

Introduction to resubmission

We are pleased to resubmit our proposal "[REDACTED]". The initial review felt that our study tackled "...an important problem," and "...may well have substantial clinical impact in the near future." Reviewers felt that our study had "...clearly stated hypotheses..." and that "The research team is spot-on to do the work." Reviews identified "...a few minor weaknesses" in the approach, which we have addressed in this resubmission as outlined below.

Improved definition of study population: Reviewers noted that bacterial vaginosis is more common in Black and Latina women, and suggested that trial population should reflect the most affected population of women. Our original planned recruitment (28% Black and 15% Latina) reflects the demographics of the Boston and MGH population, but we have now altered our recruitment plan for both donors and recipients to partner with community organizations for a goal of 40-50% of participants to be women of color (Black, Latina, Indigenous). Because our trial is one of the first to perform vaginal microbiome transplant (VMT) in humans, we place a priority on local recruitment so that any possible adverse events can be easily evaluated.

Clarification of trial procedures: We agree with reviewers that host characteristics are of significant interest as a driver of microbial colonization, and have now specified an analysis of transplant success according to concordance of race and ethnicity of donors/recipients. We have opted not to match assignment of the intervention for two reasons: 1) Matching would eliminate the possibility of evaluating the matched characteristic as a driver of microbial colonization and 2) Logistics of collecting and storing donations may mean that a donation with the necessary characteristic may not be available at all times.

Improved methods for storage and characterization of donated vaginal fluid: We agree with the reviewer that a cryoprotectant may ultimately be of use in any live biotherapeutic intervention, especially with a synthetic product composed of specifically chosen bacterial isolates. Our concern with VMT is that a cryoprotectant may alter or obscure a component of vaginal fluid which is important for microbial colonization. We have updated Figure 3 with characteristics of donated fluid microbiota, as well as stability of CFU counts of *Lactobacillus* spp in donated vaginal fluid (which includes many of the glycans and carbohydrates used as commercial cryoprotectants, such as trehalose) over 6 months in multiple different donors. We have also narrowed the window between end of metronidazole treatment and delivery of the study intervention to 48 hours in response to reviewer suggestions. We have further specified selection criteria for donated fluid to include a requirement that *Lactobacillus* species be $\geq 90\%$ of all sequences, and that *L. iners* be no more than 30% of all sequences. These two requirements should mean that any BV-associated species will be a small minority of sequences.

Characterization of host immune responses: We have expanded our flow panel to include NK cells, and have moved immune cell gene expression to future directions. Cells will be sorted and stored in a manner to allow such analyses at a later date.

Focusing and clarification of proposed analyses to define mechanisms and underlying biology: In Aim 3 we propose in depth analyses to better understand the mechanisms of microbial colonization and engraftment. As the reviewers noted, engraftment of transplanted strains is less clinically meaningful than *Lactobacillus* dominance overall. We have clarified the focus of the Aim and stated more clearly our hypothesis that there will be identifiable genetic characteristics of *L. crispatus* strains which promote a *Lactobacillus*-dominant community. In new Aim 3.1 we will perform shotgun metagenomics to compare the genetic profile of lactobacilli in all recipients (VMT or placebo) who establish *Lactobacillus* dominance vs. those that do not. In Aim 3.2 we will cultivate *Lactobacillus* isolates from donations and will perform shotgun sequencing of isolates to compare gene profiles of the lactobacilli in donations that do vs. do not lead to stable *Lactobacillus* dominance. Here we will also compare isolates that do vs. do not engraft in recipients, which will provide basic biologic insight into drivers of colonization. We will also perform characterization of metabolic capacity *in vitro* to correlate genetic and functional profiles. In Aim 3.3 we will compare the metagenomic profile of the transplanted community to identify genes or pathways associated with transplant success. As noted, the recently published VIRGO catalog of vaginal microbial genes will facilitate these analyses, and dichotomizing the comparisons will improve our power to detect differences. It is challenging to calculate power for these types of analyses, but focusing our comparison on *Lactobacillus* genes and using our whole study population will optimize our ability to detect differences between the two groups. Of our 126 participants, we anticipate 80-90 will achieve *Lactobacillus* dominance, and we anticipate isolating multiple *Lactobacillus* strains from each participant, expanding our sample for Aim 3.2. Samples will be preserved in such a manner that future transcriptomic analyses, or examination of phage burden could be performed at a later date.