Author’s response to reviews

Title: The influence of probiotics on genital high-risk human papilloma virus clearance, associated lesions and quality of cervical smear: A randomized placebo-controlled trial

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Reply to editor:

We thank to the reviewers for the valuable comments, which allowed us an opportunity to improve our manuscript. All the issues raised by the reviewers were answered in the following documents. We tried to reply every question rose by the reviewers as accurately as possible and also tried to make changes accordingly in our new version of the manuscript. All changes in the text are highlighted in red. We also confirmed that our manuscript contains all items in the CONSORT guidelines for reporting clinical trials.

Reply to reviewer 1:

1. Whether enrolled patients had a clinical diagnosis of bacterial vaginosis (BV) ?

Ans: Our main goal was to investigate the influence of oral probiotic strains on genital high-risk human papilloma virus (HR-HPV) clearance but not BV. Furthermore, a positive association between BV status and cervical HR-HPV infection had been proved in a meta-analysis by Gillet et al (Ref. 5). Therefore, whether enrolled patients had a clinical diagnosis of BV was not clear in our study.
2. Title: "The influence of probiotics on genital high-risk human papilloma virus clearance" is not entirely correct: it should be modified in high-risk human papilloma virus-associated lesions.

Ans: We have modified the title as “The influence of probiotics on genital high-risk human papilloma virus clearance, associated lesions and quality of cervical smear”

3. A definition of unsatisfactory cervical smear should be given.

Ans: We have added the definition as following: The report was considered unsatisfactory when more than 75% of the epithelial cells were obscured or could not be clearly visualized.

3. The sentence "BV was independently associated with HPV infection" is not referenced and it is not clear whether it refers to this study group.

Findings from other recent papers on the use of probiotic strains in HPV-positive patients should be discussed

Ans: A positive association between BV status and cervical HR-HPV infection had been proved in a meta-analysis by Gillet et al (Ref. 5). When writing this manuscript, there was only one paper on the use of probiotic strains in HPV-positive women. But recently we found another paper regarding the issue of probiotics and HPV. The authors reported that the clearance of PAP-smear abnormalities and HPV-clearance was higher in the treatment of metronidazole plus 6 months vaginal Lactobacillus implementation than that with 3 months use. We have added this result in discussion section (Palma et al. BMC Infectious Diseases 2018 Jan 5;18(1):13).

Reply to reviewer 2:

1. Why was vaginal pH or BV status not reported in the study? Since these are acknowledged by the authors to be important variables in HPV risk, it seems appropriate that this information would be collected? If it wasn't, this should be acknowledged as a limitation of the study design.

Ans: Because a positive association between BV status and cervical HR-HPV infection had been proved in a meta-analysis by Gillet et al (Ref. 5). Also, our main goal was to investigate the influence of oral probiotic strains on genital high-risk human papilloma virus (HR-HPV) clearance but not BV. However, it is a good idea to incorporate BV status in our study and to see
whether no effect of probiotics on HPV clearance is owing to BV status. This will be acknowledged as a limitation in discussion section.

2. Please justify selection of 50% power for power calculations. This is considerably lower than a power of 80% that is the minimum that should be used for clinical trials. Thus, they were underpowered to show a difference between the groups, and I think their negative result may be misleading. Furthermore, the main positive result from this study is the improved clearance of LSIL. The study certainly was not powered for this outcome, with only 21 women having LSIL.

Ans: Indeed, it is very difficult to calculate the optimal case number that should be included in this clinical trial because no previous similar study had been reported. Initially, we selected 75% power for calculations and a total number of 180 (90 in each arm) should be enrolled. Unfortunately, due to slow case enrollment, only 121 positive HR-HPV women were enrolled. The 55.8% power was obtained retrospectively of using 121 cases enrolled.

3. Probiotic was given orally. Justify why U-relax was selected as the product to be used? Please justify why oral administration was selected if vaginal colonization was intended? Please provide more detail about the U-relax product: dose, co-formulation, QC that was done on the product to ensure organisms were live, how long the women were intended to take the probiotics for and what proportion of them reported regular dosing/incomplete dosing? Was antibiotic treatment (for any condition) noted? As this would influence probiotic viability?

Ans: U-relax is manufactured by Chr Hansen company from Denmark. The strains, GR-1 and RC-14, are both recommended by WHO (FAO/WHO: 2001. Expert Consultation Report) to improve BV, yeast vaginitis and urinary tract infections. Therefore, we selected U-relax in this study.

Reid et al. had reported clinical evidence that GR-1 and RC-14 can be delivered to the vagina following oral intake via morphology identification and molecular typing (FEMS Immunol Med Microbiol. 2001;30(1):49-52.). Also several studies (including one from Taiwan, investigating the effect of GR-1 and RC-14 on pregnant women with GBS colonization) had proved that oral probiotics could restore vaginal flora and reducing vaginal colonization of pathogenic bacteria and yeast (Ref 9,10,11).

