Title

The vaginal microbiome following orally administered probiotic

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Keywords: Vaginal microbiome; healthy microbiome; women; public health, shotgun metagenomics

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Wordcount: 2423

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Abstract

Introduction

Here we present a longitudinal shotgun sequencing metagenomics study of 16 healthy, Danish women in the reproductive age. The aim of the study was to investigate whether lactobacilli, orally consumed, had any impact on the vaginal microbiome and its functional potential.

Materials and Methods

The 16 women aged 19-45 years were recruited from Copenhagen, Denmark. One baseline vaginal sample (day 0) and two study samples (day 25-30 and day 55-60, respectively) were sampled. The vaginal samples were analyzed by shotgun metagenomics.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/apm.13261

Results

We detected 26 species in the vaginal microbiota of the 16 women, of which six belonged to the *Lactobacillus* genus. We observed three vaginal microbiome clusters mainly dominated by *Gardnerella vaginalis*, *Lactobacillus iners* or *Lactobacillus crispatus*. The oral probiotic had no detectable effect on either the composition or the functional potential of the vaginal microbiota.

Discussion

Most of the study subjects (11 out of 16 women) exhibited only minor changes in the vaginal microbiome during the treatment with probiotics. Any compositional changes could not be associated to the probiotic treatment. Future studies may benefit from an increased number of participants, and administration of the probiotics during conditions with bacterial imbalance (e.g. during/after antibiotic treatment) or the use of different Lactobacillus spp. known to colonize the vagina.

Introduction

In the vagina, as in other parts of the body, microbes exist in a delicate mutualistic relationship which represents the first line of defence against colonization and infection by opportunistic pathogens, a phenomenon known as colonization resistance (1–3). In addition, *Lactobacillus* spp. (*Lactobacillus crispatus*, *L. gasseri*, *L. iners* or *L. jensenii*) play an important physiological role in maintaining a low pH (<4.5) in the vagina via production of lactic acid (4–6). This production of lactic acid, combined with hydrogen peroxide and bacteriocins, protect the vaginal environment from invasion by other microorganisms, infection of vaginal epithelium and has accordingly been associated with an overall well-being (2,4,5). Vaginal microbiota not dominated by a single species, but found with anaerobes and strict anaerobes species (e.g. *Gardnerella, Atopobium, Mobiluncus, Prevotella,* and *Clostridiales* spp.), have traditionally been associated with vaginal symptoms consistent with bacterial vaginosis (BV), including discharge, odor and irritation (2,3). Furthermore, women with a vaginal microbiome dominated by *G. vaginalis* and other anaerobic bacteria are considered to be at risk of adverse events. Yet, recent studies have identified *Gardnerella* spp. as a sub-dominant species in the vaginal

microbiome of healthy women in the reproductive age (7,8). Thus, the overall microbiome functional potential is important to attain a healthy vaginal flora. As such, probiotic may impact the vaginal microbiome indirectly. Furthermore, Reid et al. found that the numbers of vaginal lactobacilli increased significantly in healthy women receiving *Lactobacillus* orally (4,5,9). Only a few studies investigated the vaginal microbiota of healthy women longitudinally and very few have tested the impact of probiotics on healthy individuals. Furthermore, many of these studies have been carried out using 16S rRNA gene high-throughput sequencing (6–8). Shotgun metagenomic sequencing has become an acknowledged high-throughput technology for characterizing microbial communities (6).

The purpose of this study was to investigate, if lactobacilli from oral probiotics had any impact on the structure of the vaginal microbiome and its functional potential, using shotgun-metagenomics data analysis, a method previously validated on complex biological samples (10,11).

Materials and Methods

Participants and study design

Healthy (self-reported), reproductive-aged women were recruited from Copenhagen, Denmark through online and print advertisements. Women were interviewed and screened at Department of Clinical Microbiology at either Herlev Hospital or Rigshospitalet in Copenhagen, Denmark. Further information can be found in Supplementary material.

