Author's response to reviews

Title: Identification and genotyping of bacteria from paired vaginal and rectal samples from women at 35 weeks of gestation indicates similarity between vaginal and rectal microflora

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Version: 2 Date: 17 August 2009

Author's response to reviews: see over
Sir,

We thank both of the reviewers for their a very thorough and useful review. Their comments have been addressed in the revised manuscript. Hereby, you will find the answers of their comments.

Sincerely

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Reviewer's report
Title: Identification and genotyping of bacteria from paired vaginal and rectal samples from women at 35 weeks of gestation indicates similarity between vaginal and rectal microflora
Version: 1 Date: 10 July 2009
Reviewer: SVETLA DANова
Reviewer's report:
1. Major revision
2. General comments:
The present paper evaluates the similarity between vaginal and rectal microflora of pregnant women at 35 weeks of gestation. The title is correct and precise, but a little long. To my opinion it could be shortened: Ex. Identification and genotyping of bacteria from paired vaginal and rectal samples from pregnant women indicates similarity between vaginal and rectal microflora.
A1: We have changed accordingly, also because this title is more complete and correct.

Overall the abstract and the manuscript are well structured in accordance with journal’s instructions, but the English could be carefully checked:
• Page 2, results: “Among the 844 isolates that could be?? identified by tDNA-PCR, a total of 63 bacterial species were present,………
A2: This is not an error. Indeed, only part of the isolates could be identified. This was stated in the results section, line 194 as follows ‘Using tDNA-PCR, 844 could be identified, 103 gave no amplification and 109 gave uninterpretable patterns, due to mixed cultures.’, and has now further been clarified. Please see A34.

• Page 9 :“L. gasseri was the most frequently isolated Lactobacillus species from the rectum (20/132 subjects positive), followed by L. jensenii (16) and L. crispatus (14). L. iners could be isolated?? rectally from only 2 subjects
A3: We changed ‘could be’ to ‘was’. Line 218.

The first paragraph- “Background” is much too heavily focussed on the subject of the investigation.
A4: We have shortened to: “The vaginal microflora is important for maintaining vaginal health and preventing infections of the reproductive tract. The rectum has been suggested as the major source for the colonisation of the vaginal econiche.” Line 33.
and placed the following text: “To establish whether the rectum can serve as a possible bacterial reservoir for colonisation of the vaginal econiche, we cultured vaginal and rectal specimens from pregnant women at 35-37 weeks of gestation, identified the isolates to the species level with tDNA-PCR and genotyped the isolates for those subjects from which the same species was isolated simultaneously vaginally and rectally, by RAPD-analysis. “ in the Material and Methods paragraph.

In the same time the aims of study are clearly stated. The work appears to be carried out appropriately, however some results are presented in unclear manner. Principally the approaches are valid for the identification and genotyping of bacterial isolates. However, there are some aspects of the work that require further attention, and that would benefit from additional efforts. Overall, the applied parameters (especially the modified method of Gram staining) do not seem to provide clear cut rational and differential criteria for precise bacterial discrimination into the community of more than 50 species presented into the vagina. Therefore, more specificity could be given in the paragraph Methods, especially for the methods of identification used (see comments below). This paragraph needs a careful edition:

• The methods for identification of isolated bacteria could be briefly presented. There is no information about the primers and conditions of PCR analyses of Lactobacillus microflora. The cited references [8, 17,18] are useful for Streptococci, Enterococci and BV associated microflora.

A5: The reviewer is correct. We have previously published on the usefulness of tDNA-PCR for the identification of lactobacilli, but forgot to cite this publication. We have added the following citation: “Baele, M., M. Vaneechoutte, R. Verhelst, M. Vancanneyt, L. A. Devriese, and F. Haesebrouck. 2002. Identification of Lactobacillus species using tDNA-PCR. J. Microbiol. Methods 50: 263-271.” We hope that this is sufficient as an explanation of the method, because explaining it in detail has been done in the references now cited and would take us too much space to explain again here. Line 142 and 515.

• Kindly expand the abbreviation tDNA-PCR, at least according to the rule – “first use”

A6: We have changed to: “tRNA intergenic length polymorphism analysis (tDNA-PCR)” Line 40.

• Please, precise how the significant part of isolates were identified to the species level, before to discuss the biodiversity and similarity between rectal and vaginal microflora.

A7: We did address identification first. Now we have added some more detail in the results section. Please see A35. Also, the additional reference to Baele et al. 2002 (see A5) should help.

