Author's response to reviews

Title: Identification and characterization of vaginal lactobacilli from South African women

Authors:

Sonal Pendharkar (sonal.pendharkar@ki.se)
Tebogo Magopane (magopanet@phru.co.za)
Per-Göran Larsson (p-g.larsson@vgregion.se)
Guy de Bruyn (gdebruynmd@gmail.com)
Glenda E Gray (gray@pixie.co.za)
Lennart Hammarström (lennart.hammarstrom@ki.se)
Harold Marcotte (harold.marcotte@ki.se)

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Response to the reviewer 1

General comment

However, it is improper to conclude whether Lactobacillus colonization/hydrogen peroxide production was connected with health status with only 40 samples. A larger number of clinical samples (consider different groups of age and menstrual period) is required and sufficient data should be provided to support the conclusion.

The authors agree that the number of samples is low. However, the number of samples was sufficient to conclude that the dominant Lactobacillus species isolated in African women are similar to those isolated in women from Western countries. Furthermore, as previously reported, the absence of L. crispatus was clearly associated with BV.

The contribution of hydrogen production to the vaginal health is still a matter of debate and a higher number of samples in our study could have better clarified this issue. We have discussed more in details the role of hydrogen peroxide in the discussion (p. 12-13). Furthermore, the limitation of the study due to the sample size and single sampling occasion has been discussed (p.13, line 308-318) as follow:

“The absence of significative inverse association between BV and the occurrence of vaginal H$_2$O$_2$-producing Lactobacillus spp. in the present study could be due to different factors. Our sample size was rather small and a single sampling occasion may not properly reflect the vaginal microflora status of a woman as changes in the microflora with the menstrual cycle has been documented previously. Several studies indicated that Lactobacillus growth increases throughout the menstrual cycle, but decreases during the menses (Santiago et al. 2011; Srinivasan et al. 2010). Furthermore, we did not record any behavioral factors that might have affected the vaginal microflora status during the study. Therefore, our results need to be corroborated with larger cohort and preferably using a longitudinal study design, combined with data on subjects' behaviors.”

Specific concerns and suggestions

1. The authors should go through the paper carefully and check the details, word misspelling and misuses should be avoided. The manuscript would benefit greatly from editing by an expert in writing scientific English. Manuscript need to be prepared carefully before submitting to the journal.

   The manuscript has been checked carefully and corrections have been made.

2. Material/chemical/instrument/software information employed in the study
should be provided, include manufacturer’s name, location and software’s versions.

Missing details about the used materials, chemicals and software has been added in the respective places. Line 126-127, 135-138, 143, 154, 184-185 and 200.

3. Since other researchers may repeat or reference your work, detailed information eg. medium recipe (or cite from references), storage condition should be provided.

The media that have been used in our experiments are commercial ready to use mixtures. The brand name and manufacturer have been included in the Materials and Methods section (p. 6, Line 135-144).

4. Line 124-126, since the researchers were dealing with live microorganisms, making sure they were properly handled and stored within limited transportation time before cultivation is critical to maintain the minor bacteria populations. It is good that the researchers start cultivation of the samples upon arrival at the laboratory within 48 hours to avoid loss of bacteria species. However, the period from sampling to arrival to the lab, namely the transportation time and storage condition (4°C, 37°C or RT), is important and can direct lead to reduction of bacterial species.

We understand that the time for transportation and the storage conditions are important for maintaining the species in minority. In our presented study, the swabs were transported from South Africa to Sweden which took one to two days for its arrival to the lab in Sweden. After the arrival, swabs were streaked within 24 hours. The swabs were kept at room temperature until cultivation as cold condition is not appropriate for the conservation of lactobacilli. On the basis of our previous experiences, we have found that the Lactobacillus species recovered from the vaginal swab and their relative amount were the same 24, 48, 72, 96 hours and even one week from the time of sampling.

We have clarified this information on p. 6, line 133-135.

Response to the reviewer 2

General comment:
My main concern is that the sample size of the study is rather limited and that no efforts were made to determine the hydrogen peroxide production of L. iners. The authors might comment on that in the Discussion section. Hydrogen peroxide production of L. iners has been reported more than 10 years ago, by Antonio et al (J Infect Dis. 1999 Dec;180(6):1950-6).
The authors agree that the number of samples is low. However, the number of samples was sufficient to conclude that the dominant *Lactobacillus* species isolated in African women are similar to those isolated in women from Western countries. Furthermore, as previously reported, the absence of *L. crispatus* was clearly associated with BV. However, a higher sample size could have clarified the association between high hydrogen producers and the absence of BV.

