Reproductive Tract Microbial Transitions in First-Pregnancy Heifers: 16S rRNA Metagenetic Profiling of Vaginal Microbiota in Late Gestation and Vaginal and Uterine Microbiota in Early Postpartum

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Abstract: Studies in recent years indicate that microbial communities residing in the reproductive tract are crucial for shaping mammals' health and reproductive outcomes. Following parturition, female mammals often exhibit uterine bacterial contamination due to the open cervix allowing microbes from the vaginal microbiota or the environment to enter. This contamination may be associated with postpartum uterine inflammatory diseases, which are more prevalent among primiparous than multiparous individuals, as reported in dairy cows and women. However, investigations into the spatio-temporal microbial transitions in the reproductive tract of primigravid heifers and women remain limited. Our objective was to describe and compare the community compositions in the vagina at late gestation and in the vagina and uterus at early postpartum, in first-pregnancy heifers. Three swab samples were collected from 33 first-pregnancy Holstein-Friesian heifers: vaginal samples at gestation day 258±4, and vaginal and uterine samples at postpartum day 7±2. Each sample underwent 16S rRNA V4 region metagenetic analysis via Illumina MiSeq, with bioinformatics following Mothur MiSeq SOP. Results revealed distinct shifts in microbial composition between the prepartum vagina, postpartum vagina, and postpartum uterus. The late gestation vaginal and early postpartum uterine microbial profiles were the most distinct, with postpartum vaginal samples serving as intermediaries. The postpartum uterus exhibited lower richness and diversity compared to the prepartum and postpartum vagina. Specific microbial markers were associated with anatomical and temporal transitions, indicating a dynamic microbial landscape associated with parturition. The observed differences in phyla and genera across reproductive sites highlight the complexity of these ecosystems, expending avenues for future research in reproductive biology, microbial ecology, and reproductive tract diseases.

Keywords: reproductive tract metagenomics; 16S rRNA next-generation sequencing; uterus; vagina; pregnancy; primigravida; cow

1. Introduction

The intricate interplay between microbial communities and mammals' reproductive physiologic and pathologic dynamics has garnered significant attention in recent years [2-4]. Notably, the transition from late pregnancy to the early postpartum period, characterized by the opening of the cervix, presents a scenario where substantial uterine bacterial contamination commonly occurs, either from the vaginal microbiota or perhaps from the surrounding environment [5, 6]. This phenomenon is particularly pertinent for primigravid females, as this is their first-ever parturition. In dairy cows, postpartum uterine contamination has been documented in nearly 100% of animals [7-9], and the same was suggested in humans and other mammals [2, 10-12]. Moreover, in dairy cows and women, postpartum uterine inflammatory diseases are more prevalent among primiparous than multiparous females [13-15]. In dairy cows, traditional culture-dependent microbiological analyses have identified several microbial species in the postpartum uteri, encompassing *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, *Prevotella melaninogenica*, *Bacteroides* spp., *Pseudomonas* spp., *Streptococcus* spp., and *Staphylococcus* spp., which some have been associated with metritis [7, 8, 16-18]. However, recent years have witnessed advancements in culture-independent investigations of bacterial communities within postpartum bovine uteri, mainly next-generation sequencing (NGS) of 16S-rDNA amplicon libraries [3, 8, 9, 19]. These approaches have yielded findings that differ from culture-based studies and have enabled the identification of bacteria that are challenging to cultivate through traditional methods. Furthermore, it demonstrated that Koch's postulates are not fulfilled in postpartum uterine diseases [20].

Current research has primarily examined postpartum uterine microbial contamination in dairy cows, particularly concerning metritis. Nevertheless, there is a significant gap in our understanding regarding the transition of microbial communities from late pregnancy to early postpartum, especially in the context of first-pregnancy heifers. This is particularly noteworthy, not just because of the high susceptibility of primiparous cows to uterine inflammatory diseases, but since the first pregnancy presents a distinctive scenario, offering an unparalleled opportunity to investigate the intricate dynamics of reproductive tract physiology and pathology, along with the environment, on what is probably the first most dramatic contamination of the female reproductive tract. Accordingly, the objective of this study was to describe and compare the community compositions in the vagina at late gestation and in the vagina and uterus at early postpartum, in first-pregnancy heifers using r16S amplicon NGS data.

2. Results

A total of 99 samples were obtained from 33 first-pregnancy pregnant heifers. Heifers included in the study were those with singleton pregnancies, normal calving, and no history of pregnancy-related illness during gestation or antibiotic treatments within six weeks before sampling. Moreover, heifers were excluded if they had retained fetal membranes or signs of postpartum septic metritis, as detailed in section 4.1. Three swab samples were collected from each animal; one from the vagina ('Vag-pre') at a median gestation day 258 (Range 254-262d), and two postpartum samples, from the vagina ('Vag-post') and the uterus ('Utr-post') at 8d (Median) after calving (Range: 5-9d). The DNA from each sample was used for metagenetic analysis of the V4 hypervariable region of

the 16s rRNA gene via the Illumina MiSeq platform [21-23]. Bioinformatic analysis was performed predominantly according to Mothur MiSeq SOP [1]. In this analysis, a total of 3,887,476 sequences were initially identified, of which, 3,772,145 sequences (97%) remained after screening based on length criteria. Following uniquefication, 2,214,729 unique sequences were identified and aligned to the customized SILVA reference database targeting the V4 region of the 16S rRNA gene. Then, the additional screening step resulted in a total of 1,966,847 unique sequences (total number of sequences = 3,175,973), which were filtered to remove overhangs at both ends, and underwent an additional uniquefication step, which in turn, resulted in 1,966,162 unique sequences. Following denoising by pre-clustering and chimeras removal, 1,463,757 unique sequences (total number of sequences = 3,090,757) were selected to be classified taxonomically. After identifying and removing undesirable non-bacterial sequences, 1,404,486 high-quality unique sequences remained (total number of sequences = 2,985,052), with an average read length of 291 bp.