The results obtained are summarized in two tables and four figures, created directly from the Gene scan 3.1 software. I doubt only about this form to present the results from RAPD analyses. The authors should consider how to facilitate the results illustration. The dendrograms, derived from the raw data could be appropriate manner to prove the similarity and identity of the tested pairs of vaginal/rectal isolates (see as a possible model of presentation the article: Bernard Berger, R. David Pridmore, Caroline Barretto, Francoise Delmas-Julien, Kerstin Schreiber, Fabrizio Arigoni, and Harald Brussow (2007)Similarity and Differences in the Lactobacillus acidophilus Group Identified by Polyphasic Analysis and Comparative Genomics JOURNAL OF BACTERIOLOGY, Feb.
We constructed dendrograms, but given the large number of isolates, these are difficult to present. Moreover, tree construction correctly clusters isolates with identical fingerprints, but sometimes clusters these with isolates having clearly different fingerprints. Therefore, a dendrogram would create the false impression of some similarities which are not present. This is also the reason why we checked visually the fingerprints of those isolates that were clustered. This is practical knowledge, shared by many ‘fingerprinters’, but rarely published explicitly (but see: Van daele, S., R. Verhelst, G. Claeyts, G. Verschraegen, H. Franckx, L. Van Simaeys, C. De Ganck, F. De Baets, and M. Vaneechoutte. 2005. Shared genotypes of Achromobacter xylosoxidans strains isolated from patients at a cystic fibrosis rehabilitation center. J. Clin. Microbiol. 43: 2998-3002.). We have added this as follows: ‘Isolates of which the RAPD fingerprints were clustered together, were inspected visually to confirm similarity.’ Line 173

If requested by the editor, we are willing to omit the figures, which were added only as an illustration of some fingerprints.

Also check carefully for mistakes the data in Table 2

A8: Table 2 has been checked and corrected: Row 26 was lost, but has been added again, the vaginal genotype of S. salivarius is different from the rectal genotype, but was counted as equal, and the back of A in Rows 25 & 50 is now highlighted.

3. Specific comments
I would like to propose some minor edition:

A10: It has been changed accordingly, although we think that ‘clone’ and ‘strain’ can be used synonymously.

A11: We have changed to (Line 76): ‘It is important to establish to which degree this is also the case for lactobacilli, the predominant group of microorganisms of the normal vaginal microflora, because these bacteria are generally known to produce endogenous microbicides such as lactic acid, which acidifies the vagina, and hydrogen peroxide (H2O2), toxic to other bacteria and viruses, including HIV’

A12: We have changed to (Line 105): ‘the routine screening for Group B streptococci’

A13: We changed to (Line 180): ‘The most common genus recovered from grade Ia, Ib and Iab specimens was Lactobacillus’
• Page 9- please verify number of strains L. gasseri (40) and L. iners (14) and......... (20/132
subject positive) with the values given for the same species in the Table 2.

A14: We have changed the title of Table 2 to clarify that the numbers in the text do not
match those in Table 2.

Table 2. Old title: “Genotyping results for women which had the same 8 species present both
vaginally and rectally and for which several colonies were picked per subject”

Table 2. New title: “Genotyping results for the 50 cases in which the same species could be
isolated from vagina and rectum of the same subject.”

E.g., L. gasseri was found in 40/132 subjects, but only 16 subjects carried this species
both vaginally and rectally and therefore only 16 entries are present in Table 2 for L.
gasseri.

• Page 11- Did the authors use a referent type strain (ex. L. gasseri ATCC 9857, L. jensenii
ATCC 25258 or others) in the genotyping assay?

A15: No. We do not think this is really relevant here either. Genotyping compares
isolates from each woman separately, and the use of a type strain would not add
relevance, or would not even be a control for something.

Level of interest: An article of importance in its field
Quality of written English: Needs some language corrections before being published
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests: I declare that I have no competing interests

Reviewer's report
Title: Identification and genotyping of bacteria from paired vaginal and rectal samples from
women at 35 weeks of gestation indicates similarity between vaginal and rectal microflora
Version: 1 Date: 3 July 2009
Reviewer: Johanna Maukonen

Reviewer's report:
El Aila et al., Identification and genotyping of bacteria from paired vaginal and rectal samples
from women at 35 weeks of gestation indicates similarity between vaginal and rectal
microflora.