The limitation of the study due to the sample size and single sampling occasion has been discussed (p.13, line 308-318) as follow:

“The absence of significative inverse association between BV and the occurrence of vaginal H₂O₂-producing *Lactobacillus* spp. in the present study could be due to different factors. Our sample size was rather small and a single sampling occasion may not properly reflect the vaginal microflora status of a woman as changes in the microflora with the menstrual cycle has been documented previously. Several studies indicated that *Lactobacillus* growth increases throughout the menstrual cycle, but decreases during the menses (Santiago et al. 2011; Srinivasan et al. 2010). Furthermore, we did not record any behavioral factors that might have affected the vaginal microflora status during the study. Therefore, our results need to be corroborated with larger cohort and preferably using a longitudinal study design, combined with data on subjects’ behaviors.”

Regarding the H₂O₂ production by *L. iners*, the latter was growing poorly on MRS agar containing TMB. We thus decided not to include the data as the poor growth could affect the hydrogen peroxide production. Other methods would need to be used to measure the concentration of hydrogen peroxide in this species. This has been discussed as follow (p. 13 line 320-324):

“The production of H₂O₂ by *L. iners* isolates was not evaluated as these strains were growing too poorly on MRS containing TMB substrate. Most *L. iners* strains have been found to be for the most part non-H₂O₂ producers which might correlate with our finding that this species was isolated in both BV positive and negative women (Antonio et al 2005). The production of H₂O₂ for this species should be assessed on medium adapted for the growth of *L. iners*.”

Finally, it would omit the use of the terms “flora” and “microflora” and replace them with “microbiota”.

The term flora/microflora has been replaced by microbiota everywhere in the text.

**Minor Essential Revisions**

1. In the Abstract is stated that “The vaginal microflora of a healthy woman is
dominated by L. crispatus, L. rhamnosus, L. gasseri, L. jensenii, and L. iners,...”

L. rhamnosus was actually a minor component of the vaginal microbiota in most studies ... 

As adequately pointed out by the reviewer, L. rhamnosus is not a major component of the vaginal microbiota and it has been removed from the sentence. Line 32-33.

2. What is meant in the conclusion of the abstract with a “lesser level” in the expression “…, L. crispatus and to a lesser level, hydrogen peroxide …” – on what statistical comparison was this statement based?

Fisher’s exact test was performed to test the relation of L. crispatus and also the production of H₂O₂ with the BV free status of women.

In accordance with the other published studies, L. crispatus is significantly associated with the normal vaginal microbiota whereas it is suggestive that H₂O₂ does contribute to the normal microbiota to some extent albeit not significantly.

In order to avoid confusion we changed the sentence to: “In accordance with the other published studies, L. crispatus is significantly related to the normal vaginal microbiota. Hydrogen peroxide production was not significantly associated with the BV status which could be attributed to the limited number of samples or because other antimicrobial factors are also involved.”

3. In the Background section it is stated that “BV is associated with an increased susceptibility to other sexually transmitted diseases, …” – is BV an STD?

If BV is an STD is still an open and interesting question. It was not our intention to suggest BV as an STD in this paper but rather an error in writing the statement. The sentence has been changed to “BV is associated with an increased susceptibility to sexually transmitted diseases including infection by HIV-1” (p. 4, line 87).

4. In the results section it is stated on page 8, lines 200-201, that “[L. crispatus] ... accounted more for healthy status compared to BV or intermediate microflora (Figure 2)” – on what statistical comparison was this statement based?

The sentence was removed as only the presence L. crispatus and the genus Lactobacillus is significantly associated with the BV free status as evaluated by Fisher exact test.

