Reviewer Comments:

**Reviewer #1:** This is a very interesting paper! Here are some questions and comments that I hope you will find helpful.

**Introduction**

Does MOM really increase gut microbiome diversity? Although you state this in the background of your abstract, I don't see this specifically referenced in your paper (I may have missed this). I point this out, because, to my knowledge, there is no research on associations between newborn gut microbiome diversity and feeding type. If of course there is, it would be great if you put that reference in your introduction somewhere.

P. 1 Lines 24-P. 2, Lines 2: This sentence is an exact duplicate of a sentence earlier in the paragraph.

P. 3 line 7: Including a definition of "glycobiome" would be helpful.

It is difficult, from the introduction, to understand the differences between the different preterm feeding types. A table that clearly lists the different types of feeding along with rows that describe their differences, may be helpful. Also, it would be helpful to include non-human forms of preterm nourishment, i.e. formula here along with a brief description of what is known about the gut microbiome differences associated with each type of feeding.

**Methods**

1. What were your reasons for inclusion and exclusion criteria?
2. I see you collected abic use. Table 1, indicates that abic use was only in the first 48-72 hours. Is this correct for all babies in your study? How did you control for abic use in your analysis?
3. I understand the way that you created your feeding groups. However, I’m wondering how you controlled for the effect of changes between feeding type. You are comparing gut microbiomes between groups, but in your example on p. 6-7, an infant in the MOM+HDM group could have been in the MOM group for the first 10 days. So how would you account for the effect of previous feeding type within your group?

**Results:** Really interesting!

1. P.8, Line 5 - Need to fill in number to complete range "0 to....days"
2. Figure 2: What does PNA stand for
3. Please number your figures.
4. A brief explanation of alpha diversity and the Gini-Simpson index measures would be helpful. Why did you choose the Gini-Simpson index versus others, eg. Chao or Shannon?
5. P. 10, Line 3 - do you mean MOM here?
6. Are you able to breakdown feeding type in your Permanova analysis? Visually it seems like Formula and HDM+Formula had the biggest influence on beta-diversity.
7. I know this an exploratory study - are you powered for the permanova analysis?

**Discussion**

1. P. 11, Lines 6-8. I may be missing this, but I’m wondering where in your results/figures this statement is reflected. If it is in a figure, indicating this in brackets would be helpful.
2. Limitations?

**Miscellaneous**

1. Some grammatical/editorial corrections. Here are just a few examples, but the paper would benefit from a careful read-through.
   a) P. 8, line 4: change "varies" to "varied."
   b) P. 8, line 11: standards should be singularized
   c) P. 10, line 10: "groups" should be singularized
d) P. 13, line 10: think you can delete “are destroyed”

Reviewer #2: NARRATIVE REVIEW:
Manuscript Number NRES-D-16-00113 - Influence of Infant Feeding Type on Gut Microbiome Development in Hospitalized Preterm Infants

Thank you for the opportunity to review this interesting topic in the area of Omics in Nursing Science. Overall, this paper presented an interesting topic with an extremely complex description of DNA analysis and various advanced statistical techniques more commonly used in ecology.

Problem Statement or purpose of this study (page 3): The objectives of the study were clearly worded: “to explore the effect of different feeding types on gut microbiome development of preterm infants in the neonatal intensive care unit (NICU)” (lines 20-11, Page 3).

Background Literature (Introduction - pages 2-3): The background literature review seemed current and adequately presented research that introduced the issue and built up to the purpose statement.

Theoretical framework: The author/s did not present any theory to guide the research. There was some mention of “early programming” theory in the discussion section (line 21, page 10) but this was not explained as an actual theory and there was no citation.

Research Design and Method:

Design: On page 4 the author/s clearly state that this was a prospective exploratory study.