The manuscript describes analyses of microbial populations between two sites (vagina and
rectum) among 132 pregnant women. Isolates have been obtained from two different media
and thereafter identified and genotyped. About 35% of the pregnant women carried the same
Lactobacillus species both vaginally and rectally, and 35 out of the 50 species pairs were
identical genotypes. I found the work interesting and the number of women recruited for the
study good, but I consider that it would greatly benefit from revision, since e.g. the results do
not warrant some of the conclusions made.

A16: We do not really agree with this statement. Please see A45-A48.

In addition, the methodology and results need clarifications in several points. We thank the
reviewer for a very thorough and useful review. We address her comments below.

Major Compulsory Revisions
1. In my opinion the title would be more precise, if “microflora” would be changed to
lactobacilli”, since only lactobacilli are studied in detail. With the current title, I
expected that other culturable genera would also had been studied.

A17: We do not understand this remark, since we cultured and identified a total of 63 species (Table 1) and genotyped 5 species in addition to the Lactobacillus species present (Table 2). However, we have changed the title according to the request of reviewer 1 (see A).

2. Page 5: Were the cultivations performed in anaerobic cabinet?

A18: Yes. This was stated in the M&M of the original submission, now at Line 117: “in an anaerobic chamber (BugBox, LedTechno, Heusden-Zolder, B.)”

If so, what was the gas atmosphere (percentages of the gases included) ? Or on table in normal atmosphere?

A19: We have added the correct composition (Line 320): “Interestingly, in a previous study of our group [8], we isolated almost no L. vaginalis, using anaerobic jars and GasPak (Becton Dickinson), yielding an atmosphere of 15% CO₂, 80% N₂, and less than 1% O₂. Since we started using an anaerobic chamber, with an atmosphere of 10% H₂, 10% CO₂, and 80% N₂, the number of L. vaginalis isolations has increased significantly and this species is now among the five most abundant vaginal species.”

3. Page 5: What is meant by “resp.”

A20: We think this is a standard abbreviation for ‘respectively’. We have written this out.

4. Page 5: What is the gas atmosphere of the anaerobic chamber used for incubation?

A21: Please see reply to question A19.

5. Page 6: The explanation of how different Gram stain results are graded is somewhat difficult to read. Please rewrite it (e.g. the Figure 1 text in Verhelst et al, reference 16 is easily readable and understandable, contrary to this manuscript)

A22: We have replaced the original text: “Grade I specimens were characterized as grade Ia when only Gram positive plump, mostly short rods - mostly corresponding to L. crispatus [16] - were present, as grade Ib when only other Lactobacillus cell types were present, i.e. smaller or more elongated and less stained Gram positive rods than in Ia smears, and as grade Iab when both L. crispatus and other lactobacilli were present. Furthermore a number of samples were classified as grade I-like when gram-positive rods either quite small and short or otherwise irregularly shaped with curved edges were predominant. We have previously shown that this microflora is characterized by the presence of bifidobacteria [16]. A mixture of Lactobacillus cell types and bacterial vaginosis-associated bacteria, i.e. Gardnerella, Bacteroides-Prevotella and Mobiluncus cell types were classified as grade II, whereas samples devoid of Lactobacillus cell types with the presence of only Gardnerella, Bacteroides- Prevotella or Mobiluncus cell types were classified as grade III. Finally, samples were classified as grade IV when Gram positive cocci were predominantly present and as grade 0 when no bacterial cells were present [16].”

by the following text (largely from ref. 16)(Line 122):

“Grade Ia specimens contained mainly Lactobacillus crispatus cell types, i.e. plump, quite homogeneous lactobacilli, grade Ib contained non-L. crispatus cell types, i.e. long or short, thin lactobacilli, grade lab contained mixtures of L. crispatus and non-L. crispatus cell types, grade I-like contained irregular-shaped Gram positive rods, grade II contained a mixture of Lactobacillus cell types and bacterial vaginosis-associated bacteria (Gardnerella, Bacteroides-Prevotella and Mobiluncus cell types), whereas samples devoid of Lactobacillus cell types with the presence of only Gardnerella, Bacteroides- Prevotella or Mobiluncus cell
types were classified as grade III. Finally, samples were classified as grade IV when Gram positive cocci were predominantly present and as grade 0 when no bacterial cells were present.”

Page 6: From which dilution did you pick the colonies?
A23: No dilutions were made.