5. The authors state in the Discussion section: “However it is currently not known whether the production of H₂O₂ by lactobacilli is of clinical importance to prevent BV”: that is only partially correct, and a number of studies have found that hydrogen peroxide production is inversely related with BV risk, see for instance Hawes et al. Hydrogen peroxide-producing lactobacilli and acquisition of vaginal

It is true that the majority of clinical studies support the positive relation between H$_2$O$_2$ producers and the reduced risk of BV. However, the relative contribution of the H$_2$O$_2$ produced by the *Lactobacillus* spp. to the overall antimicrobial effect is still a matter of debate. Under the hypoxic conditions that generally prevail in the vagina, H$_2$O$_2$ production by vaginal lactobacilli is undetectable (detection threshold 10 nM). *In vitro*, cervicovaginal fluid and semen have a significant H$_2$O$_2$-blocking activity and physiological concentrations of H$_2$O$_2$ (below 100 µM) did not kill any of the tested BV-associated bacteria (O’Hanlon 2010, 2011).

We have further discussed this aspect in the Discussion section p. 12, line 287-300.

**Response to the reviewer 3**

The work is well performed and the data obtained are of interest. However, they mainly corroborate previous findings. Some suggestions follow to increase the level of novelty of the manuscript.

As pointed out by reviewer 2, very few studies of the vaginal microbiota have been performed in African populations which are at high risk for infection by HIV, making the present study worthwhile. It is anticipated that this study will generate new interest in evaluating the relation between *Lactobacillus* species, hydrogen peroxide producing lactobacilli, BV and risk for STD and HIV infection in the African population.

1. The high correlation between *L. crispatus* colonization and healthy status should be addressed by investigating possible antagonistic properties (for instance lactic acid generation rate, bacteriocin and surfactant production of the different isolates, adherence to vaginal cell cultures, etc.) and comparing them with those of other lactobacilli that did not present protection ability (*L. ruminis*?). In addition this would provide data on the variability of the *L. crispatus* strains isolated.

It is of great interest and importance to study the possible antagonistic properties of different *Lactobacillus* species and strains. However, evaluating all these parameters *in vitro* is time consuming and might not necessarily help to identify the exact mechanisms involved *in vivo*. In the present study, we chose to study only H$_2$O$_2$ production because some clinical evidences have suggested that it could be an important factor in maintaining the vaginal microbiota *in vivo*. As we have conserved all the *Lactobacillus* isolates, we hope to be able in the future to identify the possible factors that are involved in the ability of *L. crispatus* to contribute to the health status using a comparative genomic approach.
2. The data of vaginal colonization by lactobacilli should be completed by providing their proportion versus other accompanying bacteria.

Our main objective was to determine if the dominating vaginal *Lactobacillus* species were similar between women in Western and African countries and if the health status was associated with the presence of *L. crispatus*. We could clearly show that the dominating vaginal *Lactobacillus* species are similar between both populations and that the presence of *L. crispatus* is associated with the health status. We agree that to determine the proportion of *Lactobacillus* species vs other bacteria could also be indicative of the BV status, particularly since the amount of lactobacilli producing H$_2$O$_2$ (and not only their presence) is probably very important in determining the contribution of H$_2$O$_2$. However, we did not measure the proportion of lactobacilli vs other bacteria. This analysis cannot be performed on the present samples as following re-suspension in PBS, the bacteria have been stored for more than one year in glycerol 15% and this could affect the viability of bacteria and/or integrity of DNA (if molecular methods were to be used). We included the following sentence in the discussion (p. 13-14, line 325-327)

"Furthermore, the proportion of H$_2$O$_2$ producing lactobacilli (and not only their presence) might also probably be important and the relative amount of lactobacilli should be measured determined by molecular methods."

3. *L. iners* and *L. gasseri* isolates colonized women with diverse Nugent scores. Are there significant differences in their adherence and antagonistic properties that might justify this fact? Is it just a question of proportions with respect to other bacteria found in the respective samples?