One baseline vaginal sample (day 0) and two study samples (day 25-30 and day 55-60, respectively) were sampled from each participant. Participants performed swabs 7-10 days after the final day of menstruation. We used OMNIgene Vaginal kit for the easy self-collection and stabilization of DNA for microbiome analysis (DNA Genotek, Ottawa, Canada). This allowed for home-sampling. Instructions for vaginal swabs were given according to guidelines provided by the manufacturer and is briefly described in supplementary material. After collection, the samples were stored at -20˚C and mailed

(within 7 days) to Department of Clinical Microbiology, Rigshospitalet for storage at -80 °C until all samples had been collected.

Bifodan A/S determined the viability of *Lactobacillus* spp. in the oral capsules prior to our study (for further details, see Supplementary material).

Furthermore, see supplementary material for descriptions of molecular microbiology methods.

Statistical analyses

To identify differentially abundant MGSs between the sampling (visit 1, 2 and 3), we performed paired Wilcoxon signed-rank tests. We specifically tested whether the *L. gasseri* abundance changed significantly after visit 1. Where multiple hypotheses were evaluated in parallel, the Benjamini-Hochberg method was used to control false discovery rate considering significant all hits below an FDR of 5%.

Ethics

Research ethical approvals were given by the Regional Committee of Danish Data Protection Agency and the Regional Committee of Health Research Ethics for the Capitol Region of Denmark (Trial registration ID No. H-17004182). Informed consent for participation and publication was obtained from all participants prior to enrollment. Thus, all processes were performed according to the Declaration of Helsinki and its amendments.

Results

Participants

We initially recruited 21 healthy women (age range: 18-45 years), of which 3 dropped out before completing the study and 2 other completed but their swabs were lost during shipment to the department of Clinical Microbiology, Rigshospitalet. Thus, 16 women completed the study (Table 1) (they had not used either any oral or intravaginal antibiotic or

antifungal treatments 6 months prior to the study or any oral or intravaginal probiotics 3 months prior to the study).

Naturally occurring *L. rhamnosus* was not detected in any of the vaginal samples. However, the species *L. gasseri* was detected in 33 of the 48 samples (11 out of 16 subjects), ranging from 0.003 % - 32.2 % relative abundance (Figure 1). Women with *L. gasseri* detected in their vaginal microbiota displayed this species already before probiotic consumption (visit 1). We did not observe a significant difference in *L. gasseri* abundance comparing visit 1 to visit 2 (Wilcoxon signed-rank test; P = 0.69) or visit 1 and visit 3 (Wilcoxon signed-rank test; P = 0.50).

Species detection and species abundance profiles in vaginal swabs

In the vaginal microbiota of the 16 women we detected 25 individual species, of which six were *Lactobacillus* spp. Besides *Lactobacillus* spp., other dominant non-lactobacillus species were especially *Gardnerella vaginalis* and *Atopobium vaginae* (all MGS are shown in Supplementary Table 1). In Figure 1, we show the annotated metagenomic species (MGSs) abundance profiles of the top-10 most dominant vaginal MGSs. We observed three different sample clusters with one dominant species for each sample. The three predominant species *G. vaginalis*, *L. iners* and *L. crispatus* characterize the three sample groups observed. Principal coordinates analysis (PCoA) (Figure 2) based on Bray-Curtis dissimilarities among samples confirmed the existence of three different vaginal clusters and did not show any separation of samples between visits, indicating no effect of the probiotic treatment on the vaginal microbiome structure (confirmed by a Permanova test on all samples, testing the effect of visit stratification on the microbiome structure [$R^2 = 0.02$; P = 0.309]). Samples from the same subject generally showed limited compositional differences between timepoints, except for subjects 2, 4, 9, 11, and 20 who shifted from one group on visit 1 to another for sampling 2 and 3. Subject 11 and 20 were initially dominated by *G. vaginalis* (at visit 1) and moved to a group dominated by *L. crispatus* (at visit 2 and 3). Yet,

