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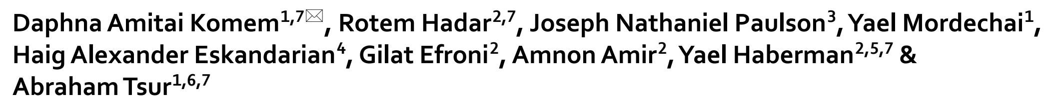
Lubricating gel influence on vaginal microbiome sampling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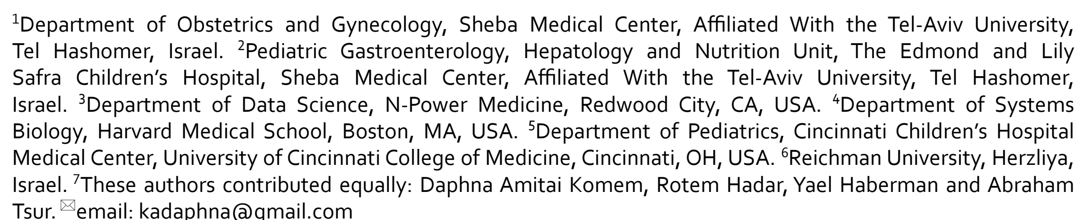
Gel lubrication is routinely used during gynecological examination to prevent or reduce pain, yet its impact on microbial composition during sampling remains unclear. This study aimed to investigate whether lubricating gel affects the microbial composition of vaginal samples. We included 31 pregnant women presenting during their third trimester to clinics or emergency room and collected 143 unique vaginal samples for 16S amplicon microbial analysis. Vaginal samples were obtained using sterile swabs under various conditions: without gel—immediately frozen (n = 30), with gel—immediately frozen, without gel—at room temperature (RT) for 5 h before freezing, with gel—at RT for 5 h before freezing, and additional sampling after 24 h without gel—immediate freezing. We found that sample collection with gel lubrication influenced specimen quality—half of the gel samples failing to meet processing limitation compared to those without gel. The effect of gel on testing quality dissipated after 24 h. However, when samples met post-sequencing filters, gel lubrication did not alter the microbial composition, individual taxa abundance or alpha and beta diversity. We recommend sampling either before gel exposure or 24 h after. These findings underscore the importance of considering sample collection methodologies in vaginal microbiome studies to ensure high-quality microbial data for accurate analysis.

Keywords 16S rRNA sequencing, Preterm birth, Bacterial vaginosis, Multiomics

The vaginal microbiome is a complex ecosystem characterized by low diversity and mainly by dominance of lactic-acid–producing bacteria. Its composition is subjected to changes due to physiological factors such as hormonal effects during reproductive life, as well as exogenous factors such as intravaginal or systemic treatments[1,2]. The vaginal microbial community has been implicated in a multitude of women's health conditions. Some studies have found that diverse, predominantly low *Lactobacillus* abundance is associated with an increased risk for preterm birth, while other studies found no link between microbial profiles and preterm birth[3–6]. Research has revealed that a non-dominant *L. crispatus* vaginal microbiota is associated with a higher risk of HPV infection and cervical dysplasia[7]. Lastly, a recent study established causality in a small group of women with recurrent bacterial vaginosis that improved after vaginal microbiome transplantations[8].

The vaginal microbiome is extracted from cervicovaginal fluid samples collected from the posterior fornix during a speculum examination and has both research and clinical implications. To prevent or reduce patient discomfort during vaginal examination, lubrication is often applied[9]. Previous studies have shown that gel lubrication significantly reduces pain without interfering with cervical cytology or GBS cultures[10–12]. However, there is no clear consensus on the sampling techniques for the vaginal microbiome, including the time lapse between sampling and freezing or the use of gel lubrication during examinations. These variables can potentially influence microbial composition and the quality of collected samples. Therefore, our study aimed to explore the effect of lubricating gel on vaginal microbiome samples.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Results