As mentioned earlier on point 2, the proportion of *Lactobacillus* species was not evaluated. The reason why *L. iners* and *L. gasseri* are isolated in women with different Nugent score might be due to their proportion as suggested or property differences between strains of the same species. Longitudinal studies of the dynamics of vaginal microflora have shown that *L. gasseri/L. iners* offer poorer colonization resistance and to some extent predispose to the occurrence of abnormal vaginal microflora (Verstraelen et al. 2009). From the study on the natural history of the normal vaginal microflora in pregnant women, it appears that *L. crispatus*, is associated with a particularly stable vaginal ecosystem. Conversely, microbiota comprising *L. jensenii* elicits intermediate stability while vaginal microbiota comprising *L. gasseri/L. iners* is the least stable. It has also been suggested that *L. iners* may become a dominant part of the vaginal microbiota when the microbiota is in a transitional stage between abnormal and normal (Jakobson and Forsum, 2007). So, a longitudinal study and evaluation of quantitative amount of *Lactobacillus* species might better answer the reason why *L. iners* and *L. gasseri* are isolated in both women. This has been discussed on p. 11-12, line 269-283.
4. Out of the 21 healthy subjects, 19 harbored lactobacilli; what happened to the other two? Related to this, diagnosis of bacterial vaginosis based exclusively on Nugent scores is arguable; is there a possibility to provide data on symptomatology of the women enrolled in the study?

Yes, out of 21 women with normal microbiota, 19 harbored cultivable lactobacilli. When Nugent scoring was performed, short gram positive rods indicative of *L. iners* were visible in the smear for these two women. But these short bacilli could not be grown on either of the culture plates. For these samples, bacterial growth was observed only on blood agar plates and none of the different colonies picked from these plates were lactobacilli. Therefore, it was understood that the short rods visible in the original vaginal smears sent for Nugent scoring was in very low number and did not grow on the culture plates. It must also be noted that Nugent score is not only based on the presence or absence of lactobacilli but on the presence of *G. vaginalis* and *Mobiluncus*. (The Nugent score for these two women was 1 with zero count for Gardnerella, clue cells, Mobiluncus).

Depending on the literature and the expert’s personal experience it could be said that the Nugent scoring is a reliable technique to define the status of the vaginal microbiota (*Zarakolu P. et al.*, Diagn. Microbiol. Infect. Dis., 2004, *Mohanty S. et al.*, Indian J Med Res, 2010). Apart from that, it was in accordance with the results that the clue cells were observed in the smears from women who were identified with. The presence of clue cells is a critical component of the Amsel’s clinical criteria for BV.

It must also be noted that whatever the system used to score BV (Amsel or Nugent score), it relies a lot on the experience of the clinician performing the scoring. In our study, the clinician (PG Larsson) performing the scoring has a long experience in the Nugent scoring method.

5. The data on H2O2 production did not significantly correlate with health status. However, Fig. 3 indicates that a significant correlation might exist with moderate hydrogen peroxide generation (score 2). In that case, the authors might argue that low production was not enough to induce protection while accumulation of high H2O2 levels might be toxic not only to the accompanying microbiota but to the producing lactobacilli as well.

We have repeated the statistical analysis by excluding women with lactobacilli scoring 3 for hydrogen production and it is not significant. The high producers (score 3) were *L. jensenii* in three women and one isolate of *L. vaginalis* with score 3 in one woman. The woman colonised by this *L. vaginalis* isolate was identified with BV whereas high H2O2 producing *L. jensenii* was colonising women with no BV (as shown in the graph). Therefore, it might also be that the species producing H2O2 is important as it might have different properties in terms of adherence and production.
of other antimicrobial substances. However, a larger number of samples would be necessary to confirm these results. We don’t find it appropriate to exclude a part of data from the analysis.

6. The terms flora and microflora should be changed to biota and microbiota throughout the manuscript.

According to the reviewers’ suggestion, the term flora and microflora has been changed to biota and microbiota throughout the manuscript.

Response to the reviewer 4

Major Compulsory Revisions
1. Through the entire manuscript women with BV are referred to as unhealthy and women who don’t have BV are referred to as healthy. There is much debate in the field about the dysbiotic condition BV, although it is true that many studies have shown that BV is associated with increased risks with many adverse health outcomes such as HIV acquisition or preterm birth. Hence, it would be preferable to re-write such that associations are made with the presence or absence of BV rather than healthy/unhealthy. For example: Lines 45-46: Can be re-worded to: “L. crispatus was significantly associated with the absence of BV (p=0.024).

Throughout the manuscript, we have changed “healthy” to normal microbiota or absence of BV, depending on the context.

2. Line 132: It is unclear from the manuscript if the swabs reached the wet lab within 48 hours post collection and were plated right away or if the swabs were plated within 48 hours after arrival at the lab. Please clarify. If the samples were not plated within 48 hours after collection, the this can have implications on isolation of strains. Were any pilot studies conducted to compare strains isolated after storage for different times? Regardless, this point needs to be addressed under limitations.