Sample: The author/s specifically identified the inclusion and exclusion criteria for the sample collection. It is implied that all parents whose infants meet the inclusion criteria during the study period were invited to participate but the parameters of the study period were not defined nor was it explicitly stated that all parents were approached. There was no indication if a power analysis was done to identify an appropriate sample size based on the number of independent variables. This is a major area of weakness since the author/s identified 6 cohorts that were assessed with multivariate analysis techniques over 3 time periods. A general rule of thumb for multivariate analysis is to have at least 35 cases per variable. In this study the independent variables seem to be the 6 feeding type cohorts: 1. MOM, 2. MOM + HDM, 3. MOM + Formula, 4. HDM, 5. Formula and 6. HDM + formula groups. The author/s also note that in 45.4% of the formula group the ‘standard’ types of formula were used and in 32.1% of the formula group an elemental formula was used. The use of numerous types of formula in any of the 3 formula groups (cohorts) may introduce error into the calculation plus it adds two additional variables to the study. It was also noted that human milk fortifier was added to 72.2% (n=24) of the infants in the second 10 days and 19 infants received this fortifier in the third 10 day study period. This adds another 2 variables (use of fortifier in human milk fed infants). Overall there seem to be at least 10 variables (6 for type of feeding, 2 for type of formula, and 2 for fortified or not fortified human milk). The sample size of 33 is not nearly large enough to study 10 different independent variables. A power analysis would have identified the minimum sample size needed to use PERMANOVA statistical analysis techniques to assess even a small or moderate effect of the type of feeding on the gut flora.

Data Collection Techniques: The DNA extraction and processing methods described on pages 5-6 were complex and seemed to be very thorough but I am not familiar with DNA extraction and testing methods so do not feel competent to make any statements about these data collection techniques. I will leave it to someone else to assess the adequacy of these techniques. The readers would benefit from a statement about how these data collection methods are tested for reliability and validity.

Statistical Data Analysis and Results: The author/s used the Gini-Simpson alpha diversity index which I presume was used to assess the diversity of gut flora in each of the feeding type groups. The author/s did
not state if the sampling estimate was determined with or without replacement. In small data sets such as this one (n=33), sampling without replacement versus with replacement could yield a substantial difference. The Simpson alpha diversity index is greatly affected by sample size and replacement. The proportional abundance of types increases as the number of types decrease and the most abundant type increases. It is recommended that the inverse Simpson index be used to transform the data from small sets and reduce the error created by these small sample sizes. The author/s did not mention the use of this transformation statistic. In the discussion on lines 1-2 page 10, the author/s noted that "the arcsine transformation was applied to Gini-Simpson diversity to obtain the normal distribution". I am not familiar with this transformation and do not know how it compares to the inverse Simpson index. The permutational multivariate analysis of variance was used to assess the effect of demographic and clinical characteristics and feeding on Beta-diversity of gut microbiome as noted in lines 15-17 on page 7. The author/s noted that "424 stool samples were collected from the 33" study participants (line 17, page 8). There was also no discussion about how to correct for autocorrelation of multiple stool samples collected from each individual case.

Summary: The author/s made the case for the importance or need for this study. This study is a complex description of DNA analysis and statistical techniques to deal with the small sample size and numerous independent variables. The author/s did not explain how reliability and validity of the DNA testing was insured or even tested. Many independent variables and numerous testing from each case can create error in analysis and the author/s did not explain if the complex statistical analyses controlled or accounted for this error.

Reviewer #3:
This is an excellent piece of work showing in-depth analysis of the developing microbiome in longitudinally collected samples from preterm infants in a NICU setting. This adds to our current knowledge base and should be published with some revisions.

When there is a switch from one type of feeding to another, microbiome developed during the first feeding should drive (to certain extent) the next phase. The only way to discern different feeding types is by comparing clean groups. However, in real life, depending on the availability of MOM, HDM, Formula, this can be controlled only to a limited extent by the caregiver. These limitations should be described in the discussion.

1. In the abstract line -4, the authors state absence of data from preterm infants. This is incorrect, and it should be changed to "limited". Although culture based, Gewolb I H et al. have described the changing microbiota pattern and the impact of BM, formula, and antibiotics in a NICU setting.

2. Since this is a feeding/breast milk study there needs to be better descriptions of and discussion on Bifidobacteriales. Why is there a paucity of Bifido in the MOM group? It is understandable to find less of Bifido in the very first days due to the gut environment (redox potential) that does not allow strict anaerobes. But, by 30 days they should be more abundant that what is expected. This reviewer is unable to attribute a reason why addition of formula will promote Bifido (Fig 1). I would like to see a separate bar graph for Bifido only so that they are better discerned and results discussed. Does not matter if we still do not know the reason for the varied levels of colonization.

3. In the discussion, the authors reasoned that-"Unlike raw MOM, HDM is considered a partial sterile product after pasteurization, impacting the presence of good bacteria present in raw breast milk such as Bifidobacterium breve and several Clostridium species are destroyed (Jost, Lacroix, Braegger, Rochat, & Chassard, 2014)". This is well taken- however, lack/traces of Bifidobacteria in infants exclusively on MOM diet in this study necessitates further investigation.