The high-quality unique sequences were assigned to 1,255 genus-level OTUs. All OTUs were taxonomically assigned to the bacteria domain and were from 30 phyla, 88 classes, 193 orders, 413 families, and 1255 genera. At the per-sample level, the vast majority of samples had >10,000 sequences assigned to OTUs; however, 19 samples had a low sequence count (≤210). Examining the rarefaction curves (Figure 1) demonstrated that these samples were unrepresentative and excluded from further analyses. Samples that remained in the downstream analyses had a high number of sequences (mean±SEM: 37,302±1760, Median: 41,244, Range: 10,240-78,602), and their rarefaction curves reached a plateau, indicating an adequate sequencing depth. The number of sequences were significantly higher among samples in the Vag-pre and Vag-post groups, as compared to the Utr-post group (41,080±2,540, 41,080±3,397, 27,722±2,578 sequences, respectively; P=0.0007, Kruskal-Wallis One-Way AOV test). Wherever relevant, downstream analyses were performed with a subsampling of 10,000 sequences from each sample.

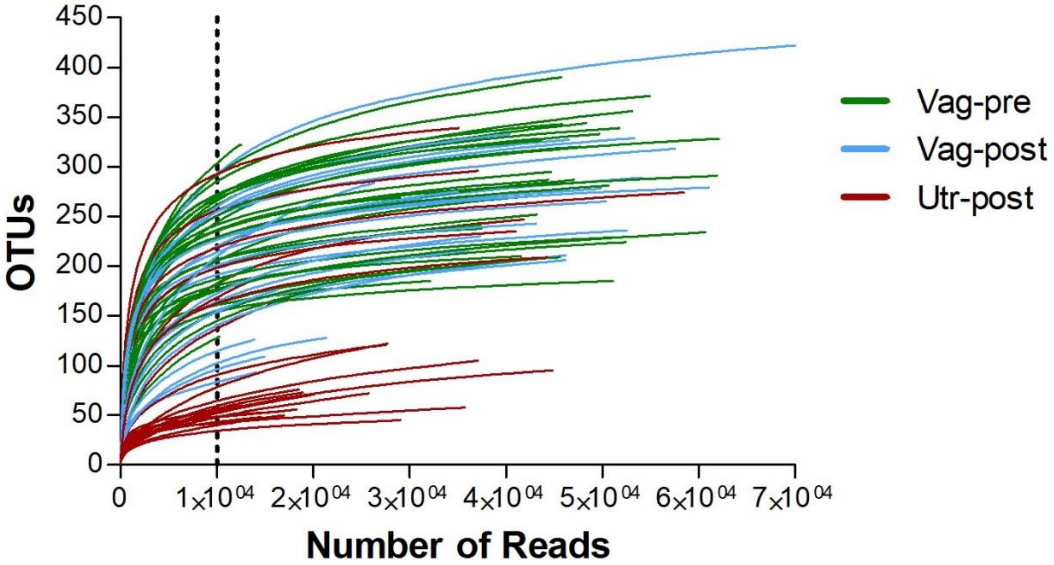


Figure 1. Rarefaction curves of 16S sequencing samples. Curves show the number of identified OTUs (Y-axis) as a function of the number of reads (X-axis). Samples from the Vag-pre, Vag-post, and Utr-post groups are represented in green, light blue, and red, respectively. Only samples with>10,000 reads (marked by the vertical dotted line) were included in further analyses.

The bacterial community compositions according to phylum in individual samples are presented in Figure 2. The mean bacterial community compositions according to phylum in the Vag-pre, Vag-post, and Utr-post groups are shown in Figure 3. Regardless of the group, the five most dominant phyla by mean relative abundance were *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria*, accounting for approximately 90% of the taxonomically assigned community components. As listed in Table 1, a comparison of mean bacterial community composition according to phylum revealed significant differences, mainly between the Vag-pre and Utr-post groups, particularly in the phyla *Proteobacteria*, which was lower, and *Bacteroidetes*, *Fusobacteria*, *Tenericutes*, which were higher in the Utr-post group. The Vag-post group had relative aboundances values that were commonly in between the values of the Vag-pre and Utr-post groups.