A prospective study of pregnant women

Thirty-one pregnant participants were recruited for the study and samples were collected during the third trimester at an average gestational age of 36 + 1 weeks. Mean participant age was 33.6 ± 3.1 years and BMI was 28.4 ± 5.7 (Table 1). In total, 152 vaginal specimens were collected, however due to technical considerations, nine samples were deemed unusable. Consequently, 143 specimens were available for analysis: 30 samples without gel and with immediate freezing, 30 with gel and immediate freezing, 28 without gel and at 5 h in RT before freezing, 27 with gel and at 5 h RT before freezing, and 28 with additional vaginal sampling after 24 h without gel and immediate freezing.

Gel exposure significantly reduced the percentage of samples reaching threshold

The number of reads per sample and group is depicted in Fig. 1A and Table S1. Samples acquired with gel lubrication and immediate freezing exhibited a pronounced reduction in the number of reads, 16 of 30 (53%) failed to meet the established threshold of 2000 reads per sample. This is compared to only three of 30 samples collected without gel and with immediate freezing (10%, Fig. 1B, Fisher's exact test $P < 0.0003$). Similarly, for samples that were left at room temperature for five hours, 14 samples of 27 with gel failed (52%) versus only two of 28 without gel (7%) (Fisher's exact test $P < 0.005$) suggesting that the presence of gel has a negative effect on the number of reads obtained. Interestingly, in samples obtained 24 h post-gel exposure, the fraction of the samples that passed our predefined threshold of 2000 reads per sample returned to levels similar to those without gel (3 of 28, 10.7%), suggesting that the negative effect of gel on the number of reads is transient.

Stability of microbial diversity and variation across collection methods

For samples that passed the threshold of 2000 reads per sample, alpha diversity was measured by the individual sample's Shannon diversity index at ASV (Fig. 2A), genus levels (Fig. 2B) and Faith's phylogenetic diversity (Fig. 2C). For each patient, we observed alpha diversity similarities of samples regardless of the method of

Subject ID	Age	BMI	Gravidity	Parity	Gestational age at sampling
P1	28	23.0	1	0	37 + 4
P2	31	38.1	2	1	38 + 4
P3	39	29.1	2	1	40 + 4
P4	31	25.9	1	0	34 + 7
P5	35	25.3	4	2	37 + 1
P6	29	30.1	3	2	35 + 9
P7	29	38.5	1	0	37 + 6
P8	35	29.6	7	3	33 + 7
P9	30	24.6	2	1	37 + 0
P10	36	25.7	6	4	37 + 4
P11	34	27.7	4	2	35 + 3
P12	33	20.7	2	1	31 + 0
P13	36	27.3	4	2	35 + 7
P14	34	20.8	1	0	34 + 6
P15	33	31.6	2	0	35 + 7
P16	30	25.8	3	0	37 + 7
P17	35	40.2	5	2	38 + 6
P18	31	28.9	4	3	37 + 9
P19	38	27.8	4	3	38 + 6
P20	39	31.6	3	2	38 + 1
P21	31	44.4	1	0	38 + 3
P22	33	31.6	2	1	38 + 3
P23	36	26.3	2	1	39 + 3
P24	31	30.1	2	1	38 + 6
P25	32	27.3	4	2	34 + 7
P26	34	19.6	4	2	30 + 1
P27	38	31.6	1	0	37 + 1
P28	31	23.7	6	5	29 + 0
P29	35	24.1	1	0	33 + 6
P30	38	25.1	5	3	32 + 4
P31	36	23.7	1	0	35 + 3

Table 1. Study Population.