7. Page 6. From which plates did you pick the colonies? It is stated earlier that you used both Columbia blood agar and MRSA, but it is not told from which plates the colonies were picked.
A24: We think this was already clear from the M&M section in the original manuscript, which stated: “Identification of isolates. From all 132 women, for both rectal and vaginal swabs, the most abundant colony types, 8 in total from each subject, were picked from both Columbia CNA and MRS agar plates”, but this should be clearer now after the changes as indicated under A26.

In addition, did you pick e.g. from subject RVS003 4 rectal colonies from MRSA and 4 vaginal colonies from Columbia blood agar?
A25: See A26 for clarification.

8. Page 6: What do you mean by “most abundant”? Please specify (see also the comment above).
A26: The sentence: ‘From all 132 women, for both rectal and vaginal swabs, the most abundant colony types, 8 in total from each subject, were picked from both Columbia CNA and MRS agar plates …‘ has been rephrased as follows (Line 138): “From all 132 women, 8 colonies per subject, i.e., one colony of each of the two most abundant colony types from both Columbia CNA and MRS agar plates and for both rectal and vaginal swabs were picked.”

9. Page 7: The tDNA-intergenic PCR is not as common as e.g. 16S rRNA PCR, so please specify the method used in more detail (all the details are not necessary, since the methods have already been published).
A27: Please see A5 and A6.

10. Page 8: Did you check and correct the profiles manually? It was said in your reference 18 (Baele et al., JCM 2001) that “visual checking of the patterns to confirm the results is advised”.
A28: The method is in use in our laboratory since 10 years now and is sufficiently robust to let us identify many different species (not all of them published, because this would be very repetitive work). Since we identify hundreds of vaginal isolates each month, and since we add newly obtained fingerprints to the reference library, most species are presented in the library by many fingerprints, and identification is usually straightforward. Only when several species names are offered as an answer, we check the fingerprints visually. However, for RAPD genotyping, we checked visually those fingerprints which were according to the similarity calculation and dendrogram construction had been clustered together. See also A8.

11. Page 9: From which samples were the isolates that did not amplify? Were they rectal? Or vaginal? Or both? Were they from same subjects? Were they from low dilutions? Please specify.
A29: We have explained now why some isolates did not yield an identification result. Please see A7. From that explanation, it is clear that this failure is not related to location or subject.

2. Page 9: Was there “a pattern” for the isolates that did not amplify? I.e. were they e.g. from same agar type? Since 20% of the isolates did not amplify, it could have a great impact on the results?
A30: Please see A7 and A29.

13. Page 9: Did you sequence (16S rRNA) any of those isolates that did not amplify with tDNA-intergenic PCR? It would be advisable to check the identity of at least some of those isolates with e.g. 16S rRNA sequencing.
A31: During revision, we have sequenced 20 of the isolates of which the tDNA fingerprints were not interpretable. We have added following sentence, explaining better why 103 isolates yielded no amplification and 109 could not be interpreted (Line 195): “A total of 103 isolates gave no amplification or tDNA-PCR patterns composed of only a few and short tRNA intergenic spacers. Most of the isolates for which no amplification or only a few fragments could be obtained, are probably corynebacteria, which yield poor tDNA-PCR fingerprints (unpublished data). Finally, 109 isolates gave uninterpretable patterns, due to mixed cultures, as was confirmed by 16S rRNA gene sequencing for 20 of these, whereby the sequences could not be interpreted because of ambiguities, pointing to mixtures.”

14. Pages 9-10: Most of the text is repetition from the Table 1. The table should be made so clear that it is understandable without two pages of explanations. In that way the text in section “Rectal and vaginal prevalence of different bacterial species” may be shortened.
A32: We think Table 1 is already understandable by itself, and that the text summarizes the data of Table 1. However, we have omitted the following text from pages 9-10. “In this study, among 132 pregnant women the prevalence of Lactobacillus species in the vagina was L. crispatus (40%), followed by L. jensenii (32%), L. gasseri (30%) and L. iners (11%).” and “Of the two women that were colonized both vaginally and rectally with combined Lactobacillus species, the first had L. crispatus, L. jensenii and L. gasseri vaginally and a combination of L. crispatus and L. jensenii rectally, the second had a combination of L. crispatus and L. jensenii vaginally and a combination of L. jensenii and L. mucosa rectally.”