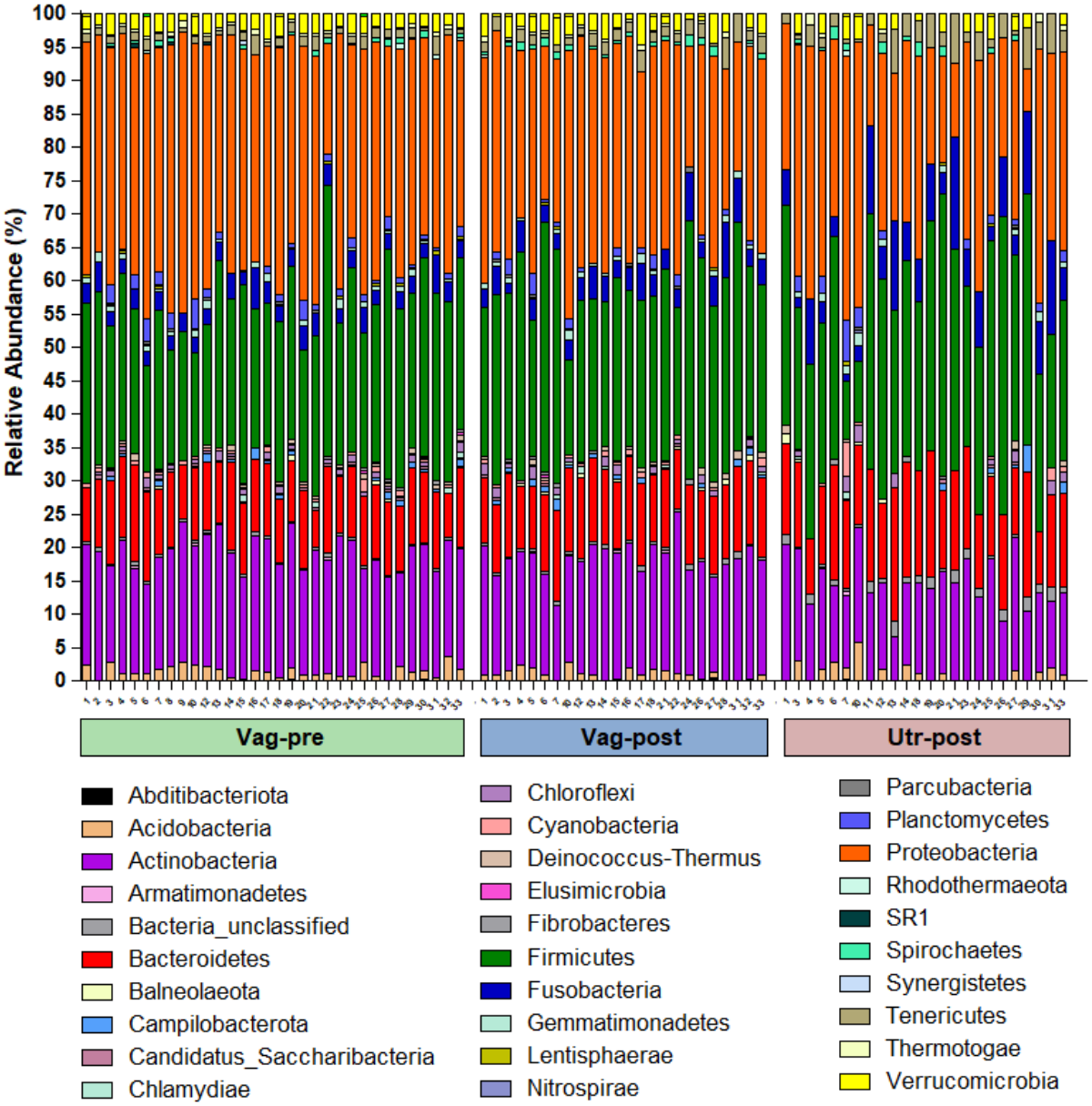


Figure 2. Individual stacked bar plots of bacterial community composition according to phylum. Each bar represents one sample, and the cow identity number is noted below the X-axis.

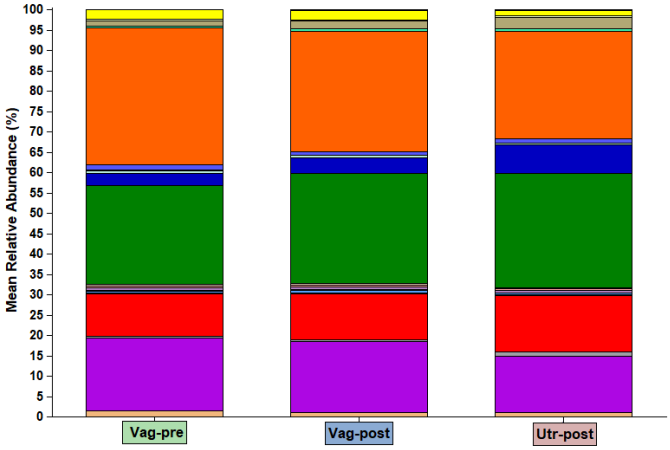
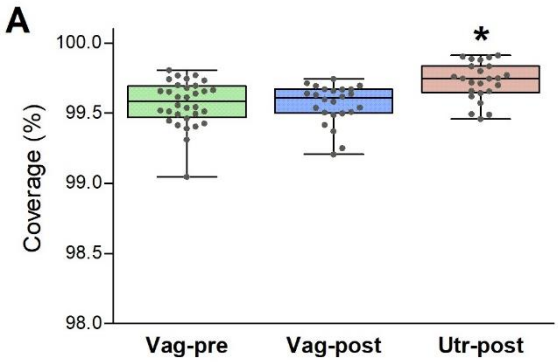


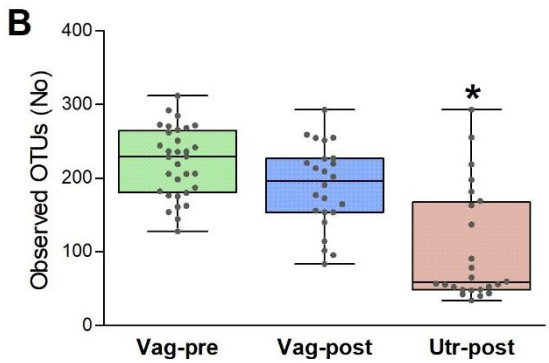
Figure 3. Group stacked bar plots of mean bacterial community composition according to phylum in the Vag-pre, Vag-post, and Utr-post groups. The color of each phylum is presented in Figure 2. The mean relative abundance values and the statistical comparisons among the groups are detailed in Table 1.

