Author's response to reviews

Title: The Lactobacillus flora in vagina and rectum of fertile and postmenopausal healthy Swedish women

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Author's response to reviews: see over
Answer to the reviewers

Thank You for valuable criticism of our manuscript “The Lactobacillus flora in vagina and rectum of fertile and postmenopausal healthy Swedish women”, MS 1470189084821803. Now we have tried to answer your questions. We think the manuscript has been improved after the revision. Now we hope You want to reconsider it for publication. The changes are highlighted in red in the manuscript. Below we give our response to the reviewer’s remarks and suggestions as follows:

Answer to the Editor,

I have also a suggestion. In your statistical analysis of hormone level you had a cut of level of LH was set to 7 I E/L. Will it be the same results using t-test? If hormone level are of interest why not FSH? Both LH and FSH are high in post menopausal women! Could it be a mistake so that LH has no influence on the colonisation of L. vaginalis. I would like the authors to discuss this and therefore not make conclusions that instead are speculations.

Reply: We have reconsidered the statistical calculations according to Your suggestions. In our previous version we had divided the subjects into two groups depending on hormonal levels. The limit for low or high level of LH was set to 7 IE/L. All postmenopausal subjects had higher levels than that. Some of the fertile women had higher and some lower levels. We have excluded this categorization, as it seems more reasonable just to divide subjects into fertile and postmenopausal women, or fertile women at day 7 and 21. The only effect this statistical revision had on the results and the discussion was that the previous assumption that presence of L vaginalis was associated with low LH levels is omitted. We agree with the referees and the Editor that the former limit of 7 was rather speculative.

1) Please spell out what "LH" stands for in the Abstract.

Reply: As we excluded the calculation with high and low levels of hormones, there was not any significant difference regarding LH and rectal microbial flora. Thus, LH is now not mentioned in the abstract.

2) We recommend that you copyedit the paper to improve the style of written English. If this is not possible, you may need to use a professional copyediting service. Examples are those provided by the Manuscript Presentation Service (www.biomedes.co.uk), International Science Editing (http://www.internationalscienceediting.com/) and English Manager Science Editing (http://www.sciencemanager.com/). BioMed Central has no first-hand experience of these companies and can take no responsibility for the quality of their service.

Reply: We have now thoroughly revised the manuscript with corrections of spelling errors, grammatical errors, errors in the reference list, tables and figures. Thus, we think it is markedly improved and more correctly written. As there have been several corrections throughout the manuscript, they are not highlighted in red as the corrections suggested by the referees.

Referee 1,

page 1: Affiliation of some of the authors are given as “Malmö University” (perhaps
University College of Malmö is meant), however to the best of my knowledge the so affiliated authors have position at Lund University. The mistake is disturbing and might suggest that some of the authors have not checked the manuscript before it was submitted.

Reply: We belong to Skåne University Hospital, Malmö, Lund University. This is now corrected.

page 2, line 7-8:
Compared with page 9 line 2-3 it seems that the authors give different aims/purpose of the study.

Reply: The purpose on page 9, line 2-3 is rewritten so it is in accordance to the aim in the abstract and background, which was the true aim in the start of the study.

page 2, line 10:
I suggest the authors reconsider the use of “smear” throughout the paper to describe the sample obtained.

Reply: Samples are exchanged to smear throughout the manuscript which is a better description. These changes are not highlighted in red.

page 2, line 19:
spell out “LD”.

Reply: As we excluded the calculation with high and low levels of hormones, there was not any significant difference regarding LH and rectal microbial flora. Thus, LH is now not mentioned in the abstract. See further point 2 above under the answer to the Editor.

page 3, line 17: By definition postmenopausal women are not afflicted with Bacterial Vaginosis, sentence must be re-written.

Reply: The sentence is rewritten to give a better description of the condition, page 3, third paragraph.

page 4, line 5.
“Twenty healthy fertile women”. this is a problematic statement if not qualified by explicit reference to observations/examinations (structured clinical data and results of i.e. Amsel or Nugent scores). If this kind of data was not collected it is a major weakness in the study design.

