Author’s response to reviews

Title: Associations between dietary micronutrient intake and molecular-Bacterial Vaginosis

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Author’s response to reviews:

Reviewer #1:

Background:

1. Line 18 has an orphaned parenthesis and is a bit lengthy. Rewording might enhance the flow.

We have revised this section as indicated below:

Line 7: BV is diagnosed in clinical settings by the Amsel’s criteria (i.e. having at least three out of four of the following: thin, homogenous vaginal discharge, pH >4.5, 20% of clue cells on saline microscopy, and a fishy odor after addition of 10% potassium hydroxide to a slide of
secretions (whiff test)). Historically, in research settings, BV has been assessed by Gram’s stain of vaginal secretions (Nugent score).(1)

2. Line 24: Consider explaining Nugent score in its own sentence as run-on sentences are difficult to follow.

Please see revisions above.

3. Page 2, Lines 9-16: Save the questionnaire name for the Methods section and you probably don't need to reiterate rRNA sequencing method here.

Line 28: This sentence now reads: “We conducted a cross-sectional analysis of the associations between dietary micronutrient intake and molecular-BV among women of reproductive age.”

Results:

1. Line 20: Report the percent as well for n=4 and n=5

Line 109: The sentence now reads, “After eliminating patients with very low (n=4, (3.6%)) or high (n=4, (3.6%)) estimated energy intakes, data from 104 female subjects were available for analysis.”

2. Line 24: Delete "young" as it is subjective

Line 111: We have revised the sentence: “Patients’ mean age was 26, and nearly 50% were using HC at the time of entry into the study.”

3. Line 31-36: Include p values

Line 114: We have revised the sentence: “Patients with molecular-BV had a higher BMI (p<0.01), were more likely to engage in vaginal douching (p<0.01) and were less likely to be using HC at study entry (p=0.01) compared to those with Lactobacillus-dominated CSTs (See Table 1).”
4. Table 2 Caption: Consider rewording the caption for clarity; I understand you created a binary variable and categorised the nutrient intake as lowest quartile or top three quartiles, perhaps just reiterate this instead of using "vs the rest". I would also spell out "ref" in its first use.

The table title now reads: “Table 2. Associations between usual micronutrient intakes and molecular-BV: bivariable and multivariable models analyzing the lowest versus the top three nutrient quartiles (reference).

5. Page 3, Line 51, why only mention zinc and selenium? Vitamin A appears significant in bivariate analysis and both vitamin A and lutein have higher significance in the multivariate analysis.

Line 143: We have added an additional sentence, “Vitamin A and lutein were also of borderline significance in the full model (p=0.10).”

Discussion:

1. Line 9-13: It is confusing to mention the CSTIII microbiota research here. I would save the opening paragraph of the discussion for your main findings (i.e. that CSTIII was not associated with lowest quartile micronutrient intake) and bring in how your findings fit with the literature in subsequent paragraphs.

We have modified the text and moved the CST III microbiota discussion section to right above the limitations section.

2. Page 2, Lines 40-47: This long sentence should be reworded for clarity and to remove the need for three sets of parentheses and "/".

Line 181: This section has been edited to read: “This may have been due to three main differences in study design and population. First, the Neggers et al. study had a much larger sample size and used the full FFQ rather than the brief FFQ. Second, the Neggers et al. study population was predominantly African-American, whereas ours was predominantly White. And third, the Neggers et al. study assessed BV as diagnosed by Nugent score and severe BV as defined by Nugent score and pH where we assess BV as defined by compositional analysis of the microbiota.
3. Page 3, Line 49: Do you have any potential explanations for why betaine supplementation decreased Lactobacillus given your findings?

At this point, we can only hypothesize why betaine might be associated with decreased Lactobacilli. There is a small amount of animal data and it may not all be applicable to humans. We have added a sentence to emphasize this point, which now reads (Line 214): “However, it is unclear how applicable this limited animal data may be to humans.”

4. Page 3, Line 54: You say your study is innovative because you utilized 16S rRNA sequencing to define BV however Molecular-BV has been defined previously (McKinnon et al 2019). You should specify how this study is unique (which is the micronutrient intake association with Molecular-BV).