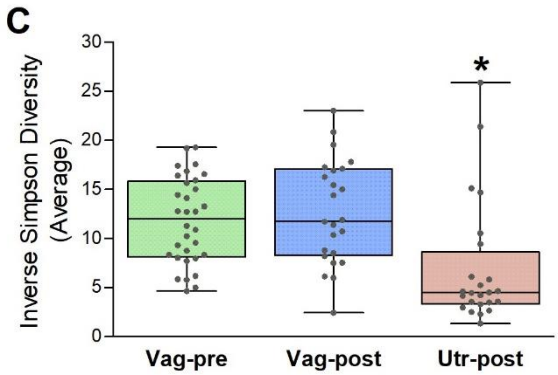
Table 1. Mean relative abundance of different phyla among the Vag-pre, Vag-post, and Utr-post groups. Data is presented as mean±SEM; Conditional formatting was applied to mean values to visually represent the data and highlight key differences.

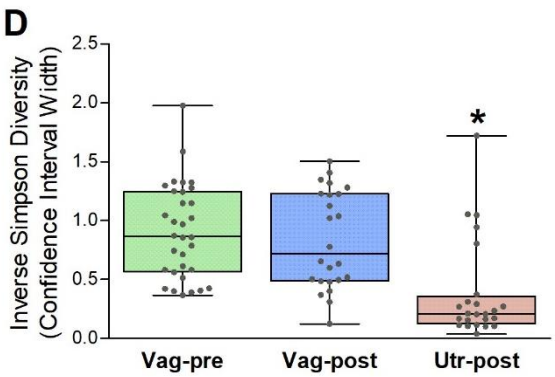
Phylum	Vag-pre	Vag-post	Utr-post	P value
Proteobacteria	33.66 ± 0.88[a]	29.57 ± 1.10[b]	26.46 ± 1.87[b]	0.0007
Firmicutes	24.25 ± 0.93	26.90 ± 1.22	28.15 ± 1.80	0.0560
Actinobacteria	17.86 ± 0.41	17.46 ± 0.51	13.70 ± 0.71	0.9373
Bacteroidetes	10.57 ± 0.34[a]	11.36 ± 0.27[a]	13.85 ± 0.66[b]	<0.0001
Fusobacteria	2.92 ± 0.17[a]	3.83 ± 0.33[ab]	6.89 ± 0.91[b]	<0.0001
Verrucomicrobia	2.28 ± 0.12	2.33 ± 0.26	1.49 ± 0.28	0.0969
Acidobacteria	1.43 ± 0.16	0.99 ± 0.16	1.13 ± 0.29	0.1318
Tenericutes	1.21 ± 0.11[a]	1.80 ± 0.21[b]	2.96 ± 0.43[b]	0.0002
Planctomycetes	1.18 ± 0.19	0.75 ± 0.18	0.90 ± 0.30	0.1083
Chloroflexi	0.60 ± 0.08	0.69 ± 0.13	0.58 ± 0.19	0.3682
Gemmatimonadetes	0.59 ± 0.08	0.60 ± 0.08	0.43 ± 0.12	0.2451
Thermotogae	0.43 ± 0.04[a]	0.36 ± 0.05[a]	0.22 ± 0.09[b]	0.0006
Cyanobacteria/Chloroplast	0.43 ± 0.06[a]	0.38 ± 0.07[ab]	0.41 ± 0.22[b]	0.0145
Campilobacterota	0.41 ± 0.08	0.44 ± 0.10	0.34 ± 0.19	0.0363
Bacteria_unclassified	0.39 ± 0.02[a]	0.47 ± 0.04[a]	1.15 ± 0.13[b]	<0.0001
Deinococcus-Thermus	0.34 ± 0.06[a]	0.41 ± 0.08[a]	0.16 ± 0.08[b]	0.0021
Spirochaetes	0.33 ± 0.07	0.66 ± 0.09	0.49 ± 0.14	0.0401
Candidatus_Saccharibacteria	0.19 ± 0.03[a]	0.11 ± 0.04[ab]	0.07 ± 0.03[b]	0.0429
Lentisphaerae	0.16 ± 0.03	0.06 ± 0.03	0.08 ± 0.04	0.0297
Chlamydiae	0.12 ± 0.05	0.13 ± 0.05	0.05 ± 0.04	0.3195
Fibrobacteres	0.11 ± 0.03	0.12 ± 0.05	0.10 ± 0.05	0.5407
Balneolaeota	0.10 ± 0.04	0.20 ± 0.06	0.14 ± 0.07	0.2824
Parcubacteria	0.08 ± 0.03	0.14 ± 0.04	0.04 ± 0.03	0.1289
Rhodothermaeota	0.08 ± 0.04	0.04 ± 0.03	0.04 ± 0.04	0.5798
SR1	0.06 ± 0.03	0.00 ± 0.00	0.01 ± 0.01	0.0648
Armatimonadetes	0.06 ± 0.03	0.06 ± 0.03	0.04 ± 0.03	0.7827
Elusimicrobia	0.04 ± 0.02	0.02 ± 0.02	0.00 ± 0.00	0.1508
Synergistetes	0.03 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.2210
Abditibacteriota	0.02 ± 0.01	0.02 ± 0.02	0.01 ± 0.01	0.9373
Nitrospirae	0.01 ± 0.01	0.00 ± 0.00	0.02 ± 0.02	0.6310