15. Page 9: I did not quite understand the line “Overall, 121 (91.6%) of 132 vaginal samples and 52 (39.3%) of 132 rectal samples were positive for lactobacilli.” By this you mean – I assume – that 121 of 132 pregnant women had vaginal lactobacilli and 52 of 132 women had rectal lactobacilli? Please clarify.
A33: Apparently, what we wrote is correctly understood. But we have rephrased as follows (Line 207): “Overall, 121 of 132 pregnant women (91.6%) carried vaginal lactobacilli and 52 (39.3%) carried rectal lactobacilli.”

In addition, how much the isolates that did not amplify with tDNA-intergenic PCR affect the results? Where there subjects that “did not have any lactobacilli”, but had only isolates that did not amplify? Please, clarify.
A34: Please see A7 and A29, and the following text, now at the start of the results section (Line 193): “For a total of 132 women 4 colonies each were picked from the vaginal and rectal sites, i.e. a total of 1056 colonies were picked. Using tDNA-PCR, 844 could be identified. A total of 103 isolates gave no amplification or tDNA-PCR patterns composed of only a few and short tRNA intergenic spacers. Most of the isolates for which no amplification
or only a few fragments could be obtained, are probably corynebacteria, which yield poor
tDNA-PCR fingerprints (unpublished data). Finally, 109 isolates gave uninterpretable
patterns, due to mixed cultures.”

16. Page 9: Sentence “In this study, among 132 pregnant women the prevalence of
Lactobacillus species in the vagina was L. crispatus (40%), followed by L. jensenii (32%), L.
gasseri (30%) and L. iners (11%).” No matter how I calculate the vaginal lactobacilli, I do not
reach the same percentages as in the manuscript. If I calculate from Lactobacillus species
“only vaginal” L. crispatus, the percentage is 32% (43/133), “vaginal + rectal” equals to 25%
(10/40), and “only vaginal” & “vaginal + rectal” equals to 31% (53/173) etc.. Please specify
what you mean, and recalculate all the percentages in this page if necessary.

A35: The calculations are correct. 43 out of 132 (not 133) women carried L. crispatus
only vaginal and 10 out of 132 both vaginal and rectal which is together 53 women with
vaginal lactobacilli out of 132 = 40%. We have omitted this sentence, because the data
are repeated later and now read (Line 211): “L. crispatus was the most frequently
identified Lactobacillus species isolated from the vagina (40%, i.e., 53/132 subjects positive
of which 10 also carried L. crispatus rectally), followed by L. jensenii (32%), L. gasseri
(30%), L. iners (11%) and L. vaginalis (10%).”

Furthermore, the sentence is poorly written, please correct the phrasing.

A36: The sentence has been omitted.

17. Page 10: Change the sentence “Rectally, the most abundant species were Streptococcus
anginosus group (47/132 subjects positive), Finegoldia magna (40), Peptoniphilus indolicus
(25) and E. faecalis (21).” to e.g. “Rectally, the most abundant species that were able to grow
on MRS agar or Columbia CNA agar supplemented with 5% sheep blood were Streptococcus
anginosus group (47/132 subjects positive), Finegoldia magna (40), Peptoniphilus indolicus
(25) and E. faecalis (21).”

A37: Since this is a culture-based study, whereby the media used are clearly listed, we
thought this was self-evident. However, we have changed as follows (Line 221): Rectally,
the most abundant species that could be cultured, were Streptococcus anginosus group
(47/132 subjects positive), Finegoldia magna (40), Peptoniphilus indolicus (25) and E.
faecalis (21).

18. Page 11: In the first sentence it is stated that: “For 35 of the 50 vaginal/rectal species
pairs, isolates with the same genotype were present vaginally and rectally.” However, there
are only 49 isolates in the Table 2 and genotype 34 is not similar (see comment 20), which
leads to 34 vaginal/rectal pairs and not to 35. Please correct in all relevant sections (including
e.g. abstract & discussion)

A38: We do not understand why one entry was lost from Table 2 during the original
submission, but the total number is 50. This probable copying error has now been
corrected in Table 2, matching the number of 50 again. Thanks for pointing out the
error with S. salivarius, so the correct number is 34/50. This has been corrected
throughout the text.

19. Page 11: In Table 2 there are 14/15 similar L. gasseri genotypes, not 14/16 as written in
the manuscript. Please, correct.

A39: This is due to one entry that was erroneously omitted. Please see also A38. There
are now again 16 subjects with L. gasseri.
20. Page 11: In the text it is said that there were same genotypes in 1/1 S. salivarius. However, in Table 1, the vaginal genotype is “A” and the rectal genotype “B” (which should not be identical).