Shipment of the swab samples from South Africa to Sweden has taken one to two days and after the arrival of swabs, they were streaked within 24 hours upon arrival. (p. 6, line 133-135). We would like to mention that from our previous experience (other studies) we have been able to recover same Lactobacillus species from the swab when streaked 24 h, 48 h, 72 h or one week after sampling.

3. Likewise, in 21 women only one type of Lactobacillus species was isolated. This is unusual as typically more than one Lactobacillus type can be cultivated from a vaginal sample when plated within 24 hours. The advantage of cultivation-based approaches is that they facilitate detection of low numbers of bacteria. This is in contrast with molecular approaches such as broad-range PCR with pyrosequencing
which tend to bias towards the more abundant sequence types and provides an estimate of relative abundance.

This is not really unusual to cultivate only one type of bacteria as it has been shown in previous studies by us and others (Vásquez et al. 2002, J. Clin. Microbiol. 40:2746; Larsson et al. BMC infectious diseases 2011, 11:223; Vitali et al. Appl. Env. Microbiol. 2007, 18:5731; reviewed by Lamont et al. BJOG 2011, 118:533). The authors would rather think that cultivable methods will allow mainly the cultivation of the dominant Lactobacillus species. The number of women colonized by more than one dominant species even tends to be higher in the present study in South Africa than previous studies in Sweden but we did not discuss this aspect due to the low number of samples in both studies (Vásquez et al. 2002, J. Clin. Microbiol. 40:2746; Larsson et al. BMC infectious diseases 2011, 11:223).

4. Figure 1 and Table 1 essentially have the same data except the numbers are cumulated in Figure 1. This does not add any significant additional value.

Figure 1 represents the overall distribution of the Lactobacillus species among these women. It is not informative of the BV status in relation to the identified lactobacilli. Whereas, table 1 is an elaborated presentation of the complete data where, you can correlate the presence of single colonising species or combination of two species and its effect on BV status.

Minor Revisions

Line 32: This is the first time the bacterium is mentioned. Change to Lactobacillus crispatus from L. crispatus and so on.

The name of the genus is added in the line before writing the species names (p. 2, line 32).

Line 32: There was no Lactobacillus rhamnosus isolated as you state in the abstract. I presume you are referring to L. ruminis.

Presence of L. rhamnosus in the line was an error in writing. L. rhamnosus has been removed from the line now. (p. 2, line 33)

Line 35: Please re-phrase. Sentence does not read correctly. Also please state how the Lactobacillus species were identified such that the reader can understand the value of the data presented (Eg. Identified by cultivation and 16S rRNA gene typing).

Suggested addition of information on how the lactobacilli were identified has been made in the line (p. 2, line 37-39).

Line 54: “It is thus likely that….restore normal microflora.” Please remove from the abstract. This is a discussion point, not a conclusion.
The line has been removed from the conclusion of the abstract.

**Line 78:** Please amend to say that “The vaginal microbiota in women is often dominated by lactobacilli.” There may be exceptions.

The sentence “The vaginal microbiota of a healthy woman is often dominated by lactobacilli” has been changed to: The vaginal microbiota of a healthy woman is often dominated by the species belonging to the genus *Lactobacillus*….(p. 4, line 78)

**Line 85:** Reference 6 is incorrect for the prevalence of BV. The most recent reference that I am aware of looking at prevalence in the US is Koumans et al. 2007 Sex Trans. Dis. 34:864-869. Likewise, please cite primary references for prevalence in Europe and Africa.

These references were used to present the status of BV in general without going into the specific geographical regions. The only purpose of having this reference here was to support the statement that BV is one of the most common vaginal syndromes. But, as suggested, more reference has been added to support the statement from studies performed in the US and Africa (p. 4, line 86)

**Line 149:** Although, it is stated that previously described techniques were used in the amplification and sequencing of the 16S rRNA gene, it would be useful to have a sentence briefly describing the method. Which region of 16S was targeted and how much sequence was obtained?

In brief, a description of the method of sequencing 16S rDNA and the region targeted has been added in the section (p. 7, lines 156-161).

