflated Beta Random Effect model to illustrated the longitudinal nature of our data, we were able to show the longitudinal nature of our data. From the model, Bacillus and Solibacillus arose to be significantly differed genus among three groups.
We plotted alluvial plot to show chronological trend in genus abundance.

Validity of the findings

Largely negative/inconclusive results. Small (but significant) differences seen between case and control groups. Conclusions from the data are not overstated. Are reasonable, given the results.

Response:
Thank you for your comments! With our updated analyses, our findings are more reliable and solid than before.

Since NEC average onset was 16 (within the 14-21d range) and LOS was 12 (within the 7-14d range), why is LOS not showing a similar significant trend for the 7-14d range in a similar way to the 14-21d range shows significant differences in NEC?

Response:
We've made a mistake here that the average onset for LOS was also 16.

In addition, we set time intervals for each patient to better analyze our data (because each patient has different onset timepoints, it is not fair to plot PCoA on a weekly basis). The intervals are: early post-partum, early pre-onset, late pre-onset, early disease, middle disease, late disease, and post disease (Detailed description is in line 183- 196).

Lines 285-287: Introducing new data in the discussion section. Why are these figures not reported in the results section?

Response:
We have moved the data from discussion to results section.

Comments for the author

Line 39: "dysbiotis" should be "dysbiosis"
Line 46: "rest17" should be "the rest (17)"
Line 67: delete "For" and start sentence with Preterm infants, who are prone...
Line 255: "less significant" -- the p value is > 0.05 so it is not a significant finding. Please correct the text.

Line 269: change "little" to "few"
Line 270: change "that" to "those"
Line 279: I believe you mean "7,472,400"
Line 317: "<Fig. S2" remove "<"

Response:
We accept all the amendment suggestions.

Figures:
S1a-c: Order of the legend is confusing. Please re-order the legend so that items in the legend from top to bottom are in order of days (i.e. 0-7 on top, and >28 on bottom)
Fig 2: Since PC1 and PC2 are such a high proportion, there is no need for S2b and S2c. S2a is sufficient to show the trend. Other figures are distracting.

Response:
We have updated our figures plotted using R with explicit illustrations.

Reviewer3 (Christopher Stewart)

Basic reporting

The English is not acceptable (see general comments for some additional information)

Response:
We rewrote the whole manuscript and got editing help from our two of Jiayi Liu's previous lab mate at City of Hope National Medical Center with full professional proficiency in English. So we believe that our manuscript is now readable and understandable.

No P values are provided in the abstract and so there is no way of knowing if the reported diversity of taxa %s are important. The same largely applies for the main results.

Response:
We amended the reported taxa% and their p values in the abstract. In the results, the diversity index values, percentage of primary coordinates, relative abundances were clearly reported with p values followed.

Sometimes the authors report 3 NEC and 4 LOS, and other times they report 4 NEC and 3 LOS.

Response:
Thank you for pointing this out. We have amended all the misleading numbers throughout our manuscript. The correct version is "4 NEC and 3 LOS patients", for your perusal.

Experimental design

With only 24 infants and a total of 192 stool samples, this study falls well short of most studies published in this area over the past years.

Response:
We acknowledge the limited sample size in our cohort. To our knowledge, this is the first study profiling NEC and LOS patients among Asian population. So our data serves as a starting point for further studies in relevant fields.

As an aside, many of these recent larger studies are not cited in the manuscript. Additionally, with only 4 NEC and 3 LOS infants, the study is greatly underpowered and fails to add substantially to the current literature.

Response:
In the new version of our manuscript, we updated our citations with larger studies, including the TEDDY study (PMID: 30356187; PMID: 30356187) and other cohorts (PMID: 28701177; PMID: 26969089).

Although our sample size is relatively underpowered, this study is the first to profile microbiota in NEC and LOS patients in Chinese population (to our knowledge, our apologies if we're wrong). Also, the high preterm birth rate increase the patient population at risk of developing NEC/LOS. Therefore, it added essential information in Chinese population and serves as a starting point for further understanding the etiology and pathogenesis of both disease in the nation.

