Author’s response to reviews

Title: Oxidative stress levels and oral bacterial milieu in the saliva from pregnant vs. non-pregnant women

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Author’s response to reviews:

To, The editor
Anne Menard,
BMC Oral Health
BioMed Central

Date: 16.02.2020

Dear Editor,

Thank you very much for giving us the opportunity to submit a revised version of our paper “Oxidative stress levels and oral bacterial milieu in the saliva from pregnant vs. non-pregnant women” in BMC Oral Health. We thank to the referees for their valuable comments and suggestions that were useful for further improvement of the manuscript.

We have submitted the revised manuscript with changes highlighted in red. An itemized response to reviewers’ comments is included in the bottom of this letter. We hope that our manuscript has now achieved a standard that is acceptable for publication in your esteemed journal.

Sincerely

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Oxidative stress levels and oral bacterial milieu in the saliva from pregnant vs. non-pregnant women

Reply to the reviewers

We sincerely thank all the reviewers who have contributed their valuable time to read and comment on our manuscript. We have tried our best to attend to all of your comments and revised our manuscript carefully taking into consideration all your suggestions as appropriate.

Reviewer 1
Sadeq Ali Al-Maweri (Reviewer 1): It was really a great pleasure reviewing this manuscript entitled "Oxidative stress levels and oral bacterial milieu in the saliva from pregnant vs. nonpregnant women". While this work addressed a very important and an interesting research question, there are some methodological limitations that should be addressed. Additionally, although the authors made a lot of efforts in writing this manuscript, the overall quality of writing is below average and hence some revisions are highly recommended. My specific comments to help improve the quality of this work are as following:

Title: Appropriate

Abstract: Overall, it needs some revisions.
Thank you for the comments.

- In the background subheading please explicitly mention the aims of the study.
Answer: The aims of the study is now included in the background section of abstract. (Page 2, lines 41-43)

- The methods should be elaborated: the procedure, techniques and statistical analyses should be highlighted.
Answer: Methods in the abstract are elaborated. (Page 2, lines 44-49)

- the Study design should be revised (please see below in the methods section).
Answer: As per comment, study design is revised. (Page 2, line 44)

Background:

- This section could have been better written. I recommend rewriting this part with more focus on the the association of oral health with pregnancy outcomes such as preterm birth and low birth weight. Additionally the rational of the study should be clearly set, and the gap in the literature should be highlighted.
Answer: We fully agree with the suggestion since our ultimate goal is to understand the association between oral health status and pregnancy outcome. The current paper is a proof of concept to observe whether pregnancy has any measurable effects on salivary oxidative stress levels and bacterial milieu. For this, we compared the saliva from healthy pregnant women with age matching healthy non-pregnant women as the control. In order to know the correlation between oral health and pregnancy outcome, one needs a larger scale longitudinal clinical study
on pregnant women that is already in progress. Therefore, we did not elaborate or discuss on the cross-talk between oral health and pregnancy outcome in this paper. However, we revised the background as per your suggestion. (Page 4-5, lines 97-132)

Methods:
major limitations of this study include small sample size; lack of any clinical data such as dental caries and periodontal parameters, both of which are potential confounding factors for both bacterial loads and oxidative stress.

Answer: Thank you for the comment. We have provided more detailed information on sample size calculation in the Methods section under Statistical analysis in our revised manuscript. Sample size required to detect 15% difference in the OS level, between pregnant and non-pregnant women with 80% power at an alpha of 0.05 was calculated to be at least 38 individuals per group on the basis of mean MDA level and standard deviation reported in the saliva of 25 healthy non-pregnant female controls in a previous report (Rai B, Kharb S, Jain R and Anand SC. World Journal of Medical Sciences 2006; 1(2):100-101) using an online sample size calculator (https://clincalc.com/stats/samplesize.aspx). (Page 8, lines 212-216)

A previous study on oxidative stress measured in the blood samples of pregnant and non-pregnant women had a sample size of 17 healthy pregnant women vs. 12 healthy non-pregnant women (Ref: Toescu, V. et al., Clinical Endocrinology, 2002, 57, 609-613). Compared to this our sample size is more than double.