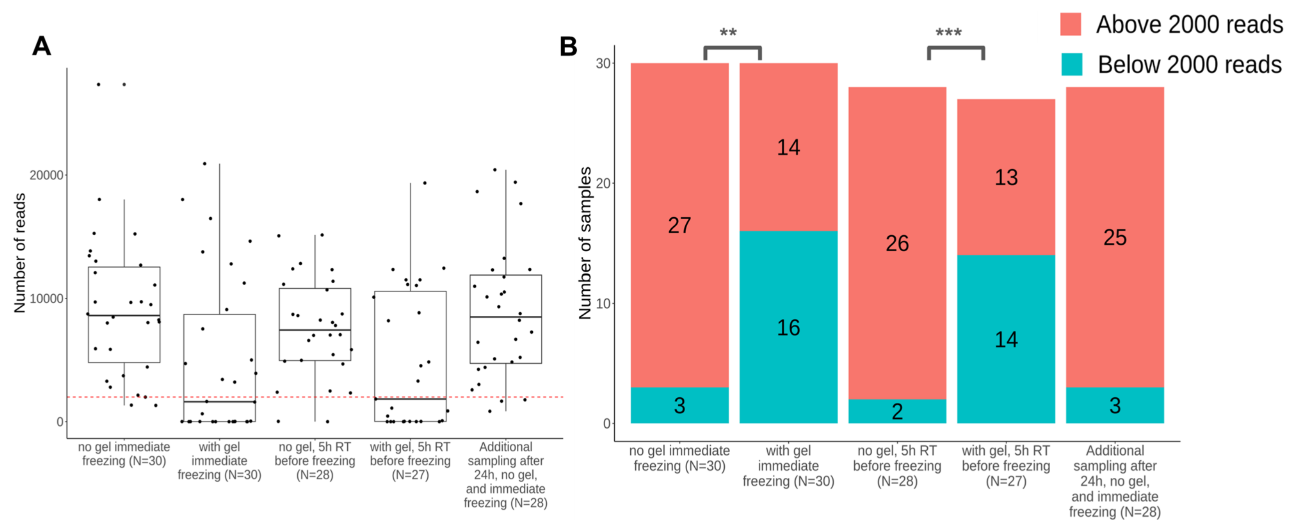

Figure 1. Samples obtained after gel application tended to have under 2000 reads, below our defined minimal threshold for analysis 143 unique vaginal samples were included from 31 women as indicated: without gel and with immediate freezing (n = 30), with gel and immediate freezing (n = 30), without gel and at 5 h RT before freezing (n = 28), with gel and at 5 h RT before freezing (n = 27), and with additional sampling after 24 h without gel and immediate freezing (n = 28). (**A**). Each point represents one sample, and the red dashed line represents the threshold selected (2000 reads/sample). (**B**). The columns show the counts of samples above (red) and under (blue) per group. Fisher test ** $P < 0.005$ *** $P < 0.0005$.

sample collection (i.e. with and without gel, immediate freezing or 5 h at room temperature, and 24 h after gel introduction). PERMANOVA analysis was used to identify significant grouping based on collection methodologies in the Bray–Curtis dissimilarity matrix. We found no significant dissimilarities between all samples when categorized according to collection method (Fig. 3). The PERMANOVA test comparing if the groups differed resulted in an R^2 of 0.0348 and a *p*-value of 0.729, indicating not enough evidence for a difference accounted for by sampling method.

Consistency of microbial composition across sampling methods

Microbial composition per subject can be observed in Fig. 4, demonstrating the presence of dominant bacteria in different subjects and the persistence of specific microbial taxa between different sampling methodologies. The heatmap displays the 25 most variable ASVs revealing a broad uniformity in the distribution of microbial abundance across different samples from the same patient. The majority of patient samples cluster according to participants in the dendrogram's hierarchical clustering, without any discernible pattern indicating a systematic influence of the collection methods. Notably, re-sampling 24 h post gel application exhibited no significant alteration in the overall microbial community structure of the dominant bacterial taxa.