Line 224: We have clarified this section: “Our study was innovative in that we related dietary measures to molecular-BV as measured by 16S rRNA gene sequencing. Importantly, molecular-BV presents a higher resolution assessment of the vaginal microbiota than Amsel-BV or Nugent-BV.(6)”

5. Page 3, Line 60: Again, be more precise than "vs. the rest".

Line 226: We have modified the text: “First, we had a relatively small sample size (n=104), which limited the analyses that could be conducted. We could not determine associations other than low-Lactobacillus CSTs (“molecular-BV”) vs. Lactobacillus dominated CSTs and to a lesser extent the L. iners-dominated CST III. Nor were we able to adjust for factors such as number of sexual partners, condom use, recent antibiotic use or menses. We were also unable to correct for multiple comparisons in the analysis.”

5b. Also, are there any limitations in examining only Molecular-BV vs Amsel-BV or Nugent-BV? These should be mentioned clearly here.

See edits above in #4.

6. Page 4, Lines 6-13: These sentences need to be reworded as the "Secondly, the study was cross-sectional in design" is a fragment. The natural fluctuations in vaginal microbiota over time seem like an important limitation, however, you do not phrase this limitation clearly. You say that vaginal microbiota fluctuates but then you say a one time sample "represents a typical
fluctuation pattern" which does not make sense. Please revise this paragraph and explain how your sample accounts for the fluctuations in microbiota and nutrition over time.

Line 231: We have revised this section: “Since the study was cross-sectional in design, we were not able to account for potential fluctuations in the vaginal microbiota or nutrition over time. It is well documented that the vaginal microbiota often fluctuate between CST III and CST IV, so there could have been some non-differential misclassification. However, we would expect that if there was non-differential misclassification, the odds ratio would have tended toward the null. Instead we observed a statistically significant point estimate, which suggests that the true risk, without the noise of misclassification, may be even stronger.

7. What should future studies focus on in light of these results?

Line 250: The results should be verified in a larger cohort with more definitive measures such as serum levels of betaine or a more detailed dietary instrument. The last sentence now reads: “However, further, larger studies which utilize more precise methods to measure betaine intake will be needed to verify and expand these results.”

Reviewer #2:

• Tuddenham et al. present a study examining the association between micronutrient intake and molecular bacterial vaginosis, finding that betaine intake was associated with BV. It is important that studies that were not optimally designed with microbiota analysis in mind (i.e. a retrofit of microbiota analysis to stored samples from a clinical trial or observational study) can still be used so long as they present novel findings and are couched in appropriate caveats (e.g. noting cross-sectional design, small sample size). I feel that the authors have achieved this.

We wanted to clarify that this study was not a retrofit of microbiota analysis to stored samples. The Parent study was designed to assess how initiation or cessation of hormonal contraception affects the vaginal microbiota. The samples were collected for 16S rRNA gene amplicon sequencing. At the baseline visit, the food frequency questionnaire was administered for this secondary aim to assess dietary intake and the vaginal microbiota at study entry.

• For those unfamiliar with the food questionnaire, it would be beneficial to provide more information on this. For example, how reliable are intake estimates from this methodology, and what period of time is captured in the result (noting that dietary changes can have a rapid impact
on gut microbiota, and vaginal microbiota can rapidly change) - these may enter the discussion as a potential limitation.

We have clarified the period of time which the dietary questionnaire covers, and will attach the list of diet analysis output variables produced by the Block Brief questionnaire as an attachment (Suppl. Figure 1). In this paper, we are only focusing on the micronutrients on this list. The text now reads “It asks participants to estimate intake of specific foods over a year and provides estimates of average daily intake of micro and macronutrients (See Suppl. Figure 1 for a list of diet analysis output variables produced by the questionnaire).

In terms of the accuracy of the FFQ, we note that they are not accurate for absolute measurements. They can only be used for relative measures. Please see the discussion section where we have modified text to note: (Line 237) “Brief food FFQs, including the one that was used in this study to estimate micronutrient intake, do not cover the full list of foods as in the Full Length FFQs. The brief FFQ likely underestimates usual intake of energy and nutrients, and can only be used to rank nutrient intakes between women in this study.”