subject 4 and 9 were dominated by *L. iners* (at visit 1) but moved to a group dominated by *G. vaginalis* (at visit 2 and 3). Other subjects with *L. iners* were all in the same group throughout the study. Subject 2 was the only participant to have three different groups based on PCoA (Figure 2). To further confirm that the probiotics did not affect the vaginal microbiome, we tested for differentially abundant MGSs between baseline (visit 1) and during-treatment (visit 2), and between baseline and post-treatment (visit 3). There were no significant differentially abundant MGSs, either between visit 1 and visit 2 (FDR adjusted $P > 0.88$), or between visit 1 and visit 3 (FDR adjusted $P > 0.23$).

We also tested the differences in functional potential between the during- and post-treatment time points (visit 2 and 3 respectively), and the baseline (visit 1) using summarized eggNOG groups and KEGG module abundances. We considered only orthologous groups and KEGG modules that were present in at least 10% of the samples, resulting in pruned tables of 7,771 eggNOG groups and 466 KEGG modules. There were no differentially abundant eggNOG groups, either between visit 1 and visit 2 (all FDR-adjusted $P = 1$) or between visit 2 and visit 3 (all FDR-adjusted $P > 0.91$). There were also no differentially abundant KEGG modules, either between visit 1 and visit 2 (all FDR-adjusted $P = 1$) or between visit 2 and visit 3 (all FDR-adjusted $P > 0.94$). These results indicate that the oral probiotic had no significant effects on the microbial functional potential of the vaginal microbiota.

Discussion

The purpose of this study was to investigate, if lactobacilli from oral probiotics had any impact on the vaginal microbiome and its functional potential in 16 healthy, non-antibiotic treated Danish women. All participants followed an oral probiotic treatment and their microbial changes were compared to their baseline sample (visit 1). This approach enabled us to characterize the vaginal microbiome of healthy women in reproductive age confirming species and species clusters previously characterized in this niche (2,3,6–8). Although *L. gasseri* was detected in the vaginal microbiota of some study participants, this microbe was an

endogenous *L. gasseri* strain and not the probiotic strain. Previous publications have shown vaginal colonization by *L. rhamnosus* following oral administration, which do not agree with our results. This could be due to a too low probiotic dose, unsuccessful translocation of the probiotic through the gastrointestinal route or signals below the detection levels.

We found three different vaginal clusters and did not show any separation of samples between visits, indicating no effect of the probiotic treatment on the vaginal microbiome structure. Overall, samples from the same subject generally showed limited compositional differences between timepoints, except for a few subjects who changed dominating species of their vaginal microbiome between sampling. Yet, the changes in these subjects were heterogenous with no indication of a specific influence from the probiotic strains. Furthermore, we found no indications that the oral probiotic had any significant effects on the microbial functional potential, since there were no differentially abundant eggNOG groups or differentially abundant KEGG modules, either between visit 1 and visit 2 or between visit 2 and visit 3. Finally, we observed no statistically significant taxonomical changes in the vaginal microbiome between visits.

Lactobacilli administered orally or intra-vaginally have been tested for their effectiveness in preventing the recurrence of bacterial vaginosis (BV) (4,5). Oral administration has been introduced in a simple attempt to use lactobacilli via a more practical route as compared to the direct administration (12). Randomized clinical trials have suggested that intra-vaginal administration of lactobacilli helped to cure women with BV more frequently than administration of a placebo or no treatment (4,5). We have too few women in this study to confirm or reject these results. Yet, our results prove the need for appropriate randomized clinical trials in order to investigate the effect of oral probiotic on the structure of the vaginal microbiome and the functional potential in women suffering from vaginosis, with and without antibiotic treatment, as well as on healthy individuals.