**A40: This has been corrected, also throughout the text.**

21. Page 13: In the text it is stated that: “incubation methods may strongly influence the outcome, e.g. incubation in an anaerobic chamber yields more L. vaginalis than in an anaerobic jar (see below)”. What is meant by “incubation in an anaerobic chamber” and “incubation in an anaerobic jar”? Are the cultivations performed in both cases in normal atmosphere? And is the subsequent incubation performed in anaerobic chamber or anaerobic jar? If this is the case, the corresponding gas atmospheres should be identified, because it is possible to have different atmospheres in anaerobic jars (and the gas atmosphere has a greater effect on the growth than the vessel in which the gas mixture is). Please, specify.

**A41: Please see A19.**

22. Page 14: See the comment 21. In the first section it is continually refered to the incubation “in anaerobic jars” and “anaerobic cabinet”. Please, specify the gas atmosphere and explain how one differs from the other. Specify also, if the cultivations were performed in the anaerobic cabinet.

**A42: Please see A19.**

23. Page 14: It is stated in the text that: “The rectal occurrence of lactobacilli in culture-based studies may be underreported.”. There are at least two articles (Dal Bello & Hertel, Syst Appl Microbiol 2006; Maukonen et al., J Med Microbiol 2008) in which intestinal and oral lactobacilli populations have been compared to each other (molecular techniques and culture-based techniques). In the study of Maukonen et al, Rogosa agar was used for the specific growth of lactobacilli, resulting in ~10^6 cfu lactobacilli / g of feces (culture performed in an anaerobic cabinet with pre-reduced media, thereafter anaerobic incubation in an anerobic jar). Many isolates were 16S rRNA sequenced, so the identity of the isolates on Rogosa was also checked. In regard of those results it is surprising that there were so many subjects in this study that did not have any rectal lactobacilli. Geographical etc. differences do not explain that big difference. The only differences that can explain the total absence of lactobacilli in so many subjects are methodological: the sampling procedure, culture conditions (including atmosphere and media) and PCR-conditions

**A43: We think that the differences can be explained by sampling. In the two publications mentioned, fecal samples are used, whereas we started from rectal swabbing. We have added this as follows (Line 334): “However, high occurrence of intestinal lactobacilli has been reported [41, 42]. The difference with our study might be explained by the fact that these studies used fecal samples, whereas we started from rectal swabbing.” We have added these references.**

24. Page 14: In the text it is stated that: “In this population of pregnant women, we isolated lactobacilli more frequently from the vagina (121 subjects, 91.6%) than from the rectum (52 subjects, 39.3%)”. See the comment above about abundance.

**A44: Please see A43**

25. Page 15-16: It is stated in the text that: “Because of the close proximity of the rectum to the vagina, the isolation of H2O2-producing vaginal Lactobacillus species from the rectum suggests that it may play a role as a reservoir for these microorganisms [2]. Vaginal colonization by Lactobacillus species was found to be transient in many females [7], and the
rectum may be a source for vaginal recolonisation by lactobacilli after a disturbance of the ecology that follows douching, menses or sexual intercourse.” This is ok, since these are just citations from other publications. However, from the results of this study it is not possible to conclude that: “the rectum may be a source for vaginal recolonization by these Lactobacillus species.” In this study, the samples were taken only ones from each individual, so there is no evidence on “recolonization”. There is only evidence that the same genotypes reside in both vagina and rectum at the time the sample was taken. Please, rephrase.

A45: Here we disagree. First our claim is moderated, and not absolute, by using the conditional tense: ‘may’. Second, we agree that we do not have subsequent sampling times, but we fail to understand how else one could explain the presence in vagina and rectum not only of identical species but also of identical strains. Still, to incorporate this remark of the reviewer, we have changed to (Line 391):

“Although we did not sample the same subjects at different time intervals, this finding …’

26. Page 16: It is stated in the text that: “This finding suggests strong dynamics of changing composition of the vaginal microflora, whereby different strains of a limited number of species replace each other continuously, and are exchanged between vagina and rectum.” This is not true within your experiment (see comments above). Please, rephrase.

A46: Again, we do not state that our findings proof this, but that they suggest this to be so. When the reviewer can suggest other explanations, we are willing to offer them as an alternative.