QC showed that U-relax has a minimum potency of 5.4 billion (5.4E+9) CFU (Colony Forming Units) per capsule. Each capsule (Gelatin, titanium dioxide) contains 180 mg of a standardized, light beige fine powder (glucose anhydrate, potato starch, microcrystalline cellulose and
magnesium stearate) consisting of freeze-dried cultures (50% GR-1 and 50% RC-14). It can be stored in room temperature 5-30C without dramatic change in CFU (see attachment files). The recommended dosing is 2 capsules/day for treatment of active vaginitis for 1 week, and then 1 capsule/day for 1 month, and finally changes to 2 capsules/week as long-term maintenance. Antibiotics could be used along with U-relax without any influence on probiotics viability (Microbes Infect. 2006 May;8(6):1450-4).

4. They don't mention how the placebo was manufactured. Please provide details of this.

Ans: The placebo capsule (Gelatin, titanium dioxide) also contains 180 mg of a standardized, light beige fine powder (glucose anhydrate, potato starch, microcrystalline cellulose and magnesium stearate) but without freeze-dried cultures (50% GR-1 and 50% RC-14). Please see attachment:

5. They only mention ethics approval but no regulatory approval for the trial. Can this be clarified? Who did the randomization? Please provide more detail on inclusion/exclusion/screening criteria.

Ans: ClinicalTrials.gov Identifier: NCT01599416. We did the randomization through the application program provided at website http://www.randomization.com/.

The inclusion and exclusion criteria have been added in the text.

Inclusion Criteria:
- Female
- Age over 30
- PAP test with negative for intraepithelial lesion or malignancy results
- HPV DNA test with positive result
- Not pregnant

Exclusion Criteria:
- Cervical intraepithelial neoplasia before conization, and within 6 months after conization
- Cervical cancer patient
• With prior GI surgery
• GI dysfunction

6. About a third of the cohort were post-menopausal. Authors should comment on whether these women were on hormone replacement therapy? Comment on BV and HPV prevalence post menopause and whether low estrogen levels would influence probiotic efficacy?

Ans: No post-menopausal women in this study had hormone therapy. A previous study showed that number of lactobacillus was increased following probiotic administration in postmenopausal women indicating low estrogen levels would not influence probiotic efficacy (PLoS One. 2014 Aug 15;9(8):e104511).

7. Discussion - page 10 line 7-10. Please provide evidence that oral probiotics make their way through the GI tract after oral administration to colonize the genital tract in women?

Ans: Reid et al. had reported clinical evidence that GR-1 and RC-14 can be delivered to the vagina following oral intake via morphology identification and molecular typing (FEMS Immunol Med Microbiol. 2001;30(1):49-52.).

8. Comparison is made in the discussion with the Verhoeven study - please provide more detail about probiotic product used by Verhoeven, dose, duration, strains etc.

Ans: Strain: Lactobacillus casei Shirota; Dose: 1X1010CFU/day; Duration: 6-month

9. Discussion page 11 line 14 - authors state that probiotics may influence different strains of HPV differently. Please provide more justification for why you think this would be likely?

Ans: Probiotics have been shown as having antiviral activity through suppression HPV 16 E6 and E7 oncogene expression in vitro but no data about other HPV types (BMC Med. 2012 Jul 12;10:72. As reference 17). Moreover, although most HPV infection will clear spontaneously, still about 10% will persist of which type 16 and 31 have the highest change of persistence (PLoS One. 2013 Nov 19;8(11):e79260). Therefore, different HPV types may have different natural history upon infection. Based on these findings, we are reasonably to assume that probiotics may influence different strains of HPV differently.
10. Limitations section in the Discussion needs to be expanded to include/acknowledge other limitations: probiotics were orally delivered, vaginal colonization was not assessed, BV status was not assessed by standard methods, vaginal pH? Study not powered for the LSIL finding since numbers were so small? Commenting on whether individuals took their probiotics for the full duration of the study or whether this information was even collected? Comment on the fact that the isolates in the probiotic were not vaginal isolates? Ie, were they appropriate for a vaginal probiotic study? HPV was measured by Hybrid capture that is not type specific, and semi-quantitative.

Ans: We have added the limitations and comments in the Discussion section as suggestion.

Only the women who took probiotics for full duration will be included in the study. They will keep OPD follow up every three months. At OPD they will receive PAP smear and HPV examination.

Healthy vaginal microbiota includes mostly microorganisms from Lactobacillus species, whose role is to prevent colonization by the pathogens. Vujic et al has reported that oral probiotics containing Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14 could re-balance the vaginal microbiota. Ho et al. also reported oral probiotic containing Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14 could reduce the vaginal and rectal GBS colonization rate in pregnant women.

11. Please include information about ASCUS/LSIL prevalence in Table 1.

Ans: We have added prevalence of ASCUS/LSIL in Table 1.