Differential abundance to test if sampling consistently affected individual ASV levels was applied, however, we did not observe any statistically significant differences in the compared groups. Different comparisons included between samples with and without gel following immediate freezing, with and without gel following a 5-h delay in storage, between the length of time for storage stratified by gel use, and between patient samples without gel use and immediate storage and those collected 24 h following gel exposure.

Discussion

We observed that lubricating gel during the gynecological examination led to fewer sequencing reads per sample. Interestingly, gel lubrication did not modify the taxonomic diversity nor individual differential microbial abundance composition of samples following normalization. We did not observe any statistically significant differences in microbial abundance for individual ASVs between the collection methodologies. We hypothesize that the reduced number of reads in gel samples may be attributed to either a dilution effect of the gel or its inhibitory effect on one of the processing steps. However, it appears that gel does not inherently alter microbial composition per se.

Pivotal studies have pointed out methodological aspects potentially hampering vaginal microbiome investigation. Huang et al.[13] proposed that the varying findings across studies investigating the link between the vaginal microbiome and preterm birth could be due to the diverse racial composition of the study populations. Additionally, Graspeuntner identified biases introduced by differences in profiling methodologies, in particular the sensitivity of primers for the V4 hypervariable region of the 16S rRNA gene for important species inhabiting the lower urogenital tract (e.g. Bifidobacterium *bifidum*, Gardnerella *vaginalis* and Chlamydia *trachomatis*) when compared to V1–V3 primers[14]. Our study contributes an additional methodological consideration, emphasizing the transient effect of gel lubrication on sample quality.