The sample coverage, the number of observed OTUs, and the Inverse Simpson diversity estimate parameters (average, 95%CI width, and Std) were evaluated for each sample; the analysis was standardized by subsampling 10,000 sequences from each sample, 1000 times, and the results were compared among the Vag-pre, Vag-post, and Utr-post groups. As illustrated in Figure 4, the sample coverages were all above 99%, indicating that all samples are highly representative. The mean sample coverage was slightly, but significantly higher in the Utr-post group (99.57±0.03%, 99.56±0.03%, 99.73±0.03%; P=0.0002). The mean number of defined OTUs did not differ between the Vag-pre and Vag-post groups; however, it was significantly lower in the Utr-post group (221.7±8.4, 189.1±11.6, 103.7±15.8 OTUs, P<0.0001). Furthermore, the Inverse Simpson diversity estimate parameters (average, 95%CI width, and Std), also known as 1/D, did not differ between the Vag-pre and Vag-post groups but were significantly lower in the Utr-post group (1/D average: 11.8±0.8, 12.7±1.1, 6.9±1.3, P<0.0001; 1/D 95% width: 0.91±0.07, 0.84±0.08, 0.38±0.08, P<0.0001; 1/D Std: 0.19±0.02, 0.18±0.02, 0.08±0.02, P<0.0001). Overall, these results indicated that the postpartum uterus had lower bacterial richness and diversity compared to the vagina before and after parturition.









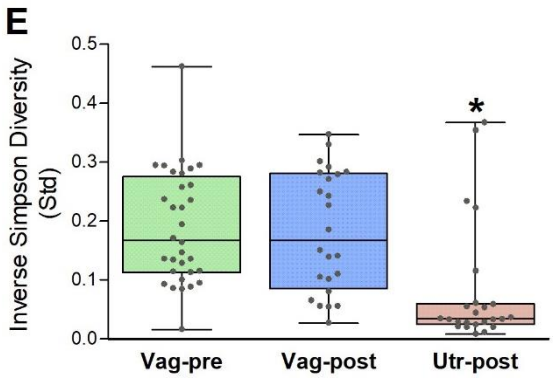


Figure 4. Alpah diversity metrics. The sample coverage, the number of observed OTUs, and the Inverse Simpson diversity estimate parameters (average, 95%CI width, and Std) were evaluated for each sample [1]; the analysis was standardized by subsampling 10,000 sequences from each sample, 1000 times, and the results were compared among the Vag-pre, Vag-post, and Utr-post group. Box and whiskers plots are presented with the overlap of scatter plots. The median (central white line) is shown in the middle of each box, with the 25% and 75% percentile ranges (box depth) and the maximum and minimum (whiskers); each dot represents a value of a single sample. Kruskal–Wallis one-way AOV, followed by Dunn's all-pairwise comparisons. * P ≤ 0.0002.

The non-metric multidimensional scaling (NMDS) ordination [24] of the bacterial communities from the Vag-pre, Vag-post, and Utr-post samples is shown in Figure 5 (3-dimensions, Stress=0.197, R2=0.77). To explore the observed spatial separation of community structures, the AMOVA test was applied and indicated that the centers of the clouds representing each group significantly differ from each other (P<0.001). The HOMOVA test analysis revealed that the bacterial community variations among samples in the Vag-pre and Vag-post groups did not differ (P=0.099), but it was significantly higher in the Utr-post group (SSwithin/(Ni-1) values = 0.170, 0.208, 0.325, respectively, P<0.001; Bonferroni pairwise error rate = 0.0167).

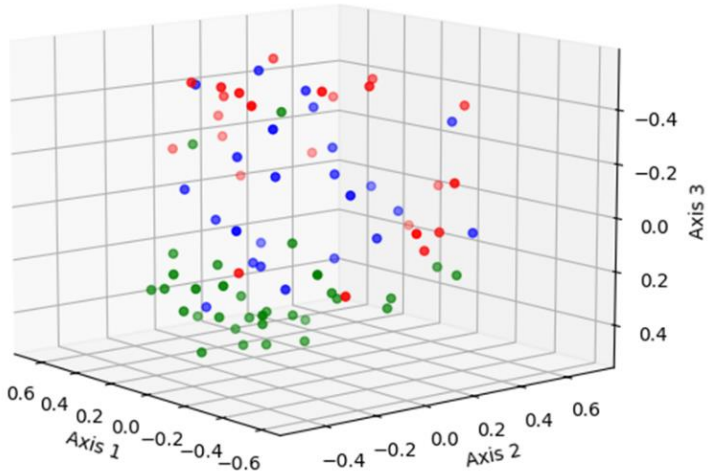


Figure 5. Non-metric Multidimensional Scaling (NMDS) ordination plot of the bacterial communities composition from the Vag-pre (green), Vag-post (blue), and Utr-post (red) samples (3-dimensions, Stress=0.197, R2=0.77).