4. Recent literature now suggest stringent stool extraction protocols (with extensive bead beating for recovery of gram positive Bacterial DNA), and utility of specific primer combinations in the 16SrRNA-V3-V5 regions that may help in effective coverage of Bifidobacterium proportions for microbiota analyses.

5. Did the formula contain Fructo-oligosaccharides? Some new ones do. FOS promote selective growth of Bifido/Lacto.

6. It looks like HDM or formula promote Enterobacteriales. This should be emphasized. I would expect this in
formula-fed infants, but the results of HDM should be noted.

7. I would like to see some discussion on use of antibiotics. While this has been added in the multiple regression model, almost all infants would be expected to have received antibiotics during different stages for different durations. Antibiotics very seriously affect gut colonization. Especially when the naïve gut is getting colonized for the first time with limited number and types of bacteria, elimination or reduction of one or a few species may have very significant repercussions. Although we have no control over it (except using antibiotics judiciously, which we do not do), this aspect should be discussed and kept in context for the readers.

8. I am intrigued by the impact of gender, but using interaction terms the authors have addressed this to the best of their ability.

9. Figure legends need to have some description of seminal points. Fig 1 has none. Fig 2 has some etc.

10. The discussion is too long. There is no need to so much emphasize the positive aspects of fresh breast milk (it reads alike a nice BM review), most readers would know these points. The authors could touch upon the points and provide references to reduce verbiage. However, the description and discussion on HDM appear good. Results of this paper tend to show that HDM is no better than formula, in fact may be better. While the comparisons have not been done using clean recipients (mixed feeding and stool samples were lumped), it is hard to establish any strong correlation. The authors could describe the "associations" stating the limitations and leave the interpretations to the readers.

11. Concluding para: Instead of "Potentially pathogenic Gut microbiota" emphasis should be on - "preventing colonization due to pathogenic bacteria".


Reviewer #4: Summary-
The manuscript describes a study concerning the effects of feeds on the microbiota of premature infants over the first month of life. As the authors indicate, and much to my surprise, this has not been the subject of a specific microbiota-based study, although much information is available in term infants. The results are consistent with term cohorts, as one would expect, although a number of important methodological processes need to be clarified - some data is missing (the Jacard analysis), whilst elsewhere confounders appear to have been ignored (microbiota taxa analysis) and I have concerns over what I think is use of multiple stools per baby within a single time category.

Major corrections
As far as I can tell, the statistics performed on the microbiota-order level descriptors (as opposed to alpha diversity) have been conducted without consideration of the confounders (delivery method, sex, gestation etc). Given the very small group sizes of some feeds groups, it is essential to consider these other factors. Please repeat the microbiota analysis with this in mind- I suspect some groups will be too small to offer concrete conclusions.

P9 - lines 6 to 11 - After consideration of confounders (as above) - are these differences statistically significant?

Figure 2 - Looking at this figure, I am guessing that multiple stools were tested (where available) for a baby at a single time category? (days 11-20 show multiple points for formula, despite this being data from one infant). If this is true, I would be concerned that babies with more frequent stools will have a greater influence over the analysis, and casts some doubts on the validity of the results in my eyes. I would strongly encourage the authors to perform the analysis with the same numbers of stools per baby per time category.

Demographics of the different feed groups would also be very useful to allow critical appraisal of the data- ie. a breakdown of table 1 into feed groups.
From p8, line 12-13, it appears that some infants are supplemented with BM fortifier. I could not determine which infants these were in table 1, nor how it was distributed amongst the groups- please could this be
clarified. If the fortifier is concentrated in a particular group (e.g. all MOM babies are supplemented) the
effects of the particular feed will be more difficult to interpret (I think fortifier was only included as a
confounder in the alpha diversity analysis?)

Chimera slaying has not been mentioned in methods, yet apparently has been performed (p8, line 22).
Please could you clarify this in the methods.
The terms "microbiome" and "microbiota" are used interchangeably- given this is a 16s study, the paper
concerns the microbiota. Please correct where appropriate.
P9, line 4- the text describes "order-level species quantifications" (I assume this means order-level relative
abundances)- I'm not sure what "species" is doing there?
P10 - The beta diversity results are presented for the Bray-Curtis method- where are the results for Jaccard?

Table 3 - Coefficients for the different factors would be useful, as the provided data doesn't tell you the
direction of the relationship.