27. Page 17: It is stated in the text that: “indicating that many species can colonize the vagina from the rectum or migrate to the rectum from the vagina.” It is not proven in this study that the bacteria migrate between the sites (again, see above). Please, rephrase.

A47: Again, we fail to see how else one can explain the simultaneous occurrence of the same strains in two places, moreover closely linked to each other. Our results moreover confirm what is already considered as general knowledge, but which has not been studied thus far in such detail as in our study. Our findings simply confirm previous studies, e.g., Reid G, Charbonneau D, Erb J, Kochanowski B, Beuerman D, Poehner R, Bruce AW. Oral use of Lactobacillus rhamnosus GR-1 and L. fermentum RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. FEMS Immunol Med Microbiol. 2003 Mar 20;35(2):131-4. (note the erroneous use of ‘vaginal flora’).

28. Page 17: It is stated in the text that: “All these data indicate strong dynamics of the vaginal and rectal microflora, influencing each others species composition to some degree and containing several genotypes per species, which also may be exchanged between both sites, whereby different genotypes of the same species are continuously replacing each other. It is possible that the rectal colonization by lactobacilli may function as a reservoir for the maintenance of a normal vaginal flora and that this may be associated with a decreased incidence of BV-associated adverse effects, as has been suggested [3]”. See the comments 25-27. Please, rephrase.

A48: Please, see A45-A47

29. Page 25: Table 1. Please modify the table legend in a way that the table is understandable even without the text of the results section.

A49: We think the Table is understandable as it is. We slightly modified the legend to: “Number of pregnant women carrying this species.” and we shortened the text. Please see A32.
30. Page 27: Table 2. Please modify the table legend in a way that the table is understandable even without the text of the results section.

**A50:** Again, we think the table is understandable as such.

31. Page 29: Table 3. Please modify the table legend in a way that the table is understandable even without the text of the results section. The whole table is difficult to understand, please modify it. e.g. Are the numbers percentages or isolates? I would guess that they are percentages, since there are decimals, but I shouldn’t have to guess. In addition, in several columns there are no decimals and in others there are. Please use one or the other format.

**A51a:** We have changed the title as follows: “Vaginal and rectal occurrence of *Lactobacillus* species, expressed as percentage of subjects positive, according to different studies “.

We have provided the following legend:
“a: The column entitled ‘Vagina all’ presents the sum of subjects with the species in the vagina only and those with the species in both vagina and rectum. The column entitled ‘Rectum all’ presents the sum of subjects with the species in the rectum only and those with the species in both vagina and rectum.”

We have presented all percentages in a similar format.

Furthermore, in the table 3 the abundance of *L. crispatus* in vagina is 32.6 (see comment 16), even though in text it is said to be 40%. Please, cross-check and correct.

**A51b:** This number is correct. *L. crispatus* was found in the vagina only in 32.6% of the women and in vagina overall (= women with *L. crispatus* in vagina only + women with *L. crispatus* in both vagina and rectum) is 40%. This should be clearer now from the added legend, although the column heading was already explanatory, stating ‘vagina only’.

Minor Essential Revisions

1. A term “microflora” has been used throughout the manuscript. “Microbiota” would be a more accurate term (I personally would place bacteria rather to “fauna” than “flora).

**A52:** This is an ongoing discussion. First, personally we would place bacteria neither in fauna nor flora, since bacteria are prokaryotes while both animals and plants are eukaryotes. We think the term ‘microflora’ can stand on its own, not relating to ‘flora’. We avoid strictly to use the clearly erroneous term ‘vaginal flora’, used by some authors.

We are well aware that the terms microflora and microbiota are used interchangeably, though none of both terms unambiguously describe what is meant, i.e. the assembly of all bacteria in a certain habitat. “Flora” may indeed lead to confusion with the Plant Kingdom. “Biota” on the other hand is defined by ecologists as the total collection of organisms of a geographic region, a time period or a habitat. Biota is the superdomain that contains all life, and hence refers collectively to flora, fauna and other forms of life such as bacteria, and fungi. Hence, “microbiota” – though not properly defined would encompass all life in certain habitat, which is again, not what is meant.

So basically, though the use of a particular term has been advocated by some, none of the available terms – microflora, microbiota, microbiome, etc – actually defines unambiguously what we all try to designate.’Microbial community’ might do better, but is somewhat cumbersome.