Reply: The subjects were examined by a basic clinical examination to exclude disease. A gynaecological examination including PAP smear and smear for a bacterial culture were carried out. All patients had normal cytology and a normal bacterial flora. This is now mentioned in the manuscript, page 4, line 7-10.

page 5, line 14:
PCA, give a reference to the specific method used in applying PCA to the data set.
**Reply:** As both referees as well as the editor suggested statistical review of the manuscript, this has been performed. PCA did not add any further to the manuscript compared to what was already found by Fisher’s exact test and Spearman’s correlation, why we have decided not to apply PCA in this manuscript. The results are written after the two other statistical tests. The sentence about PCA is therefore deleted.

Page 10, next last and last para.
The discussion on collagenous colitis is speculative and does not add much to the paper. What is really meant by “Treatment with probiotics may have a benign effect on the clinical course”?

**Reply:** As this study was a negative study, not confirming our hypothesis about collagenous colitis and changes in rectal microbial flora as a possible etiological factor in ageing women, we have deleted these paragraphs, and only briefly mentioned other speculations to GI diseases in ageing women. Page 10, second paragraph.

Page 11, line 1:
“amount” change to “number” (and in tables etc.).

**Reply:** Amount is changed to number throughout the manuscript. These changes are not highlighted in red.

Table 1-3.
Reword “… woman did not fulfill the analyzes”

**Reply:** We have exchanged fulfill to complete, Table 1-3.

Figures.
Seems to be submitted in duplicates.

**Reply:** The figures are now deleted from the manuscript file according to instructions to authors.

Tables and figures:
Presentation could be done in a more compact format; especially the tables could be condensed without loss of information.

**Reply:** We have formatted the tables. Subjects or lines with no detectable bacteria are omitted.

Authors’ contributions
Unusual format and wording. Reconsider the wording, contributions are given individual per individual not task per task which is more easy to read. What does “financially supported” mean as written for some authors. Financial and other disclosures should be clearly spelled out per individual author and financial support to the study by granting organizations (and/or companies) clearly be spelled out.
Reply: We have used the format suggested by the BMC papers, with the name of the author first and then the tasks. We have added the financial supports in the paragraph mentioned, and also in acknowledgements.

General:
The paper does not add much new information to the accumulating body of knowledge in Lactobacilli in the vaginal and rectum. The lack of data on L. iners is disturbing and the authors should have seriously considered non-culture methods for the study.

Reply: We certainly do agree and we are well aware of the shortcomings of this study while not cultivating *Lactobacillus iners*. At the time for the study, it was not possible for practical reasons. When it comes to the choice of methods, the best scenario would of course have been to use both culture-depending and nonculture-depending ones such as 16S rRNA gene sequencing or quantitative PCR. But, also here, practical conditions were deciding.

Quality of written English: Needs some language corrections before being published

Reply: The language is corrected.

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Reply: The statistical calculations are reviewed as suggested by both referees and the editor. In the previous version the subject were divided into two groups depending on the levels of the measured hormones. This is now deleted, and the calculations are only performed between fertile and postmenopausal subjects, or between fertile women at day 7 and 21. PCA did not add any further to the manuscript compared to what was already found by Fisher’s exact test and Spearman’s correlation, why we have decided not to apply PCA in this manuscript. The results are written after the two other statistical tests. The only effect this statistical revision had on the results and the discussion was that the previous assumption that presence of *L vaginalis* was associated with low LH levels is omitted. We agree with the referees and the Editor that this was rather speculative.

Referee 2

Comments to “The Lactobacillus flora in vagina and rectum of fertile and postmenopausal healthy Swedish women.”

General comments. The paper should have a minor revision, though the groups are so small. Usually less than two bacterial strains in each group and still very hard conclusions’ are drawn.

Reply: We have deleted the last sentence on page 8, about *L acidophilus*.