Discussion
P12, lines 10- 13 - I think that the WHO recommendation is being misinterpreted here. Where MOM is not
available, the choice is between HDM and formula- stating that MOM is better is both obvious and irrelevant
to the WHO statement. Unfortunately, the study isn't powered to make the relevant comparison of HDM vs
formula- there are insufficient babies in either group to perform a statistical comparison and decide which is
preferable.

Line 16-17- "Variability within naturally occurring microbial communities is often strongest between
samples," - This is a very odd statement. One would very much expect that different samples have different
microbial communities. I assume it was meant as a statement that there is high variability between samples
(perhaps comment on intra- and inter-baby differences?)

Lines 18- 20 -"Feeding type, gender, and the interaction of feeding type and gender explains a substantial
23% of the variability of the communities." - this implies that a good portion of the variability has not been
explained by the analysis and that something may potentially have been overlooked.

P13- "Our finding of a higher abundance of Clostridiales, Lactobacillales, Bacillales and Pasteurellales taxa in
the MOM cohort corroborates with the earlier work by Jost and colleagues" - To my knowledge, Jost et al
were investigating vertical transmission of bacteria, not the abundance of the different taxa.

P14- "Although it is still relatively unclear how different neonatal feeding types influence the gut
microbiome" - Many studies have investigated this- some reviewed in Coppa et al 2006 (Prebiotics in human
milk: a review) for example. It would be very easy to theorise why lactic acid bacteria might grow well in
the presence of breast milk.

The many studies of the effects of breastmilk on the term infant faecal microbiota are not mentioned in the
discussion- please compare these to your findings!

General Statistics comments
Were multiple hypothesis corrections made for each series of tests?

What distributions were used for the MELMs? A normal distribution isn't very suited to 16S count data (and I
assume wasn't used for the microbiota).

Minor corrections
Abstract
Line 12- "was estimates" should be "were estimated". Also, weren't the measures were calculated, not
predicted?
Line 13 - "Preterm infants fed MOM at least 70% total diet had highest abundance" - grammar.

Page 2
Line 12 - "Some research suggest" - should be "suggests". Also, this comment implies that there is other research indicating that it begins at other times - please expand on this if you wish to raise it as a point.

Lines 24-2 are a repeat of lines 18-21, word for word.

Page 3 -
Line 3 - Feeding mode implies the mode of feeding rather than the foodstuff being fed (which is what the authors are discussing).
Line 6 - "Raw breast milk" is a very odd term! I would prefer the terms pasteurized or unpasteurized.

Line 18 - "microbial composition associated pasteurized human banked milk and other alternate feeding modalities" - very difficult to follow.

Page 6
Line 1 - "visualization" should be "visualized"

Page 7
Line 4 - unnecessary comma.
Line 6 - QIIME will not calculate clinical data, and please correct the phrasing around "QIIME process".
Line 9 - "organism" should be "organisms".

Page 8
Line 5 - Missing the number of days in "from 0 to days"

Page 9
Line 17 - "The progression of microbial development seems different" - this is clear from figure 2 - this sentence should either expand on the differences or be removed.

Page 10
Change "were not significantly contributing" to "did not significantly contribute"
DOL is mentioned on line 15, but, unlike the other indicated factors, does not appear in Table 4.

Table 4 - Birth GA has a p value of <0.

*****
EDITOR’S COMMENTS:
--Please use (M = xx.x, SD = xx.xx), not M +/- SD
--On the reference list, please spell out journal names in full.

REQUESTED REVISIONS FOR STYLE

TITLE PAGE --
--Supply running head of less than 50 characters.

ACKNOWLEDGEMENTS --
--Supply the titles of those acknowledged.

TEXT --
--When your revision has been completed, run a final spell check. Also, proof to be sure that spelling errors not picked up by the spell check have been corrected.

REFERENCES --
When your revision has been completed, verify consistency between references cited in the text and references included on the reference list.

Update REFERENCE LIST using APA 6th Ed. format. In particular:
--Do not include the journal issue number if the journal is paginated consecutively.

Update IN-TEXT CITATIONS using APA 6th Ed. Format. In particular:
--Several citations/references were introduced in the Discussion (Civardi; Collado; Hamosh; Jost; Koenig; Landers; Li; McPherson; Montjaux-Regis; MM Nelson; WHO; Reeves; Silvestre; Tully; Untalan). Please include them before the Discussion or delete them.