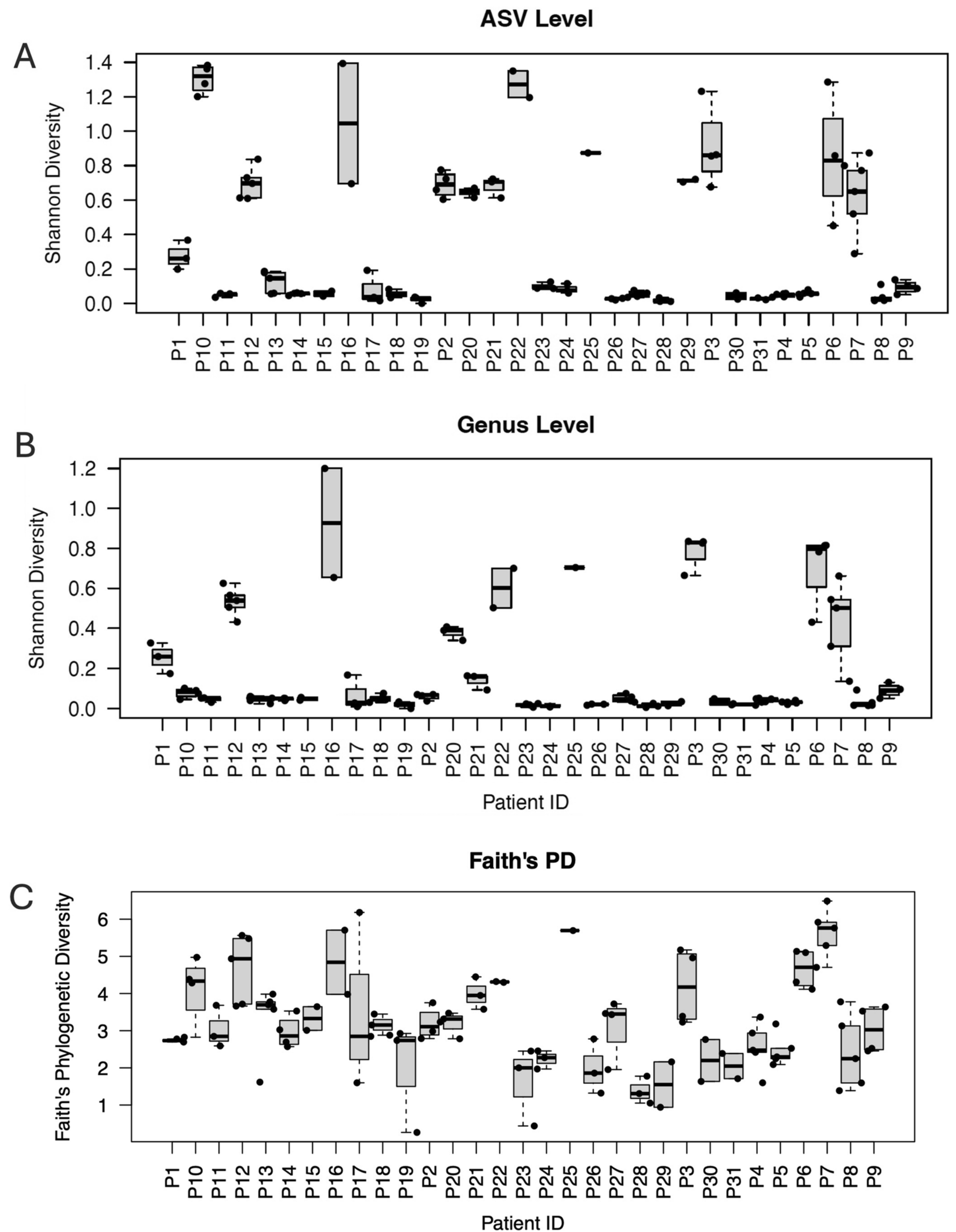

Figure 2. No difference in alpha diversity is observed for samples with and without gel sampling. Boxplots of an individual samples Shannon diversity index at the (**A**) ASV, (**B**) genus level and (**C**) Faith's phylogenetic diversity for each patient. All patient samples (above 2000 reads/sample) showed consistency in alpha diversity regardless of sampling method (i.e. with and without gel, immediate freezing or 5 h at room temperature, and 24 h after gel introduction). Boxes show median and interquartile range; whiskers extend from above and below the 1.5 interquartile range or maximal value above or below.

The absence of significant differences observed in sample storage conditions, whether stored immediately at − 80 °C or after a five-hour delay suggests that immediate freezing is not a requisite for preserving microbial integrity in vaginal samples. Furthermore, the lack of discernible alterations in microbial reads or composition 24 h post-gel exposure implies that the effect of gel on the quality of microbiome samples is transient. These results highlight the microbiomes' capacity to maintain stability even under temporal delays and exposure to external factors like gel application. However, inability to detect a significant effect of the sampling on the microbial populations does not indicate that such a difference does not exist, but rather that the effect is small enough