Unfortunately we are unable to report any clinical data as such data were not collected. However, none of these women reported to have any medical problem including oral health issues.

Other specific comments are as following:
- Kindly follow STROBE guidelines
Answer: There is always a general advantage in following STORBE guidelines even though ours is not an epidemiological study. We appreciate your suggestion and have followed the guidelines.

- Please double-check the study design
Answer: Thank you for this. Indeed, this was a cross-sectional study with two comparing groups. We have made changes to our manuscript reflecting this. (Page 5, line 135)

- Inclusion and exclusion criteria should be extensively highlighted
Answer: Thank you for the comment. Inclusion and exclusion criteria are now clearly listed in the manuscript. (Page 5-6, lines 140-149)

- Please mention how sample size was estimated.
Answer: Sample size required to detect 15% difference in the OS level, between pregnant and non-pregnant women with 80% power at an alpha of 0.05 was calculated to be at least 38 individuals per group on the basis of mean MDA level and standard deviation reported in the saliva of 25 healthy nonpregnant female controls in a previous report (Rai B, Kharb S, Jain R and Anand SC. World Journal of Medical Sciences 2006; 1(2):100-101) using an online sample size calculator (https://clincalc.com/stats/samplesize.aspx). (Page 8, lines 212-216)
- I wonder why only pregnant women in their second trimester were recruited. Why did not you include other pregnant women at different pregnancy stages so as to compare the levels of bacterial milieu and OS between pregnant women based on the stage of gestation.
Answer: Thank you for the comment. We agree that including women at different gestational ages could have been another possibility. However, our choice of second trimester was a pragmatic one. Firstly, taking samples at different gestational ages from different pregnant women could increase the variability of measurements as the level oxidative stress and oral bacterial milieu may change during the course of pregnancy. In fact, we are currently conducting a longitudinal study to investigate this hypothesis. Secondly, in Norway, free ultrasound screening is offered to all pregnant women during gestational week 18-20. At this stage the placental function and pregnancy associated physiological changes are fully established, women have been already screened for any medical problems/risk of complications and fetal anomalies. Therefore, we consider this to be an ideal time point for a cross-sectional study to compare whether differences exist between pregnant and non-pregnant women in regards to OS and oral bacterial milieu.

- Again as stated above, I wonder why no any clinical data such as dental caries, tooth loss, and gingival inflammation indicators were established. Such factors might have responsible for the differences in the bacterial load and OS found in this study rather than the pregnancy status. Additionally, a part form the subject's age, no other relevant data were obtained including smoking status, BMI, nutritional status, systemic health, medications use, oral hygiene practices.... etc
Answer: Thank you for the comment. Unfortunately, we do not have clinical data on the non-pregnant participants of this study, although self-reported oral health status including dental caries, tooth loss and gingival health status have been collected for the pregnant population. Therefore comparing clinical oral health status between groups was not possible. However, no participants reported to have any chronic medical condition or oral health problem at the time of sampling.

Result: Relatively good, though some sentences need some refining for better clarity.
- Captions of figures should be moved from this section.
Answer: Thank you. The captions were included for the purpose convenience to improve readability, but they are now removed as suggested.

- I wonder why no statistical analysis was conducted to determine if there is any correlation between OS and bacterial load.
Answer: Thank you for the suggestion. We have now calculated the correlation between oxidative stress and bacterial load among the groups of pregnant and non-pregnant women population separately using Pearson correlation coefficient. No statistically significant correlations were observed.
Table 1