The comparative analysis among the Vag-pre, Vag-post, and Utr-post groups, utilizing non-parametric LEfSe analysis, uncovered distinct microbial profiles associated with each anatomical niche. In the comparison between Vag-pre and Vag-post, 65 discriminative features were identified, as listed in Table 2. Notable genera with the highest logMaxMean values (>4) and LDA scores (>3.5) in the Vag-pre group included *Trinickia, Micrococcaceae_unclassified, Intrasporangiaceae_unclassified, Staphylococcus,* and *Betaproteobacteria_unclassified*, among others. On the other hand, Vag-post exhibited higher logMaxMean values for *Fusobacterium*, *Leptotrichiaceae_unclassified*, *Histophilus*, *Mycoplasmopsis*, *Parvimonas*, *Bacteroidales_unclassified*, and *Fusobacteriaceae_unclassified*, among others. When comparing Vag-pre with Utr-post, 113 discriminative features were uncovered, as listed in Table 3. Significant top genera in the Vag-pre group included *Trinickia*, *Micrococcaceae_unclassified*, *Intrasporangiaceae_unclassified*, *Ruminococcaceae_unclassified*, *Glutamicibacter*, *Micrococcales_unclassified*, and *Streptococcus*, among others; while Utr-post showed higher logMaxMean values and LDA scores for *Gemella*, *Leptotrichiaceae_unclassified*, *Trueperella*, and *Streptobacillus*, among others. Lastly, in the comparison between the postpartum vagina and uterus, 71 discriminative features were identified, as listed in Table 4. Genera such as *Trinickia*, *Leptotrichiaceae_unclassified, Micrococcaceae_unclassified, Ruminococcaceae_unclassified, Fusobacteriaceae_unclassified* and *Clostridiales_unclassified* had the highest logMaxMean values and LDA scores in the Vag-post group, while *Trueperella*, *Streptococcus*, and *Peptoniphilus* were more prominent in the Utr-post group. The distinctive genera identified in each of these comparisons provide insights into the specific microbial dynamics associated with these anatomical niches.

Table 2. Discriminative genera obtained from LEfSe comparison between Vag-pre and Vag-post samples. The logMaxMean represents the logarithm value of the maximum mean abundance of the OTU across the specified group (indicated as a class). LDA, Linear Discriminant Analysis score. Conditional formatting was applied to logMaxMean and LDA values to visually represent the data.

Phylum	logMaxMean	Class	LDA	P value
Trinickia	5.28	Vag-pre	4.86	<0.0001
Micrococcaceae_unclassified	4.87	Vag-pre	4.18	0.0071
Intrasporangiaceae_unclassified	4.42	Vag-pre	3.75	0.0019
Staphylococcus	4.00	Vag-pre	3.65	0.0009
Betaproteobacteria_unclassified	4.06	Vag-pre	3.58	<0.0001
Paraburkholderia	3.89	Vag-pre	3.48	0.0002
Burkholderiales_unclassified	3.85	Vag-pre	3.44	0.0001
Kocuria	4.02	Vag-pre	3.40	0.0040
Micrococcales_unclassified	4.14	Vag-pre	3.38	0.0030
Uruburuella	3.65	Vag-pre	3.28	0.0006
Burkholderiaceae_unclassified	3.62	Vag-pre	3.24	0.0001
Lactobacillales_unclassified	3.73	Vag-pre	3.23	0.0001
Chitinophagaceae_unclassified	3.86	Vag-pre	3.20	0.0347
Pseudomonadaceae_unclassified	3.63	Vag-pre	3.16	0.0015
Proteobacteria_unclassified	3.64	Vag-pre	3.12	0.0003
Enterobacteriaceae_unclassified	3.43	Vag-pre	3.06	0.0002
Rhodanobacter	3.49	Vag-pre	3.03	0.0005
Corynebacterium	3.56	Vag-pre	3.02	0.0012
Rhizobiales_unclassified	3.73	Vag-pre	3.00	0.0432
Vibrionaceae_unclassified	2.53	Vag-pre	2.94	0.0168
Tersicoccus	3.56	Vag-pre	2.92	0.0079
Oceanotoga	3.27	Vag-pre	2.91	0.0039
Enterococcus	3.18	Vag-pre	2.89	0.0035
Mycobacteriales_unclassified	3.24	Vag-pre	2.89	0.0002
Gammaproteobacteria_unclassified	3.53	Vag-pre	2.87	0.0072
Micrococcus	3.28	Vag-pre	2.87	0.0403
Boudabousia	2.87	Vag-pre	2.87	0.0165
Burkholderia	2.95	Vag-pre	2.83	0.0001
Staphylococcaceae_unclassified	3.27	Vag-pre	2.82	0.0110
Bradyrhizobiaceae_unclassified	3.49	Vag-pre	2.81	0.0283
Undibacterium	2.67	Vag-pre	2.80	0.0004
Rhodanobacteraceae_unclassified	3.18	Vag-pre	2.79	0.0009
Enterobacterales_unclassified	2.71	Vag-pre	2.79	0.0051
Paracoccus	2.61	Vag-pre	2.78	0.0082
Neisseriaceae_unclassified	3.31	Vag-pre	2.78	0.0156
Enterococcaceae_unclassified	3.07	Vag-pre	2.77	0.0090
Sphingomonadaceae_unclassified	3.23	Vag-pre	2.77	0.0047
Bacilli_unclassified	3.18	Vag-pre	2.77	0.0089
Salinivibrio	2.50	Vag-pre	2.75	0.0047
Carnobacteriaceae_unclassified	2.57	Vag-pre	2.71	0.0015
Lawsonella	2.79	Vag-pre	2.71	0.0034