Specific comments.
The sample were treated in an ultrasonic bath and diluted, before plating on Rogosa agar. How many dilutions were plated out and in which volume then it is possible to isolate as few as 400 CFU/g discharge? And at the same time avoiding confluent growth then it could be as many as up to 1,8 10^8 CFU/g?

Reply: The amount of sample (faeces as well as vaginal smear) was between 0.2 and 0.4 gram. As it was put in 5 ml transportmedium and undiluted 0.1 ml of this was used for plating the detection limit was 125-250 CFU/g, depending on the weight of the very sample. Five tenfold dilutions were made and all were plated. With a cut off at 250 colonies per plate, the upper limit of CFU was 3.1-6.2 x 10^9 cfu/g.

The species identification were performed by multiplex PCR or sequencing. How many were sequenced, how long fragments, and which gene were used?

Reply: The multiplex-PCR identified isolates of the following species: *Lactobacillus crispatus*, *Lactobacillus acidophilus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactobacillus reuteri*, *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, *Lactobacillus salivarius* and *Lactobacillus paracasei*. Isolates (altogether 13) of *Lactobacillus vaginalis*, *Lactobacillus coleohominis* and *Lactobacillus ruminis* were identified by sequencing of approximately 800 bp long fragments of the 16S rRNA gene. This is now stated in the manuscript under “cultivation and identification of lactobacilli”, page 5.

M and M Page 5.
1. Statistical analyses. The authors introduce three different methods in 8 lines. Have some expert check on this!

Reply: The statistical calculations are reviewed as suggested by both referees and the editor. In the previous version the subject were divided into two groups depending on the levels of the measured hormones. This is now deleted, and the calculations are only performed between fertile and postmenopausal subjects, or between fertile women at day 7 and 21. PCA did not add any further to the manuscript compared to what was already found by Fisher´s exact test and Spearman´s correlation, why we have decided not to apply PCA in this manuscript. The results are written after the two other statistical tests. The only effect this statistical revision had on the results and the discussion was that the previous assumption that presence of *L. vaginalis* was associated with low LH levels is omitted. We agree with the referees and the Editor that this was rather speculative.

Results Page 8.
1. Subject 2,3,4,5,6 and 30 are the one that have *L. vaginalis* in rectal samples, these patients has at the same time serum levels of LH which are 5.8, 1.4, 5.6, 2.2, 3.5 respectively 15.7. I don’t see the statistically significance, either in number of patients or in exceptional low LH levels!

Reply: See answer above and answer to the Editor. This is now deleted.
2. L. crispatus in fertile women (7 subjects) out of which 6 have high levels of oestrogen, compared to two subjects in the post menopausal group which all have low levels of oestrogen! Also statistically significant p=0.036! It would be nice with bigger groups!

Reply: This was designend as a pilot study as the methods are very expensive, and we wanted to have some indications of changes before performing the study in larger cohorts. But we fully agree that the groups are small.

3. L. acidophilus (2 subject) were usually identified in patients with low levels of oestrogen, luckily not significant!

Reply: We have deleted this sentence.

Discussion page 9.
1. L. crispatus was more often found in fertile women. Previous studies demonstrate similar results. Do reference 5,17,18 study post menopausal women?

Reply: The former ref 5, 17 and 18 only describes the effects in fertile women. Now ref 18 is exchanged to a reference describing postmenopausal women.

2. The main finding of this study is that L. vaginalis is more prevalent in patients with low LH levels! Se Results 1.

Reply: This is deleted, see comments above under M&M, page 5

Discussion page 10.
Depletion of (L. vaginalis) may be an etiological factor contributing to GI dysfunction! This is far-reaching conclusion on this small number of patients!

Reply: The discussion on page 10, second paragraph is rewritten.

Discussion page 11.
The association between LH and presence of L. vaginalis , indicate that the rectal mucosa is under influence of sex hormones is something that warrants further investigations in larger cohorts, is something that I fully agree to!

Reply: Agree. This was a first pilot study.

References page 16.

Reply: The names are spelled out.