Our study had several other limitations that may have influenced the outcome. Firstly, we had a limited number of participants and the period was 2 months, within which only 3 vaginal swabs were taken. Secondly, all participants performed the vaginal swabbing with no control of how it was performed, nor did we control time from the last day of menses or have any detail of the cycle of the menstrual phase. However, no women took swabs during menstruation. Furthermore, we did not include a control group and did not have access to participants´ data to build a phenotypic profile. We chose to use swabs as sampling was performed by participants, as cytobrush and swab sampling has been found to provide a comparable microbiome composition and detect a small proportion of underlying species (13). We have too few women in this study to confirm or reject results of previous studies, although we are convinced our method of detection is more meticulous. Several studies have illustrated that colonization by species such as *L. crispatus, L. gasseri, L. iners*, and *L. jensenii* is essential for a healthy vaginal microbiota, whereas *L. rhamnosus* and *L. reuteri* are commonly used as ingredients of oral probiotics but are species more prone to colonize the gut, and as transient colonizers of the vagina only (14). We cannot rule out, that our results had been different had we used a strain of a *Lactobacillus* spp. known to colonize the vagina and not *L. rhamnosus* (2,3,5). Of note, the strains used here had been isolated from human vaginal swap samples. Of note, in a randomized placebo-controlled study including 100 women, Larsson et al. showed that the vaginal installation of *Lactobacillus gasseri* EB01-DSM 14869 and *Lactobacillus rhamnosus* PB01-DSM 14870 (EcovagFlora™), lengthened the time to BV relapse significantly, why intra-vaginally administration may be beneficial, but is beyond the scope of our study (15). Nonetheless, our conclusion agrees with Birte J. Wolff et al. that on young, self-reported healthy women, the oral probiotic had no detectable effect on either the composition or the functional potential of the vaginal microbiota.

Future clinical studies would benefit from administering the probiotics during conditions of low bacterial presence, e.g. during/after antibiotic treatment, or on other occasions where

microbial vaginal imbalance would occur. We would also recommend a clinical trial with increased number of study participants, and the probiotic administered by the vaginal route to test whether these strains can thrive and colonize the vaginal niche.

Conclusion

Thus, the results indicate that the oral administration of the probiotics did not cause changes taxonomically or functionally in the vaginal microbiome. Although few subjects changed vaginal microbiome composition clusters, there were no significant taxonomical or functional changes associated to the treatment, suggesting an overall stable microbiota during the study timeframe. Thus, through shotgun sequencing we could not reliably confirm that the orally administered probiotic strains have any indirect or direct impact on the vaginal microbiome of healthy women.

Declarations and Acknowledgements

Authors' contribution: FBH was the primary investigator and NFM was the primary supervisor of the project. *Concept and design:* FBH, LSM, EB and NFM.

Handling of vaginal and rectal swabs: MKB. *Acquisition of data:* FBH, JBH and MKB. *Analysis or interpretation of data:* FBH, AP and JBH. *Drafting of the manuscript:* FBH. *Critical revision of the manuscript for important intellectual content:* MKB, NFM, LSM and EB. *Statistical analysis:* JBH and AP. *Administrative, technical, or material support:* JBH, MKB, LSM, EB. *Supervision:* NFM. All authors take responsibility for the integrity of the data and the accuracy analysis and interpretation of data and have approved the final version of the manuscript.

Consent to publish: Given prior to enrollment in the study.

Conflicts of interest: FBH and NFM are medical doctors employed by the public health care system in Denmark. FBH and NFM declare no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous three years, no other relationships or activities that could appear to have influenced the submitted work.

LSM and EB are employed by Deerland Probiotics & Enzymes A/S a commercial company producing and selling probiotics, including probiotics containing *Lactobacillus* spp. Deerland Probiotics & Enzymes A/S had no role in conducting the study, analysis or interpretation the data.