• Is there a standard list of micronutrients that are assessed in the questionnaire? How many micronutrients were considered in this study? Table 2 lists 16 nutrients but there are probably more (e.g. iodine, B2, copper) which are not listed - were these also examined?

We examined all micronutrients listed in Supplementary Figure 1, however, we only list micronutrients which have a p<0.10 in Table 2. Table 2 does also include calcium and vitamin C as these have been evaluated in previous BV publications. We have revised the methods text to state (Line 87) “We assessed intakes of all micronutrients listed in Supplementary Figure 1, vitamins, minerals, methyl donors, carotenoid-derived antioxidants, essential fatty acids, and selected phytoestrogens.”

• For methods, vaginal microbiota characterisation, How many samples underwent a second round of extraction/sequencing, and could this have affected results? Is there validation data indicating that the latter extraction method gives improved results, noting that it has a lower input volume? Is the suggestion that these samples contained PCR inhibitors, or they had low DNA content?

Only three out of 104 samples were sequenced on the MagAttract Microbial DNA kit/Hamilton platform. The lab transitioned to using this platform because it uses a lower sample starting
volume, the method offered higher efficiency, with less sample loss along the steps of the extraction, and it was higher-throughput. With the QiaSymphony protocol, it took about 3 days for a person to process 90 samples, while it takes about 6 hours to process 180 samples on the Hamilton platform. There are several possible reasons for samples to have failed—indeed we typically see about a 5-10% failure rate for samples in vaginal microbiota studies. There could be low microbial content in the samples, the presence of PCR inhibitors, or mistakes in pipetting/sample transfers that resulted in microbial DNA below our detection limit. Importantly, based on a multiple sample series, we see no differences in sequence profiles between the two extraction methods. Bead disruption and complete lysis are key to consistent results and both protocols are very similar in that regard.

For clarity, we have modified this section (Line 49) to read: “All vaginal Eswabs (n=104) were first extracted with the QS DSP Virus/Pathogen Midi Kit (Qiagen) on the QiaSymphony platform. Three samples were reprocessed with the MagAttract Microbial DNA Kit (Qiagen) using a custom automated protocol on the Hamilton Microlab Star because the samples resulted in less than <15,000 reads with the first round of sequencing. Bead disruption and complete lysis are similar in both DNA extraction approaches.”

- Can the authors please indicate the year of recruitment of participants and the duration of storage of samples prior to extraction and analysis. Is there a reference for the parent study?

The study was recruited between the years 2011-2015. We have modified Line 35 to reflect these dates. This manuscript will be the first study to be published from the parent study, so there is not another reference currently. We could cite conference proceedings if the editor would like (Tuddenham S, Ghanem KG, Gajer P, Robinson CK, Ravel J, Brotman RM. The Effect of Hormonal Contraception on the Vaginal Microbiota Over 2 Years. The International Society for Sexually Transmitted Disease Research, 23rd Biennial Congress, July 14-17, 2019, Vancouver, Canada).

- There could be some more detail on the sequence results (e.g. what was the range and average read number for samples).

- For negative controls, presumably there were no swabs from the original study to use as negatives? The methods should detail how the negative controls were used to inform the analysis of study samples - i.e., what was present in these samples, and what was subtracted from the results of study samples. This information will help others when performing similar analysis.
We have included the following sentence in the Methods section (Line 72) “Samples included in this analysis had a median of 55,162 and a mean of 59,110 sequences (range of 17,313-235,834).”

Regarding the questions on negative controls, we have added the following text to the Methods section: (Line 57) “Water was processed in parallel with samples through the DNA extraction process and added as template during the first round of PCR. These acted as quality control for the PCR steps, so if a band was detected in negative controls, the PCR would be redone. If bands persisted in the second PCR, samples on that plate would be re-extracted to try to eliminate the contamination. Negative controls were not used here to remove any taxa from analysis.”

- Other factors associated with BV were not included in the analysis (e.g. past history of BV, female sexual partner, partner condom use), and factors that may have been important (e.g. recent antibiotic use, menses). Data may not have been collected for these factors, nevertheless these data should be mentioned as unavailable.