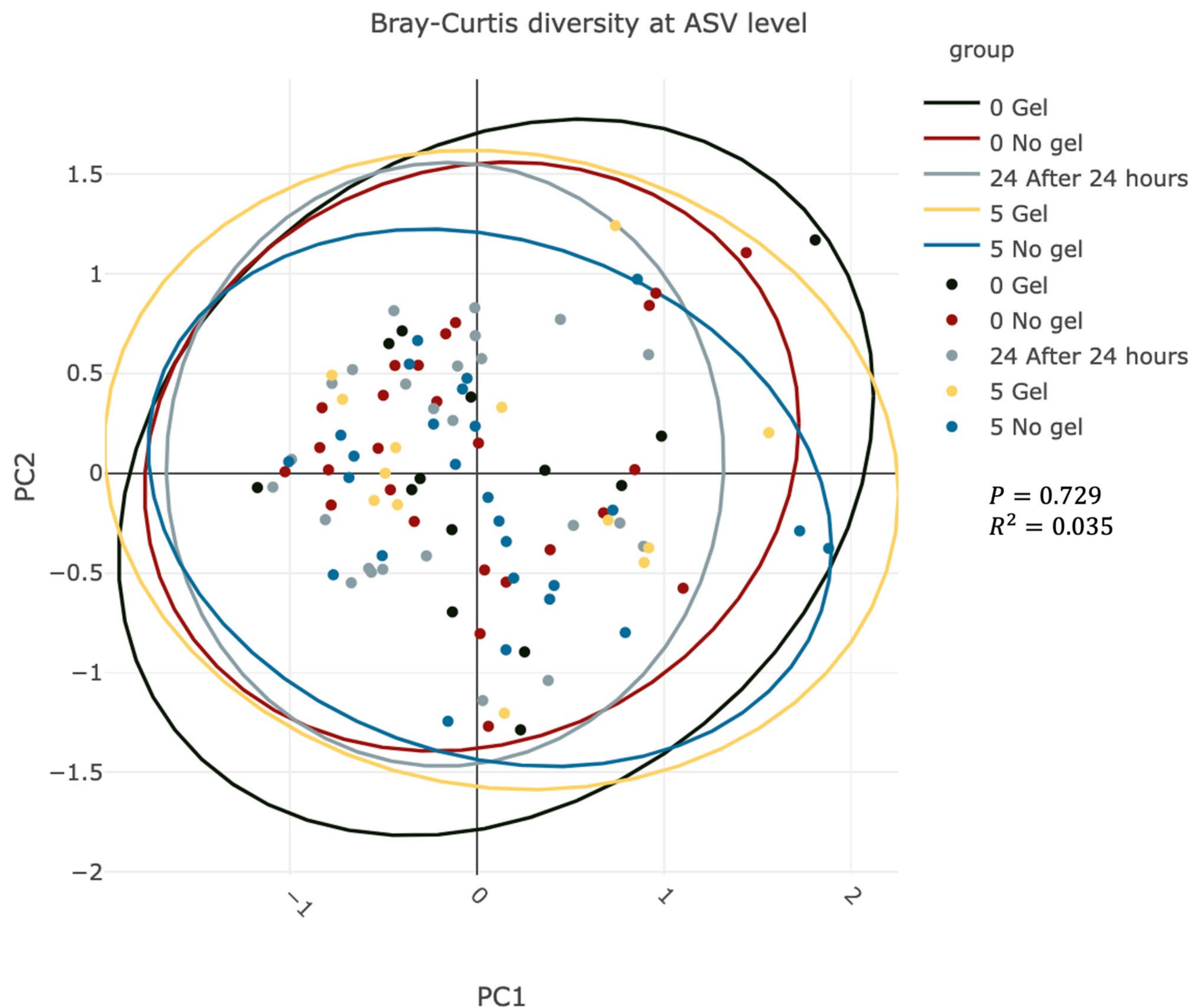

Figure 3. Bacterial composition is largely unaffected by sampling method. Principal Coordinate Analysis (PCoA) based on Bray–Curtis similarity matrix was calculated at the ASV level for 105 samples that passed the threshold of 2000/sample. Scatterplot. Each point represents a sample and colors represent the different collection methods (i.e. with and without gel, immediate freezing or 5 h at room temperature or 24 h after gel introduction). The first and second principal components represented 24.85% and 17.56% of the variability in the data. PERMANOVA (permutational multivariate analysis of covariance) ellipsoids with 95% confidence intervals are displayed for each of the different strata. The PERMANOVA test comparing if the groups differed resulted in an R^2 of 0.0348 and a *p*-value of 0.729.

to be undetectable in the number of samples used in our study. Hence, while we did not detect a significant effect of sampling, we recommend using a persistent mode of sampling for all samples in each study (i.e. sampling with or without gel throughout and with similar interval pre-freezing) or at least a random distribution of the sampling across the different sample groups studied.

Our study implies that the optimal vaginal microbiome sampling is either before gel application or at least 24 h afterward, to minimize the impact on microbial read count and sample quality. A key strength of our study was its prospective design, in which different sampling methods were obtained from the same women, serving as their own controls, thereby preventing different confounders, and allowing isolating the sampling procedure from affecting the results. However, this dataset was not powered to identify small differences in microbial composition.