<table>
<thead>
<tr>
<th>Test</th>
<th>Group</th>
<th>r (Pearson corr. coff)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>Pregnant</td>
<td>0.20764</td>
<td>0.2386</td>
</tr>
<tr>
<td>LB</td>
<td></td>
<td>0.10593</td>
<td>0.5509</td>
</tr>
<tr>
<td>SM</td>
<td>Non-pregnant</td>
<td>0.10082</td>
<td>0.4860</td>
</tr>
<tr>
<td>LB</td>
<td></td>
<td>0.11193</td>
<td>0.4389</td>
</tr>
<tr>
<td>MDA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>Pregnant</td>
<td>0.11009</td>
<td>0.5105</td>
</tr>
<tr>
<td>LB</td>
<td></td>
<td>0.29464</td>
<td>0.0725</td>
</tr>
<tr>
<td>SM</td>
<td>Non-pregnant</td>
<td>0.17653</td>
<td>0.2200</td>
</tr>
<tr>
<td>LB</td>
<td></td>
<td>0.01280</td>
<td>0.9296</td>
</tr>
</tbody>
</table>

- Discussion
This section is too long, so I would recommend to make it concise and focused, as per the STROBE guidelines; in the first paragraph you may restate the main objective along with a brief summary of the key findings of the study. Additionally, the findings of this study should be interpreted and compared with other previous studies. Finally, the limitations of this study should be elaborated.

Answer: The discussion section has been updated according to the comments.

Figures and tables: Please indicate which statistical test was used
Answer: Statistical tests used described in the Statistical analysis section and also indicated in the figures. (Page 9, lines 218-223, Table 1, figure 1, 2, 3 and 4)

Language:
This work would highly benefit from extensive English editing by a native speaker
Answer: The revised manuscript has been proof-read by a native English speaker with editorial experience.

Reviewer 2
Esam Halboub, PhD (Reviewer 2): With reference to the manuscript entitled "Oxidative stress levels and oral bacterial milieu in the saliva from pregnant vs. nonpregnant women" and Manuscript Number: OHEA-D-19-00565, I highly appreciate the authors' efforts in conducting this study. You will find below my comments.

Abstract:
1. Elaborate more in the methodology in order to be a standalone abstract.
   Answer: The methodology is updated in the abstract.( Page 2, lines 44-49)

2. Explain the abbreviation "ABTS" upon its first appearance.
   Answer: Thank you. The full form for ABTS is provided when it first appears in the text. (Page 2, line 45-46)
3. Explain what OD731 stands for.
Answer: Full form for OD731 is added in abstract. (Page 2, line 53)

Introduction:

1. Many sentences were not referred to relevant citations.
Answer: We re-checked the cited references where relevant and appropriate.

2. It is not clear whether there are previous studies on the topic or not.
Answer: Thank you. So far to our knowledge no such study has been published previously. There is one paper on oxidative stress measurement in the blood sample of pregnant and non-pregnant women with a sample size of 17 healthy pregnant women vs. 12 healthy non-pregnant women (Ref: Toescu, V. et al., Clinical Endocrinology, 2002, 57, 609-613). Oxidative stress in saliva in association with bacterial milieu in pregnant women has not been reported. We have clarified this in the revised manuscript.

Methodology:

1. The "cross sectional design" is acceptable. But "case-control design" confuses the readers about the disease (cases versus controls) and the exposure (risk factor). Did you consider the physiologic condition like pregnancy as a disease for which you recruited healthy women as controls? What about the bacterial milieu, OS, TAC? Did you consider them as exposures (risk factors), or they were resultants secondary of pregnancy? Really, it is confusing design.
Answer: Thank you for this comment. Indeed, it was a cross-sectional study with comparison two groups and not a case-control study. To reflect this, we have revised the study design in the methods section of our revised manuscript. (Page 5, line 135)

2. Why did you collect stimulated saliva? Why not unstimulated saliva was collected instead, which reflects the oral health condition most of the time?
Answer: Thank you for the comment. In the commercial kit protocol of measuring bacteria milieu suggested to use stimulated saliva. Therefore, we used stimulated saliva. The same method was applied to both groups.

3. The inclusion and exclusion criteria were not detailed except for: "Woman with a history of any acute or chronic illness requiring regular medical treatment", a criterion that is vague.
Answer: Thank you. The inclusion and exclusion criteria are now listed more clearly in the manuscript. (Page 5-6, line 140-149)

4. Provide the full term of TBA solution.
Answer: Thank you. Full form for TBA is added. (Page 8, line 203)
5. The details on the sample size calculation were not clear. There must be means and standard deviations of both groups derived from previous studies or obtained from a pilot study. In addition the calculation program or equation must be provided.