JBH and AP are employed by Clinical Microbiomics, a private company. Clinical Microbiomics have been involved in the study to ensure correct microbiological analyses. Clinical Microbiomics have had no influence on the purpose.

***Transparency declaration**: All authors affirm that this manuscript is conducted honest, accurate, and transparent.*

Availability of data and materials: Online supplementary contains all analyzed data. All the anonymous stored raw data will be available on reasonable request after all sub-analyzes are done from the corresponding author upon request.

Funding sources: Exclusively funded by Deerland Probiotics & Enzymes A/S. Deerland Probiotics & Enzymes A/S was the primary financial contributor for the project and covered all expenses including, vaginal swabs, compensation for the participants, the microbiological analyses and assessment of the generated data.

Role of funders: The funding agencies had no role in designing and conducting the study, analysis and interpretation the data, or the writing, review, and approval of the manuscript.

Additional contributions: We would like to thank Department of Clinical Microbiology, Rigshospitalet, Copenhagen[1] and the laboratory technicians.

Ethics statement: Research ethical approvals were given by the Regional Committee of Danish Data Protection Agency and the Regional Committee of Health Research Ethics for the Capitol Region of Denmark (No. H-17004182). Informed consent for participation and publication was obtained from all participants prior to enrollment.

Compensation to individuals in the study: Compensation for the participants was provided according to standard guidelines on compensation for volunteers in clinical studies in Denmark. The participants received a gift card for 250 DKK to Matas (a Danish health shop).

Table 1. Study participants overview

Participant number	Ethnicity	Status	Age (years)	3 vaginal swabs 7-10 days after last day of menstruation	2 rectal swabs performed with 1st. and 3rd. vaginal swabs
1	Caucasian	Completed	26	X	X
2	Caucasian	Completed	25	X	
4	Caucasian	Completed	23	X	
5	Caucasian	Completed	36	X	X
6	Caucasian	Completed	24	X	
8	Caucasian	Completed	24	X	X
9	Caucasian	Completed	34	X	
10	Caucasian	Completed	31	X	X
11	Caucasian	Completed	27	X	
14	Caucasian	Completed	32	X	X
15	Caucasian	Completed	37	X	X
16	Caucasian	Completed	34	X	
17	Caucasian	Completed	26	X	X
18	Arabic	Completed	35	X	X

19	Caucasian	Completed	45	X	X
20	Caucasian	Completed	24	X	
7	Asian	Lost in mail	33		
13	Caucasian	Lost in mail	36		
3	Caucasian	Dropped out	19		
12	Caucasian	Dropped out	22		
21	Caucasian	Dropped out	27		

Supplementary Table 1. All species detected in the vaginal samples.

Supplementary Table 2: Strain-level analysis of *L. gasseri* in during-treatment and post-treatment samples.

Supplementary Table 3: Primer sequences and amplified DNA fragments

Supplementary Table 4: Concentration of selected primers and probes used in individual reaction mixtures.

Supplementary Table 5: qPCR cycling programme for each qPCR reaction mixture. The qPCR amplification was performed on a MyGo Pro instrument (IT-IS Life Science Ltd.).

Supplementary Table 6: Volume of primers, probes, DNA templates for amplification and qPCR reagents (Ampliqueen) in each qPCR reaction mixture.

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Figure 1.