Halomonas	2.57	Vag-pre	2.69	0.0480
Pseudomonas	2.93	Vag-pre	2.66	0.0150
Chryseobacterium	2.50	Vag-pre	2.63	0.0245
Devosiaceae_unclassified	2.95	Vag-pre	2.60	0.0314
Actinobacteria_unclassified	3.03	Vag-pre	2.56	0.0307
Cyanobacteria	2.74	Vag-pre	2.55	0.0292
Xanthomonadales_unclassified	2.91	Vag-pre	2.55	0.0020
Oxalobacteraceae_unclassified	2.65	Vag-pre	2.52	0.0354
Rhizomicrobium	3.36	Vag-pre	2.49	0.0372
Lactococcus	2.72	Vag-pre	2.43	0.0343
Sphingobacteriaceae_unclassified	2.51	Vag-pre	2.43	0.0488
Phyllobacteriaceae_unclassified	2.62	Vag-pre	2.36	0.0293
Fusobacterium	5.10	Vag-post	4.53	0.0003
Leptotrichiaceae_unclassified	4.67	Vag-post	4.38	0.0123
Histophilus	4.56	Vag-post	4.23	0.0223
Mycoplasmopsis	4.78	Vag-post	4.20	0.0425
Parvimonas	4.29	Vag-post	3.93	0.0056
Bacteroidales_unclassified	4.33	Vag-post	3.77	0.0083
Fusobacteriaceae_unclassified	4.43	Vag-post	3.65	0.0179
Caviibacter	3.95	Vag-post	3.52	<0.0001
Acinetobacter	3.89	Vag-post	3.22	0.0120
Peptostreptococcus	3.64	Vag-post	3.15	0.0149
Fusobacteriales_unclassified	3.56	Vag-post	3.05	0.0001
Mollicutes_unclassified	2.95	Vag-post	2.68	0.0017

Table 3. Discriminative genera obtained from LEfSe comparison between Vag-pre and Utr-post samples. The logMaxMean represents the logarithm value of the maximum mean abundance of the OTU across the specified group (indicated as a class). LDA, Linear Discriminant Analysis score. Conditional formatting was applied to logMaxMean and LDA values to visually represent the data.

Phylum	**logMaxMean**	**Class**	**LDA**	**P value**
Trinickia	5.28	Vag-pre	4.93	<0.0001
Micrococcaceae_unclassified	4.87	Vag-pre	4.57	<0.0001
Intrasporangiaceae_unclassified	4.42	Vag-pre	4.10	<0.0001
Ruminococcaceae_unclassified	4.47	Vag-pre	3.92	0.0003
Glutamicibacter	4.26	Vag-pre	3.91	<0.0001
Micrococcales_unclassified	4.14	Vag-pre	3.84	<0.0001
Streptococcus	4.30	Vag-pre	3.76	0.0003
Kocuria	4.02	Vag-pre	3.76	<0.0001
Oerskovia	4.02	Vag-pre	3.75	<0.0001
Lachnospiraceae_unclassified	4.37	Vag-pre	3.68	0.0011
Betaproteobacteria_unclassified	4.06	Vag-pre	3.65	<0.0001
Paraburkholderia	3.89	Vag-pre	3.56	<0.0001
Staphylococcus	4.00	Vag-pre	3.56	<0.0001
Burkholderiales_unclassified	3.85	Vag-pre	3.50	<0.0001
Acinetobacter	3.82	Vag-pre	3.49	<0.0001

Uruburuella	3.65	Vag-pre	3.33	<0.0001
Lactobacillales_unclassified	3.73	Vag-pre	3.33	<0.0001
Burkholderiaceae_unclassified	3.62	Vag-pre	3.30	<0.0001
Chitinophagaceae_unclassified	3.86	Vag-pre	3.29	<0.0001
Tersicoccus	3.56	Vag-pre	3.28	<0.0001
Pseudomonadaceae_unclassified	3.63	Vag-pre	3.28	<0.0001
Corynebacterium	3.56	Vag-pre	3.23	<0.0001
Actinobacteria_unclassified	3.81	Vag-pre	3.22	0.0001
Rhizobiales_unclassified	3.73	Vag-pre	3.14	<0.0001
Rhodanobacter	3.49	Vag-pre	3.11	<0.0001
Alphaproteobacteria_unclassified	3.65	Vag-pre	3.09	0.0001
Proteobacteria_unclassified	3.64	Vag-pre	3.08	<0.0001
Cellulomonadaceae_unclassified	3.33	Vag-pre	3.08	<0.0001
Phocaeicola	3.69	Vag-pre	3.07	0.0068
Dietzia	3.30	Vag-pre	3.05	<0.0001
Neisseriaceae_unclassified	3.31	Vag-pre	3.05	0.0003
Devosia	3.45	Vag-pre	2.99	0.0001
Micrococcus	3.28	Vag-pre	2.98	<0.0001
Rhodobacteraceae_unclassified	3.39	Vag-pre	2.97	<0.0001
Gammaproteobacteria_unclassified	3.53	Vag-pre	2.96	0.0004
Bradyrhizobiaceae_unclassified	3.49	Vag-pre	2.96	0.0004
Mycobacteriales_unclassified	3.24	Vag-pre	2.94	<0.0001
Oceanotoga	3.27	Vag-pre	2.93	<0.0001
Enterococcus	3.18	Vag-pre	2.92	<0.0001
Staphylococcaceae_unclassified	3.27	Vag-pre	2.91	<0.0001
Enterobacteriaceae_unclassified	3.43	Vag-pre	2.85	<0.0001
Turicibacter	3.28	Vag-pre	2.85	0.0002
Enterococcaceae_unclassified	3.07	Vag-pre	2.84	<0.0001
Romboutsia	3.30	Vag-pre	2.83	0.0001
Burkholderia	2.95	Vag-pre	2.83	<0.0001
Rhizomicrobium	3.36	Vag-pre	2.82	0.0006
Clostridiales_Incertae_Sedis_XIII_uncl	3.30	Vag-pre	2.82	0.0001
Moraxellaceae_unclassified	2.90	Vag-pre	2.80	<0.0001
Pseudomonas	2.93	Vag-pre	2.80	<0.0001
Paeniclostridium	3.22	Vag-pre	2.79	0.0007
Sphingomonadaceae_unclassified	3.23	Vag-pre	2.78	<0.0001
Psychrobacter	2.73	Vag-pre	2.77	0.0002
Salinivibrio	2.50	Vag-pre	2.76	0.0001
Boudabousia	2.87	Vag-pre	2.76	<0.0001
Acetoanaerobium	2.82	Vag-pre	2.75	<0.0001
Asticcacaulis	3.13	Vag-pre	2.75	0.0126
Nocardioidaceae_unclassified	2.33	Vag-pre	2.74	0.0082
Paracoccus	2.61	Vag-pre	2.74	0.0002
Opitutaceae_unclassified	3.22	Vag-pre	2.74	0.0003
Undibacterium	2.67	Vag-pre	2.73	<0.0001
Halomonadaceae_unclassified	2.39	Vag-pre	2.73	0.0002