Answer: The details of sample size calculation now are provided in the revised manuscript:
Sample size required to detect 15% difference in the OS level, between pregnant and non-pregnant women with 80% power at an alpha of 0.05 was calculated to be at least 38 individuals per group on the basis of mean MDA level and standard deviation reported in the saliva of 25 healthy non-pregnant female controls in a previous report (Rai B, Kharb S, Jain R and Anand SC. World Journal of Medical Sciences 2006; 1(2):100-101) using an online sample size calculator (https://clincalc.com/stats/samplesize.aspx). (Page 8, lines 212-216)

6. No data on oral health of the participants. Such data are so important owing to their confounding effects.

Answer: Thank you for the comment. We do appreciate the importance of this and have discussed it as a limitation of our study. Unfortunately, we do not have clinical data on the non-pregnant participants of this study, although self-reported oral health status including dental caries, tooth loss and gingival health status have been collected for the pregnant population. Therefore comparing clinical oral health status between groups was not possible. However, no participants reported to have any chronic medical condition or oral health problem at the time of sampling.

Results:

1. With presentation of data as medians, the Inter-Quartile-Range (IQR) must be presented.
Answer: We have provided IQR when data are presented as medians. (Page 9, line 227)

2. For SM (text and table 1), why did you compare as subcategories (Pregnant women who had score 0, 1, 2, and 3 versus the same subgroups of nonpregnant women)? There were statistical significance values for each subcategory!!!! What is the statistical test which you used for this purpose? In case you used Chi squared test, there must have been only one significant value.
Answer: Thank you for the comment. We did compare the overall status of SM and LB by conducting $\chi^2$ (chi-squared) test (SM: p= 0.003, LB: p = 0.267) and for categorical variables, chi-squared test with Bonferroni adjustment to determine the significant subcategory.

3. Also for SM (Figure 1), you have to either present your results for a given outcome (SM in this case) as either a table or a figure, not both. It is not acceptable to duplicate the results even the ways are different. In case you decide to delete the table and retain the figure, do one bar graph that includes both pregnant and nonpregnant women using bars with different colors. This will ease the reading of the graph and contrast the statistical differences if any.
Answer: We deleted the Table, and the figures for pregnant and non-pregnant were combined as suggested.

4. The above last two points are applied to results of LB with regard to table 1 and Figure 2.
Answer: Same as in comment no 3 (above).
5. With regard to the following sentence: "The average ABTS radical scavenging capacity in the saliva of pregnant women were 46 % lower comparing to that of non-pregnant women (OD732: 0.118 ± 0.01 vs. 0.063 ± 0.02; p&lt;0.001).," explain how "lower" although the numerical value is "higher".
Answer: Thank you. We presented our data as direct measurement of UV spectrophotometer optical density values after the reaction of saliva sample with ABTS free radicals. Numerical data in Figure 3 shows quantitative expression of free radicals. Free radicals decrease when antioxidant capacity is increased or vice versa. In the methodology section, with the subheading measurement of total antioxidant capacity of saliva, we have mentioned that higher OD731 value represents lower level of TAC. (Page 8, line 194) We measured mean optical density value as the ABTS radical scavenging capacity in saliva of pregnant and non-pregnant women as 0.118 and 0.063, respectively. Based on these values we calculated and expressed that saliva of pregnant women has lower TAC by 46%.

6. The following sentence: "TAC was found to be 46 % lower in pregnant women's saliva compared to non-pregnant women's saliva (OD732: 0.118 ± 0.01 vs 0.063 ± 0.02; p&lt;0.001)." is just a repetition. Delete it.
Answer: Thank you. The sentence is now deleted.