Given the small sample size, we unfortunately were not able to correct for many additional confounders. We feel that factors such as past history of BV, female sexual partner and partner condom use are less likely to be true confounders (i.e. associated both with diet and molecular BV). However, we agree that recent antibiotic use and menses could have an important short term impact on the vaginal microbiota. Therefore, we conducted additional analysis including these two factors in our models. This did not alter our results. We have added a line in the results section which now reads: Line 125: “We conducted additional analyses in which we also included menses in the last week and antibiotic use in the last 30 days in our models, however this did not substantially alter our results, and we present the more parsimonious model in Table 2.”

- Were samples used in this study also assessed for BV by another method, and if so, how did that correlate with the "molecular BV" designation?

Vaginal smears for Gram stain assessment were collected, but they have not yet been read. Nugent scores are not yet available for this study. Ravel and Brotman et al (PNAS 2011) have previously published on Nugent score and CST and they have consistently found high concordance across their studies.
• For the designation for each community state type, can the authors indicate the proportion of the main taxon in each group

Thank you, we have now included Supplementary Table 2 with this information, which we have referenced in the text.

• In the discussion, it would be helpful to include some more information on some of the studies - for example, samples sizes and strength of associations could be indicated in parentheses "(n=X, aOR=Y, p=Z)" to help the reader assess the outcomes of the study without downloading the supplementary table.

We appreciate this comment, however, it would be cumbersome to include this data on all studies in the discussion section. However, we have modified the discussion section to include this information on the Neggers study (which is most comparable to ours in terms of methods of dietary assessment, and was the most heavily discussed in this section-see below). The relevant sentences now read: “Finally, one study published by Neggers et al.(22) assessed dietary intake in a sample of N=1521 primarily lower socioeconomic status African-American women from Alabama. In that study, significant associations were found between severe BV (defined as Nugent score ≥9 and vaginal pH>5) and low intakes of folate (aOR 0.4, CI: 0.2-0.8), vitamin E (aOR 0.4, 0.2-0.8) and calcium (aOR 0.4, CI: 0.3-0.7).”

• Could the authors expand their discussion of betaine slightly? E.g. is it fully digested/absorbed by the gut, or does a substantial amount pass through? Is it secreted/available for bacteria at skin and mucosal surfaces?

We were not able to find information on whether dietary betaine is secreted/available for bacteria at skin and mucosal surfaces, though this is an intriguing question. We have modified the discussion to read: (Line 188)“There are several plausible mechanisms for why betaine may be associated with BV. Betaine is a small zwitterionic compound found in plants, animals and microorganisms. Dietary sources of betaine include seafood (especially marine invertebrates), wheat germ or bran, and spinach; in mammals it can also be obtained by endogenous synthesis from choline.(33) Dietary betaine is quickly absorbed, primarily in the small intestine.(34, 35)

• The authors note that betaine may enhance the osmotollerance of Lactobacillus species, but could this be a common phenomenon, i.e. there evidence of betaine supporting other species, and could this extend to G. vaginalis and other BV-associated bacteria?
We searched the literature but were unable to find further information on this. We have therefore added a sentence “Whether betaine plays a role in osmotolerance of BV associated bacteria is not known.

Minor comments:

• It would be good to list the number of CST (line 8, paragraph 2 of background)

We felt this sentence was confusing and modified to read: Line 14: “The CSTs are dominated by different species of Lactobacillus, or are characterized by a paucity of Lactobacillus spp. The latter CSTs are comprised of a variety of anaerobes such as Gardnerella vaginalis and Sneathia spp., and are consistent with BV.(5) The low-Lactobacillus CSTs have been collectively termed “molecular-BV,” as they reflect similar low-Lactobacillus states that are captured by Nugent score and Amsel’s criteria.(6)”

• A few minor errors in Table 1: 49/78=62.8%, 12/26=46.2% "FFQ" should be defined (used in Discussion)

We have made these corrections.

• In the discussion about the study by Neggers et al., the direction of the associations with folate, Vit E and Ca could be indicated.

We have clarified this to read “In that study, significant associations were found between severe BV (defined as Nugent score ≥9 and vaginal pH>5) and low intakes of folate (aOR 0.4, CI: 0.2-0.8), vitamin E (aOR 0.4, 0.2-0.8) and calcium (aOR 0.4, CI: 0.3-0.7).”

• This is a well written and easy to read manuscript

Thank you!