**Line 173:** Sentence confusing – please re-phrase.

The line has been re-phrased as: “Since it was difficult to culture *L. iners* on MRS agar medium, *L. iners* isolates were not included in the H$_2$O$_2$ production test” (p. 8, line191-193).

**Line 184:** In the methods, the study enrolled 40 women. Were no lactobacilli isolated from 10 women? If so, was it because they had BV? Please provide an explanation. It would be very helpful to have a Table 1 describing the study participant characteristics and demographics of the study population. Please include data such as: prevalence of BV in the cohort, menses at time of collection, use of vaginal products or antibiotic use prior to collection of samples etc. This would alleviate confusion such as the question above on why lactobacilli were isolated from just 30 women in spite of enrolling 40 women in the study.

40 women were enrolled in the study and 30 women were identified with cultivable lactobacilli. Of the 10 women without lactobacilli, two had normal microbiota, two had intermediate microbiota and six had BV. When Nugent scoring was performed, short
gram positive rods indicative of \textit{L. iners} were visible in the smear from the two women with normal microbiota. These short rods visible in the vaginal smears were however in very low number which could explain why we could not isolate them on blood agar. Study participant characteristics have been added in Table 1. Women were non-menstruating and were not taking antibiotics at the time of sample collection (p.5, line 124-125). We however don’t know the last time they have taken antibiotics before sampling.

\textbf{Lines 185-187}: On first reading, I understood the sentence to be: \textit{L. crispatus, L. iners and L. gasseri} were the dominant Lactobacillus species in 10, 8 and 7 women respectively rather than \textit{L. crispatus, L. iners and L. gasseri} were present in 10, 8 and 7 women and represent the most common Lactobacillus species isolated from South African women. Please edit for clarity.

As suggested, these lines have been edited for clarity.

“\textit{L. crispatus, L. iners and L. gasseri} were identified in 10 (33\%), 8 (27\%) and 7 (23\%) women respectively, whereas \textit{L. vaginalis} and \textit{L. jensenii} colonised five (17\%) women each. These five species were the most common \textit{Lactobacillus} species identified in South African women” (p. 9, lines 213-216).

\textbf{Line 192}: Edit to Lactobacillus colonization and BV status rather than health Status

The heading has been edited to “BV status and \textit{Lactobacillus} colonisation” (p. 9, line 203).

\textbf{Lines 197-201}: \textit{L. crispatus} was isolated…This sentence is confusing to read. Please edit to something like: \textit{L. crispatus} was only isolated from women without BV. Other predominant lactobacilli (…) were isolated from women with and without BV, but were more often isolated from women who did not have BV. The lines have been edited for clarity as follows:

“\textit{L. crispatus} was isolated only from women without BV whereas the other predominant \textit{Lactobacillus} species (\textit{L. jensenii}, \textit{L. gasseri}, \textit{L. vaginalis} and \textit{L. iners}) were isolated both from women with and without BV” (p. 9, lines 219-222).

\textbf{Lines 220-224}: Please re-phrase. Confusing

The paragraph has been re-phrased as below:

“\textit{The isolation of high level of H}_2\textit{O}_2\textit{ producers (either \textit{L. jensenii}, \textit{L. crispatus} or \textit{L. vaginalis} with a score of 2 or 3) was in general more frequent in women with a normal microbiota than in the women with BV. However \textit{H}_2\textit{O}_2\textit{ production as an individual factor did not contribute significantly to the normal vaginal microbiota (Fisher test,} \textit{P=0.064}) (Figure 3)” (p. 10, lines 241-246)

\textbf{Line 235}: It is either the vagina or the genital tract, not the vaginal tract.
The term had been corrected by writing vaginal microbiota (p. 10, lines 256-259).

**Line 402:** Re-phrase to: Association between colonizing lactobacilli and BV status defined by Nugent Score

The line has been re-phrased as suggested (p. 21, line 504-505).

**Discretionary Revisions**
While microflora is the terminology that has been often used in the literature, the current trend to refer to the bacteria in/on humans is “microbiota.”

The term has been changed to microbiota as suggested by the reviewers.

Lines 83-84: While there is a decrease in the numbers of lactobacilli, it would be more accurate to state something like “there is a shift in the microbiota from mostly lactobacilli to diverse anaerobes, which is associated with the common condition, BV.”

The suggested change has been made (p. 4, Line 83-85)