It is not clear which samples were from the diaper or from the perianal skin surface. Furthermore, the latter represents a unique and none validated sample – is this representative of gut or stool? I also wonder about the ethics of collecting perianal samples using a spatula from extremely preterm infants.

Response:
We checked with the personnels who collected samples during the collection time and confirmed that: all the samples are collected from the diaper within 30 minutes after defecation.Therefore, we amended the time after defecation and deleted the "perianal skin" part in the manuscript, to eliminate the ethics concerns.

With read lengths of ~500bp there is likely to be a high error rate in the data. Pat Schloss explains this perfectly in his excellent blog post - http://blog.mothur.org/2014/09/11/Why-such-a-large-distance-matrix/

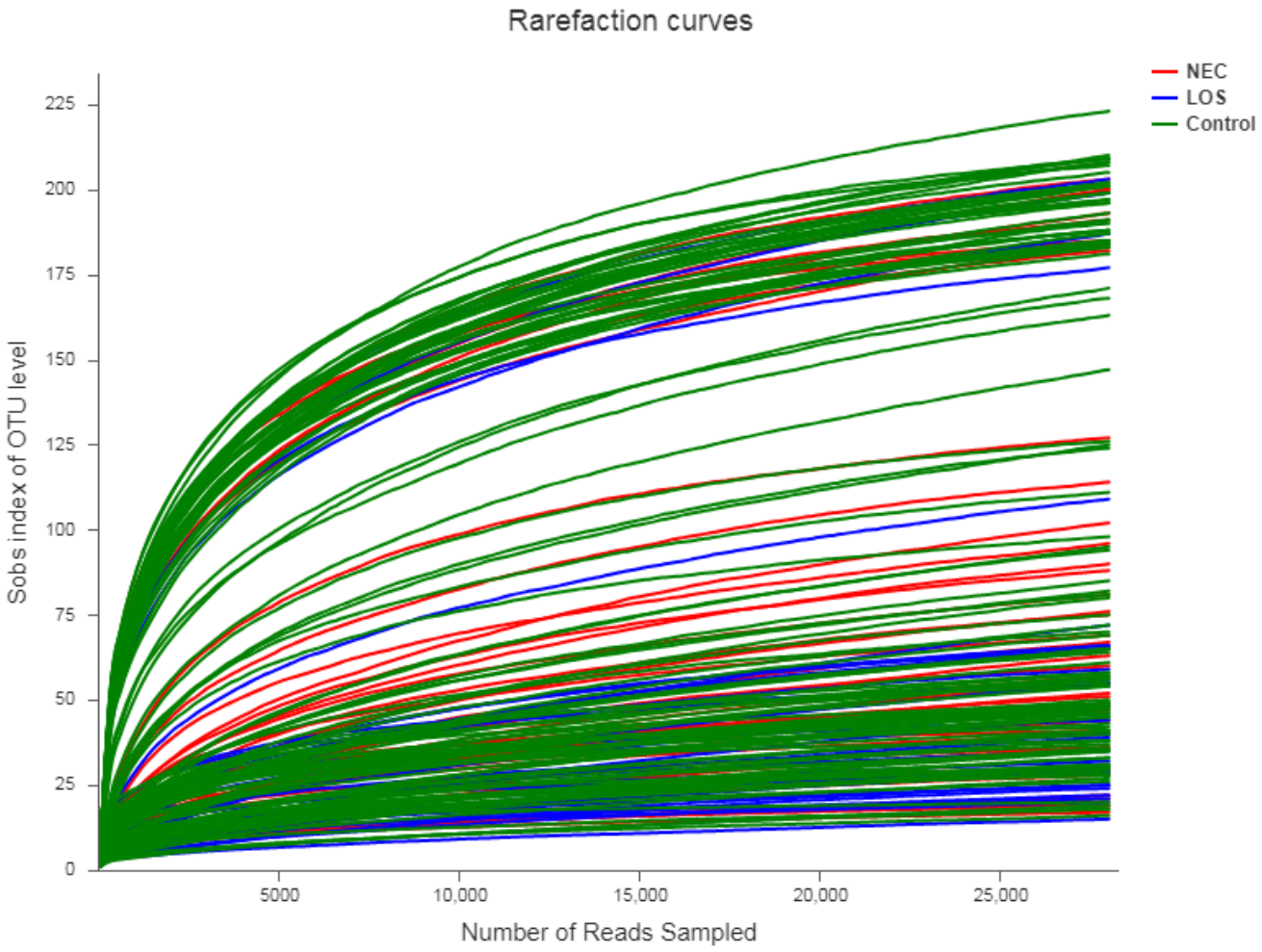
Response:
For our ~500 bp read length: We used primers 338F and 806R used (with the supposedly ~460bp read length) and illumina MiSeq (PE300) sequencing platform so that we could get >100 bp redundancies, which could satisfy our QC standards. The procedures of QC, assembling and merging reads would allow us to control the qualities of bases and reduce error rates.