Our findings reveal that sampling the vaginal microbiome post-gel lubrication may impact the results of microbiome processing, an effect that dissipates 24 h after gel exposure. Consequently, we advocate that for optimal microbiome research methodology, vaginal microbiome should be sampled either before gel lubrication exposure or at least 24 h afterward.

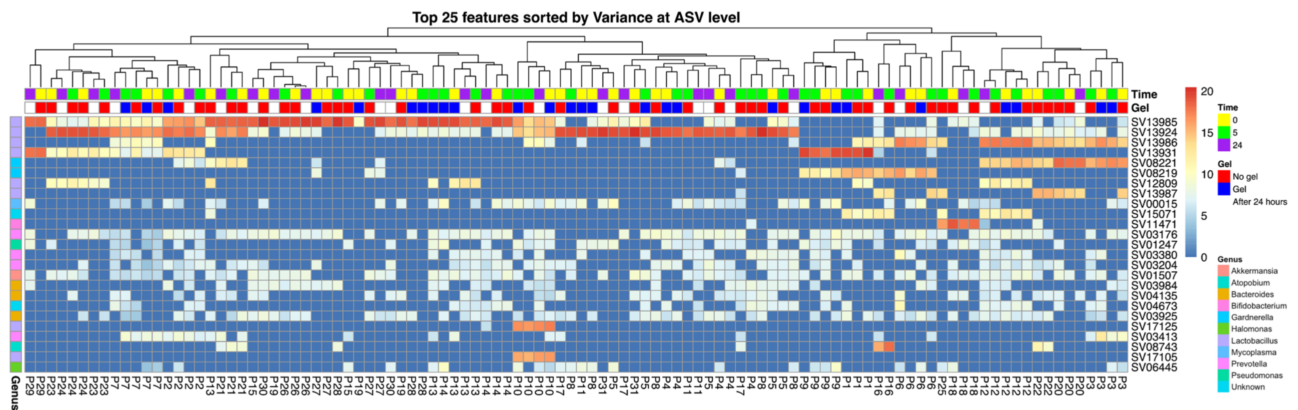

Figure 4. A consistent bacterial signal within each subject is detected with the different sampling. Heatmap of the 25 most variable ASVs. Columns represent patient samples and rows represent specific ASVs. Colors in the heatmap cells are the log2-normalized count abundances ranging from low (blue) to high (red). Legends on the right for the genus annotation for each specific ASV row. Sample metadata is annotated at the top for time and gel depicting the collection method. Patient samples were clustered using hierarchical clustering on the Euclidean distance matrix represented by the dendrogram above.

Materials and methods

Cohort and sampling

Pregnant women presenting to the obstetric clinics or emergency room of a university-affiliated tertiary referral center for prenatal care were invited to participate in the study. Inclusion criteria consisted of age 18–40 years, singleton gestation, and the ability to provide informed consent. Exclusion criteria consisted of current hormonal or steroidal treatment, coitus during the 72 h prior to sampling, known allergy to gel products, vaginal bleeding, rupture of membranes, and any of the following occurring within one week of sampling: fever, vaginal infection, antibiotic treatment, any intravaginal product use, vaginal examination, or transvaginal ultrasound. Specimens were collected from the posterior fornix using sterile sample collection swabs (Danyel Biotech. Rehovot, IL) during a speculum exam in the following order: two swabs without gel, two swabs immediately after gel lubrication (sterile, water-soluble contact medium for ultrasound transmission) was applied, and a fifth swab collected 24 h later. Two samples (one with and one without gel) were stored at − 80 °C within one hour of sampling, and another set was stored at room temperature for five hours after sampling prior to freezing. Samples collected 24 h later represent in vivo interactions between microbiome and gel lubrication. All participants provided written informed consent prior to completing a medical questionnaire and sample collection. The study was approved by the institutional review board at Sheba Medical Center (IRB-8413-21). In addition, all experiments were performed in accordance with relevant guidelines and regulations.