7. With regard to the following sentence: "The average OS levels in the saliva of pregnant women (n = 38) and non-pregnant women (n = 50) were 0.92 nM and 1.07 nM; p = 0.023), respectively", I think the reverse is true. Pregnant women had 1.07 nM while nonpregnant had 0.92 nM. Revise it.
Answer: Thank you for the correction. It was a mistake and this sentence is now corrected in the manuscript. (Page 11, line 269)

8. The following sentence: "MDA was found to be significantly higher in pregnant women's saliva compared to nonpregnant women's saliva (0.92 nM vs. 1.07 nM; p = 0.023)." is a repetition. Delete it.
Answer: Thank you. The sentence is now deleted.

Discussion:

1. The first paragraph is redundant. Delete it.
Answer: Thank you. It is revised.

2. Try to introduce your main results early in the discussion and put them into the context of what such results mean and how they contrast to other studies.
Answer: We revised the manuscript accordingly

3. Try to make your discussion more focused and to the relevant points.
Answer: We tried the make changes according to your guidance.
4. Most of the second paragraph is redundant explaining completely different topic. The remaining part of this paragraph which started discussing the SM and LB is just a repetition of the results.
Answer: It is revised.

5. I am wondering why the authors discussed a topic not related to their study and its results: salivary vitamin C.
Answer: We did not measure vitamin C levels directly in the saliva. However, we compared antioxidative capacity of vitamin C as the standard and saliva sample with same lots of ABTS free radicals. Based on these results we made a discussion in the manuscript (Page 13, lines 326-337). We have revised the manuscript to make it clearer.

6. Many paragraphs are not related to the topic; instead they were discussing other things like preterm birth.
Answer: It is also revised.

Reviewer 3
Nejat Nizam (Reviewer 3): The authors of the current study evaluated and compared the oxidative stress levels and oral bacterial milieu in the saliva obtained from pregnant vs. non-pregnant women in a cross-sectional study design. The topic is of interest to the readers of the journal however some sections, especially the materials and methods section needs to be rewritten, and the results section should be revised accordingly.

1- The pregnant women were those who attended to hospital for routine screening, however the healthy controls were working at the university or the hospital. The major problem with the test and the control groups is that they do not represent the same population and therefore a comparison between these groups is not appropriate. The education level, brushing habits etc. may differ between the groups and may affect the outcomes. The authors should explain how these groups may represent the same population.
Answer: Thank you for the comment. We understand concern that the different results in oxidative stress and bacterial milieu between pregnant and non-pregnant groups might be due to other reasons, such as socioeconomic differences rather than the pregnancy itself. However, in Norway, socioeconomic differences are small, and education level and affordability between pregnant and non-pregnant groups are not significantly different. Furthermore, unemployment is rate is less than 4%, and education and healthcare is free. As the university and the hospital are one of the biggest employer in the city, several of the pregnant women also worked there. Our study groups were matched for age. Therefore, we believe that the groups are comparable. However, we have discussed your concern as the limitation of the study. (Page 14, lines 360-362)

2- The inclusion and exclusion criteria are missing. How about the systemic conditions, body mass index, smoking status etc. How about the oral inclusion criteria? Did they include participants with removable partial dentures? what was the minimum number of teeth required? How was the dental and periodontal status of the patients? When was the last time they used antibiotics?
Answer: Thank you for the comments. Detailed inclusion and exclusion criteria are provided in the revised manuscript. Since the study population were pregnant women and non-pregnant women of similar reproductive age, we did not have any specific oral inclusion criteria and participants did not undergo a clinical oral examination before saliva sampling. However, none of the woman participating in the study reported to have a Removable Partial Denture (RPD). (Page 5-6, lines 140-149)

3- Especially the periodontal status should be addressed in detail. There is no data regarding the number of periodontally healthy and diseased individuals. Data for probing pocket depts, clinical attachment levels, Full mouth plaque and bleeding scores should be present. Since these variables affect the level of bacteria and OS markers in saliva, the results cannot be interpreted with these missing data.
Answer: Thank you so much for the comment. As stated before, a detailed clinical oral examination was not performed before saliva sampling in this study. However, all the participants were healthy and none of them included in this study reported any significant medical illness or oral health problem.

