Response to Review Comments
(Manuscript ID nutrients-552980)

Reviewers' comments:
Reviewer #1: This observational study examines nervonic acid (NA) in human milk and infant formula in China. The methods are relatively well described, and the authors make note of existing literature examining NA content in milk. This study is well written however it is unclear how the analytical method employed compares to current methods and reporting of NA in this population adds to what is currently known. Therefore, the novelty of the study in the context of the current literature and methodology is not clear and should be emphasized in this manuscript. Suggestions are revisions are included below.

The authors thank the reviewer’s comments. We have addressed the reviewer’s comments from point by point as follows. The corresponding modifications are now included in the revised version of the manuscript.

1. Please state in the abstract and line 212 how NA content in infant formula compares not only to colostrum, but mature milk as well.
The authors thank reviewer’s comments. Added as suggested (line 26).

2. In the introduction, please ensure the correct citation is used (reference 15), and state associated, rather than caused.
The authors thank reviewer’s comments. This part has been rewritten (line 50-51).

3. Please provide further details on the mixture of 37 FAMEs used as a standard.
A standard mixture of 37 kinds of fatty acid methyl esters (FAMEs) was bought from Sigma-Aldrich (St. Louis, MO, USA). The information has been added into the Materials.

4. From the sample size of n=8, it is unknown how this was used to formulate the values in Table 1. How many samples and timepoints were taken from each woman? And how was this variation in the number of samples considered in the statistical analysis? Moreover, how was missing data or difference in sample size for each of the lactation days considered in the statistical model?
This study was designed to analysis the NA and fatty acid composition in human milk during every lactation day in the first month. More than 10 volunteer mothers were enrolled. However, only 8 participants were successful exclusive breastfeeding. The human milk samples were collected from 8 participants every day from 3 to 30 days postpartum. Therefore, a total of 224 samples were obtained as shown in Table S1.

5. Further details are how samples were obtained are needed. Were women given specific instructions on expression on milk, and how were the samples stored and transported prior to storing at -80°C?
More detailed information was added to the manuscript where related (line 88-90).
6. How do the stages of infant formula (infant, follow-on and growing up) compared to lactation stages?
We have added a principal component analysis (PCA) to determine the differences in NA content in three lactation stages of human milk and three stages of infant formulae (Figure 5).

7. The authors give a reference (20) for how lipids were extracted, but please briefly describe in the manuscript.
The authors thank reviewer’s comments. Added as suggested.

8. Line 86 – was the 24:1 n-9 FAME standard the mixture of 37 FAMEs mentioned in 2.1? They are of different standards. The mixture of 37 FAMEs contains 24:1 n-9. However, for absolute quantification of 24:1 n-9 concentration, a calibration curve was set up using 24:1 n-9 FAME standard and 13:0 FAME as an internal standard.

9. Please explain the rationale for using a 23:0 FAME as an internal standard.
The internal standard needs to be absent in human milk and has similar physicochemical properties with NA which requires that the retention time of NA and internal standard is close [1]. Based on it, 23:0 FAME was chosen as the internal standard in this study.

10. Explain the statistical method used in analyzing the differences between women (Table 1). Additionally, please explain the rationale for examining differences between the n=8 women. Was the sample size large enough to detect meaningful differences in NA content?
Differences between women were tested by one-way analysis of variance (ANOVA) for continuous variables which has been added to the manuscript. We agree to the reviewer that the sample size is small (eight mothers), but we think the final set of samples were qualified (224 samples over 3-30d postpartum). We have some conditions to filter the human milk samples, 1) the mothers are exclusive breastfeeding in at least the first month of lactation, 2) the samples were collected every day during lactation, 3) the sample volume were enough for the NA analysis and the lipids in human milk were extracted as soon as the sample collected. The samples were difficult to obtain.

11. Figure 1 - please add the FA names to either footnote of on the figure.
Added as suggested (line 172-175).

12. Figure 2- was this a mean of NA content for each of the lactation days?
Yes, this is a mean of NA content in human milk fat of the 8 mothers. We also labelled the standard deviation of NA content of the lactation days.

13. Table 2- how were the n=181 infant formulae represented in this table?
A total of 181 infant formulae were analyzed, and 97 (53.59%) were found to contain NA. The n-9 FA composition of 97 infant formulae are presented in Table 2. And the 97 infant formulae were divided into three stages (infant formula, follow-on formula and growing-up formula) and three sources (cows' milk formula, goats' milk formula and plant-oil formula). This detailed information has been added to the manuscript where related.
14. Line 219 – states NA decreases over days of lactation, but then states concentrations remain stable after day 15. As this is contradictory, please provide more details on the pattern of NA over the course of lactation days.

The concentration decreased markedly during the first ten days and then declined slowly after 15 d. From 15 to 30 day, the concentration of NA still decreased, but the trend was slow down. This part has been rewritten.

15. The range of 0.06-0.020% in this study is noted as marginally different than previous studies reporting a range of 0.19-0.99%. However, this is more than just marginally different, and on the lower end of all other studies. The authors explain the difference could be due to analytical methods and the objectives state that the aim is to develop an effective method for quantification of NA. However, the authors fail to discuss in the introduction or discussion what the current issues are with quantifying NA are, and why this proposed method is effective.

The authors thank the reviewer’s comments. Follow the reviewer’s comments, and more discussion has been added into the Introduction (line 52-28).

16. In the discussion, please include what factors may be considered in explaining the discrepancies in the NA content between populations.

Besides the dietary habits, the individual variation also may be related to maternal age [2], and BMI [3]. These have been added to the discussion (line 266-267).

17. The abstract and discussion conclude that interindividual variation in NA content may be due to endogenous factors rather than dietary intake. However, the authors did not look at either of these factors to make this conclusion. Please revise and expand on why diet was not examined.

This study failed to exam the impact of maternal diet on the NA concentration of human milk. However, as far as we know, the NA concentration in the normal diet is very low. NA generally occurs in some plant oils. We previously analyzed the fatty acid composition in commonly edible vegetable oils using the same analytical method we used in this study [4]. The results indicated that NA was not detected in all 18 edible vegetable oils. According to the publications, NA has been found in some plant species [5] for example *Malania oleifera* [6] and *Lunaria annua* [7], which are very uncommon edible oils. However, the NA has been detected in all human milk samples, and the same decreased trend were observed. Therefore, we raised a hypothesis that NA in human milk may be influenced by the endogenous factors. Further study is needed.

18. Line 133 – abbreviated to ANOVA.

Corrected as suggested.

References