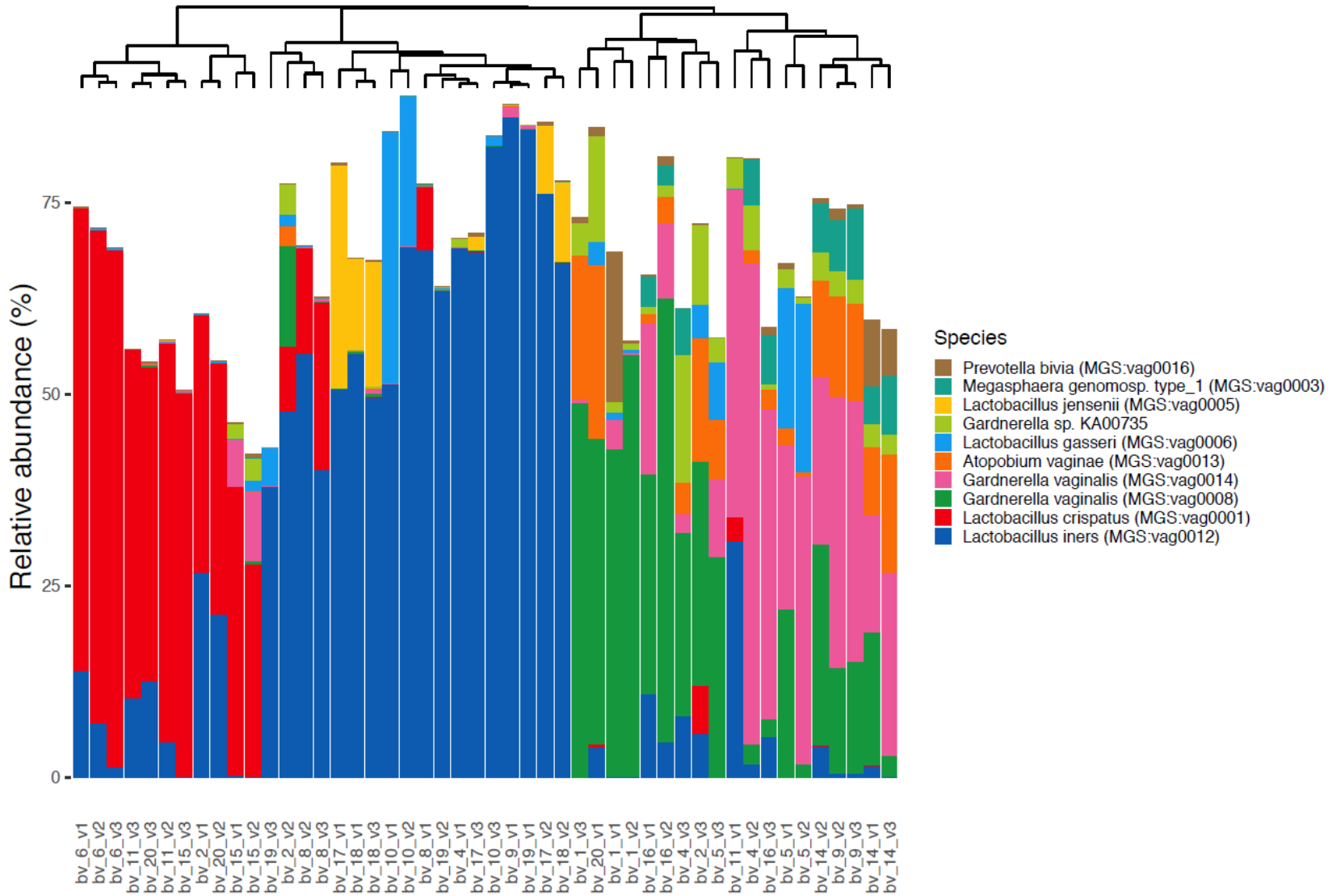

Figure 1. MGS abundance profiles of the top-10 most dominant MGSs in the study vaginal samples. The samples are clustered based on Bray-Curtis dissimilarity and a phylogenetic tree on top of the plot exhibits the different species clusters. Bars do not sum to 100% because we represent only the 10 most dominant species across samples, and because some reads mapped to non-MGS-annotated genes.

Figure 2.