4- Based on the statistical analyses the minimum number of patients needed was 38 in each group and one group of the current study had exactly 38 patients. Were the authors able to analyze all the samples? Why more patients were not included in order to compensate for possible sample dropouts?
Answer: Thank you for the comment. This is part of a larger ongoing study on oral health during pregnancy. We included 38 consecutive pregnant women in this cross-sectional study based on our sample size calculation. Fortunately, we were able to successfully analyze all samples that were collected from these women.

5- Detail regarding the saliva sampling is needed. What was the time of saliva sampling? Did the participants have any instructions for brushing and food consumption before the sampling?
Answer: Thank you for the comment. Saliva samples were collected from participants in the daytime between 09:00-15:00. Since the participants especially pregnant women were in the waiting room for their routine pregnancy ultrasound control and the saliva sample were taken after they were finished with their appointment, they were not brushing or eating minimum 1-1:30 hrs before sampling. Suggestion is incorporated in the revised manuscript.

Reviewer 4
Takayuki Maruyama (Reviewer 4): Major comments:

1. Why did the authors select and analyze only Streptococcus mutans and Lactobacillus? As you know, periodontitis is associated with preterm birth. Also, periodontal pathogens such as Porphyromonas gingivalis is associated with oxidative stress. Why didn't you analyze periodontal pathogens? Please explain about this.
Answer: Thank you so much for the comment. Our main focus in this study was on dental caries related bacteria specially SM and LB and their correlation to oxidative stress. Periodontal pathogens will be taken into account in future studies.
2. Page 11: The association between bacterial colonization of oral cavity and oxidative stress / total antioxidant capacity is unclear. Please discuss about it with reference to other literatures.
Answer: Thank you. We have included this discussion citing appropriate references. (Page 12-13, lines 314-321)

3. Page 12: "OS was found to be 16 % higher in the saliva of pregnant women compared to nonpregnant women." Please discuss about this result with reference to other literatures.
Answer: Thank you for the suggestion. So far to our knowledge, there are no other published reports on the OS measurement in saliva comparing pregnant and non-pregnant women. There are some papers where OS was compared in the blood samples of pregnant and non-pregnant women. It is included in the discussion of our revised manuscript.

Minor comments:

1. Page 2, Results: Please describe the official name of "ABTS".
Answer: Thank you. This is now provided in the manuscript. (Page 2, lines 45-46)

2. Page 4, Methods: Please describe that non-pregnant controls was age matched with pregnant group.
Answer: Thank you. This is now mentioned in methods section. (Page 6, line 146)

3. Page 9, TAC in saliva: "The average ABTS radical scavenging capacity in the saliva of pregnant women were 46% lower comparing to that of non-pregnant women (OD732: 0.118 ± 0.01 vs 0.063 ± 0.02)." Is the Figure 3 correct? Please check.
Answer: Thank you for the comment. We checked the data and Fig 3 once again. We presented our data as direct measurement of UV spectrophotometer optical density after the reaction of saliva with ABTS free radicals. Decrease in free radicals means increased antioxidant capacity and vice versa. In the methodology section, with the subheading measurement of total antioxidant capacity of saliva, we have mentioned that higher OD732 value represents lower level of TAC. (Page 8, line 194) Fig 3 shows the number of free radicals left after reaction in the saliva. We measured mean optical density value as showing ABTS radicals left after reaction in the saliva of pregnant and non-pregnant women as 0.118 and 0.063, respectively. Based on these values we calculated and expressed that the saliva of pregnant women has lower TAC by 46%.

4. Page 14, Abbreviations: Please add "ABTS" and "OD".
Answer: Thank you. ABTS and OD are added in the abbreviations. (Page 15, line 379-380)

5. Table 1: You should describe "p&lt;0.05" instead of "p=&lt;0.05". Also, what is the difference between * and **?
Answer: Thank you for the comment. Based on the suggestion of Referee 2, Table 1 is deleted. Your suggestion is included in Figure 1 and 2.
Reviewer 5
Renita Lorina Castelino (Reviewer 5): The manuscript is well written but requires few language corrections. The results are promising for further research.

Answer: Thank you so much for the feedback. The revised manuscript has been proof-read by a native English speaker.