Validity of the findings

How were the samples normalised? There is no mention of rarefaction or other?

Response:
We randomly subsampled to the lowest number of sequences produced from any sample to normalize our OTU dataset (PMID: 15592412) and we attached the

rarefaction curve for your perusal. Please let us know if it should be shown or omit in the manuscript.



No P values are reported for the alpha diversity or taxonomic comparisons, with the exception of the random P value on lines 234 and 235.

Response:
In our updated manuscript, p values are reported for every comparison in diversity indices both in text and figure descriptions (Fig 2, 3, &4). P values for taxonomic comparisons are all mentioned in the text.
We apologize for unprofessional data illustrations previously.

For the second P value the authors report "weakly significant", but this P value was 0.11 and is therefore not significant.

Response:
We agree with the reviewer's opinion and have deleted this part. Apologies for our unprofessional descriptions.

Comments for the author

The English is generally poor throughout making the manuscript challenging to read. While I cannot go through the entire text line-by-line and improve the English, from the abstract alone here is a selection of some errors:

- line 14 I think the authors mean "the remaining 17 were..", not "the rest17".

- Sometimes abbreviations are used and other times they are wrote in full, e.g., line 50 Late-onset sepsis should be LOS as it has already been abbreviated, in the conclusion for both NEC and LOS

- Line 50-51 "…held the least diversified gut microbiota" is poor English, similarly line 52 "with the control group held the most diversified one" is also poor English.

- Line 54 "Both two groups"

Response:

We rewrote the whole manuscript and got editing help from our two of Jiayi Liu's previous lab mate at City of Hope National Medical Center with full professional proficiency in English. So we believe that our manuscript is now readable and understandable.

It is not clear why sometimes rRNA is used and other times rDNA is used. The authors should be clear this is 16S rRNA gene sequencing and use this phrase throughout (e.g., in place of rDNA).

Response:

We changed all wrongly expressed terms into "16S rRNA gene sequencing" and use this phrase throughout. Thank you so much for pointing this out.

It is not stated what organism was cultured in the third LOS infant.

Response:

For the third late-onset sepsis patient, the following chief complaints and past medical history explained why she is grouped in the LOS group:

The patient presented with constant fever, tachypnea, poor feeding and white blood cells >20 cells/microL; she didn't react well with empirical antibiotics usage for more than 7 days. Thus, according to the principles of beneficence, she was given vancomycin before the result of hemoculture could be available. After receiving vancomycin, the symptoms alleviated, CBC parameters went normal so that her diagnosis was traced back as"Late-onset Sepsis". This explanation part is included in the correspondent table (Table S3).

* This is the translation version of the medical records and prescription charter. We apologize if anything is confusing. We would like to provide her whole medical records if necessary.

Centroids should be added to Figure 4, otherwise it is impossible to see what is going on.

Response:
We've updated our figures and discarded Figure 4. Instead, Figure 5 serves as the original Figure 4.