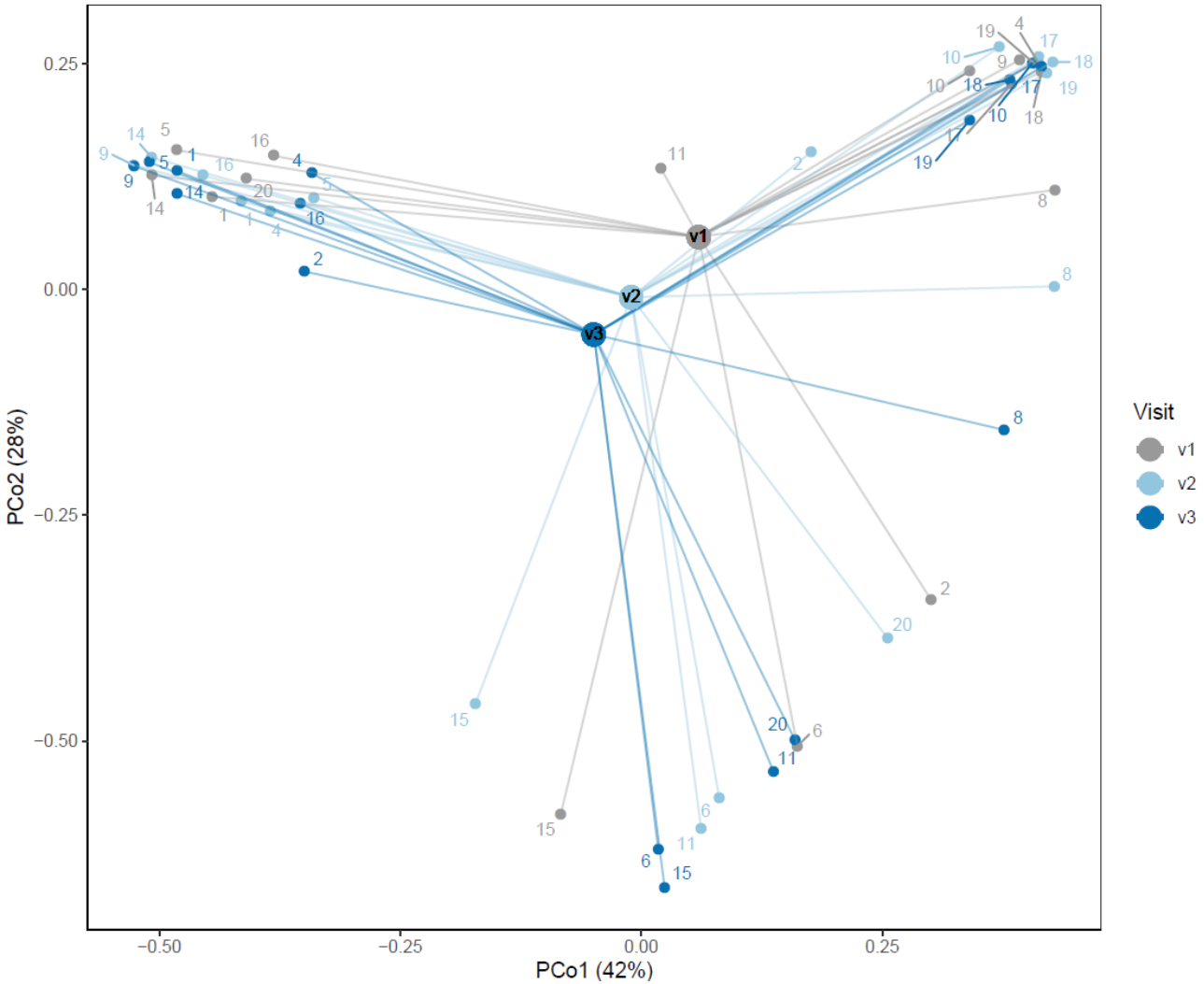

Figure 2. Vaginal samples. PCoA based on Bray-Curtis dissimilarities between vaginal samples calculated using the MGS abundances in vaginal samples. Numbers denote the subject, and the samples are color coded by visit. The median, of all the samples per visit, is expressed with a larger dot
(centroid).