DNA extraction and 16S amplicon sequencing

DNA extraction and PCR amplification of the variable region 4 (V4) of the 16S rRNA gene using Illumina adapted universal primers 515 F/806 R was conducted using the direct PCR protocol [Extract-N-Amp Plant PCR kit (Sigma-Aldrich, Inc.)]. PCRs were conducted and amplicons were pooled in equimolar concentrations into a composite sample that was size selected (300–500 bp) using agarose gel to reduce non-specific products from host DNA. Sequencing was performed on the Illumina MiSeq platform with the addition of 15% PhiX, and generating paired-end reads of 175b in length in each direction.

Reads were processed in a data curation pipeline implemented in QIIME2 version 2019.4. Reads were demultiplexed according to sample-specific barcodes. Quality control was performed by truncating reads after three consecutive Phred scores lower than 20. Reads with ambiguous base calls or shorter than 150 bp after quality truncation, were discarded. Amplicon sequence variants (ASVs) detection was performed using Deblur and duplicate samples from different runs were joined, resulting in 143 samples, of which 105 passed the expression filtering threshold of 2000 reads/sample, and these had a median of 8773 reads/sample (IQR 5440–11,456). Reads were then truncated to 150 bp and taxonomic classification was performed using the 2022.10 Greengenes 2 (gg2) databases. All ASVs and taxonomies are indicated in Table S1.

Bray–Curtis similarity indices were calculated as a measure of between-sample beta diversity and Shannon diversity index was calculated as a measure of alpha diversity. Diversity metrics were calculated at both the ASV and genus level and outcomes remained the same. Cumulative sum scaling was used for normalization to account for technical artifacts in sequencing variability[15]. Normalized counts were used to perform multidimensional scaling (MDS) and calculate ANOVA and PERMANOVA statistics comparing the diversity between sampling techniques. Normalized counts were plotted in a heatmap using pheatmap[16].

Statistical analysis and data availability

Fisher's exact test and a linear fixed effects model including a covariate for patient identifier were used for the analysis of the number of reads and alpha diversity comparisons between treatment groups respectively.

PERMANOVA (Permutational Multivariate Analysis of Variance) was used to assess differences in microbial community composition between sample collection methodologies.

Differential abundance testing was performed using limma[17] in a paired model like above while accounting for individual normalization effects. Multiple testing was accounted for using Benjamini–Hochberg FDR correction and results were considered significant at an alpha of 0.05.

All statistical analyses were performed using R version 4.3.

Data availability

Sequencing results are available at SRA BioProject ID: PRJNA1050665. All other data are available in the article and its Supplementary files or from the corresponding author upon request. All data generated and analyzed during this study are included in this published article.

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Author contributions

D.A.K., A.T., H. A. E., A. A., and Y.H. designed the study; D.A.K. and Y.M., and G.E. collected and processed samples; R. H., A. A., Y. H., and J. N.P., analyzed data; A. T., and Y. H. supervised; D.A.K., J. N.P., and A.T. wrote the manuscript. All authors approved the final version of the manuscript.

Competing interests

The authors declare no competing interests. AT reports the following financial disclosures: primary medical inventor of a mechanical device for the prevention of preterm birth and holds minority shares in PregnanTech, a company commercializing the invention under the name "The Lioness"; cofounder and chief medical officer of Shela Ltd. developing Maternal–Fetal Precision Medicine; Medical advisor and holds minority shares in Signalife Ltd., developing direct measurement of fetal pH in the labor and delivery ward; serves as consultant of Pollie Ltd., developing digital interventions for polycystic ovary syndrome. Medical advisor of Ciconia Medical developing a medical device providing digital vaginal examination during labor.

Additional information

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