Illustrative to this lack of consensus is that leading researchers in this field, leading scientific journals like Nature and Science, and authoritative organisations like the American Society for Microbiology continue to use both microflora and microbiota as established terms.
While we are awaiting some definitive consensus on this issue, for historical reasons, there is a slight preference to denote the bacterial communities of the skin, the oral cavity, the gut, and the vagina as “flora” or “microflora”.

2. This manuscript is mainly written in American English. However, the use of commas varies. Please make sure that you use American punctuation systematically (ie comma before “and” in lists).

A53: We think these details will be dealt with by the typesetter and language editor.

3. Page 8: Change “and”, which is currently in italics (last row) to normal text.

A54: Has been changed.

4. Page 12: Change “culture free” to “culture independent”

A55: Has been changed. Line 270.

5. Page 9, last row: “Lactobacillus cellobiosus fermentum” In J.P. Euzeby’s “List of Prokaryotic names with Standing in Nomenclature” (http://www.bacterio.cict.fr/index.html), there is no “Lactobacillus cellobiosus fermentum”. Please check the correct nomenclature.

A56: We have changed to ‘Lactobacillus fermentum’

6. Page 13: In this page there is “et al.” written in italics and with normal font. Please check through out the text that “et al.” is written in italics.

A57: Has been changed.

7. Page 13: In sentence “More technically related factors concern variations in the way that samples are taken, transported and treated, the fact that the culture media and incubation methods may strongly influence the outcome, e.g. incubation in an anaerobic chamber yields more L. vaginalis than in an anaerobic jar (see below), the use of MRS agar precludes isolation of L. iners) and that identification has often been based on phenotypic methods [32, 33].” there is no parenthesis matching the one after “of L. iners)”. Please, correct.

A58: Has been changed.

8. Page 19: Reference 1; there are two dots in the end – omit the other.

A59: Has been changed.

9. Page 25: Table 1, Row 28; “Lactobacillus casei paracasei” In J.P. Euzeby’s “List of Prokaryotic names with Standing in Nomenclature” (http://www.bacterio.cict.fr/index.html), there is no “Lactobacillus cellobiosus fermentum”.

A60: This has been changed. Please see A61.

There are only Lactobacillus casei subsp. casei and Lactobacillus paracasei subsp. paracasei.

A61: We are not sure anymore after consulting the confusing history of these names. We opted to change to ‘Lactobacillus casei’ and ‘Lactobacillus fermentum’.

10. Page 25: Table 1, Row 29; “Lactobacillus cellobiosus fermentum” In J.P. Euzeby’s “List of Prokaryotic names with Standing in Nomenclature” (http://www.bacterio.cict.fr/index.html), there is no “Lactobacillus cellobiosus fermentum”. Please check the correct nomenclature.

A62: Has been changed to ‘L. fermentum’.
11. Page 25: Table 1, Row 31; Overall count is calculated as 55. However, 43+4+10=57. Please, correct. 
A63: Has been corrected.

12. Page 26: Table 1, Row 50; Overall count is calculated as 15. However, 3+13+0=16. Please, correct.
A64: Has been corrected.

13. Page 26: Table 1, Overall column; after corrections 11 & 12, recalculate the column.
A65: Has been changed to ‘513’.

14. Page 26: Table 1, Row 58; change “group” currently in italics to normal text.
A66: Has been changed.

15. Page 27: Table 2, Rows 25 & 50; add highlight at the back of “A”
A67: Has been highlighted.

16. Page 27: Table 2, Row 49; The vaginal genotype is “A” and the rectal genotype “B”, which should not be identical. Please correct.
A68: Has been corrected.

Discretionary Revisions
1. Abstract: It is stated in the conclusions that your results support the hypothesis “that the rectal microflora serves as a reservoir for colonisation of the vaginal econiche”. Since you took samples only once, please remember that you know the situation only at that time point (see Major revision number 25-28)
A69: Please see A45-A48.

2. Did you make plate counts from the cultivations? The results from those would also be interesting. It would be nice to know what was the cfu on MRS as compared to Columbia blood agar. In addition, it would be nice to see, if the plate count results (e.g. low number of lactobacilli on the agars you used) correlate with genotypic results, or with those isolates that did not amplify with tDNA-intergenic PCR.
A70: We did not perform quantitative culture. Given the large number of samples and the two different culture media, this would have been a work too enormous. However, we have very interesting qPCR results now for the same samples for six species, which we intend to publish.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests: I declare that I have no competing interests.