Clostridiaceae_1_unclassified	3.16	Vag-pre	2.72	0.0081
Rhodanobacteraceae_unclassified	3.18	Vag-pre	2.72	<0.0001
Paenibacillaceae_1_unclassified	3.09	Vag-pre	2.71	0.0001
Halomonas	2.57	Vag-pre	2.70	0.0002
Aerococcus	2.86	Vag-pre	2.70	0.0005
Brevundimonas	2.90	Vag-pre	2.69	0.0022
Brachybacterium	2.83	Vag-pre	2.67	<0.0001
Oxalobacteraceae_unclassified	2.65	Vag-pre	2.66	<0.0001
Lactococcus	2.72	Vag-pre	2.64	<0.0001
Xanthomonadales_unclassified	2.91	Vag-pre	2.64	0.0012
Ornithinimicrobium	2.55	Vag-pre	2.64	0.0002
Enterobacterales_unclassified	2.71	Vag-pre	2.63	0.0001
Planococcaceae_unclassified	2.57	Vag-pre	2.63	0.0029
Vibrionaceae_unclassified	2.53	Vag-pre	2.62	0.0003
Clostridium_sensu_stricto	3.29	Vag-pre	2.62	0.0091
Gp6_unclassified	2.85	Vag-pre	2.62	0.0008
Lawsonella	2.79	Vag-pre	2.61	<0.0001
Bacilli_unclassified	3.18	Vag-pre	2.61	0.0414
Chryseobacterium	2.50	Vag-pre	2.60	0.0010
Enhydrobacter	2.62	Vag-pre	2.60	0.0123
Actinobacteria_unclassified	3.03	Vag-pre	2.60	<0.0001
Opitutus	3.07	Vag-pre	2.60	0.0007
Dokdonella	3.03	Vag-pre	2.60	0.0133
Carnobacteriaceae_unclassified	2.57	Vag-pre	2.60	<0.0001
Mogibacterium	2.67	Vag-pre	2.57	0.0004
Cytophagales_unclassified	2.97	Vag-pre	2.57	0.0035
Comamonadaceae_unclassified	3.05	Vag-pre	2.56	0.0006
Deltaproteobacteria_unclassified	2.85	Vag-pre	2.55	0.0225
Devosiaceae_unclassified	2.95	Vag-pre	2.54	0.0053
Sphingopyxis	2.92	Vag-pre	2.54	0.0011
Cyanobacteria/Chloroplast_unclassified	2.74	Vag-pre	2.54	0.0019
Rhizobiaceae_unclassified	2.92	Vag-pre	2.53	0.0048
Olsenella	2.87	Vag-pre	2.53	0.0025
Erythrobacteraceae_unclassified	2.64	Vag-pre	2.51	0.0080
Brevibacterium	2.63	Vag-pre	2.51	0.0009
Micropepsaceae_unclassified	3.10	Vag-pre	2.51	0.0187
Anaerobutyricum	2.60	Vag-pre	2.50	0.0037
Polyangiaceae_unclassified	2.95	Vag-pre	2.49	0.0182
Streptomycetaceae_unclassified	2.90	Vag-pre	2.47	0.0153
Rhodospirillales_unclassified	3.04	Vag-pre	2.46	0.0016
Sphingomonadales_unclassified	2.62	Vag-pre	2.46	0.0144
Streptacidiphilus	2.77	Vag-pre	2.43	0.0334
Flavobacteriaceae_unclassified	2.62	Vag-pre	2.43	0.0083
Phyllobacteriaceae_unclassified	2.62	Vag-pre	2.39	0.0076
Microbacteriaceae_unclassified	2.52	Vag-pre	2.34	0.0412

Sphingobacteriaceae_unclassified	2.51	Vag-pre	2.30	0.0039
Gemella	4.94	Utr-post	4.59	0.0241
Leptotrichiaceae_unclassified	4.56	Utr-post	4.18	0.0095
Trueperella	4.12	Utr-post	3.90	0.0063
Streptobacillus	4.16	Utr-post	3.73	0.0039
Caviibacter	3.26	Utr-post	2.94	0.0009
Gemmatimonadaceae_unclassified	3.11	Utr-post	2.76	0.0336

Table 4: Discriminative genera obtained from LEfSe comparison between Vag-post and Utr-post samples. The logMaxMean represents the logarithm value of the maximum mean abundance of the OTU across the specified group (indicated as a class). LDA, Linear Discriminant Analysis score. Conditional formatting was applied to logMaxMean and LDA values to visually represent the data.

Phylum	logMaxMean	Class	LDA	P value
Trinickia	4.79	Vag-post	4.41	<0.0001
Leptotrichiaceae_unclassified	4.67	Vag-post	4.24	0.0021
Micrococcaceae_unclassified	4.38	Vag-post	4.11	<0.0001
Ruminococcaceae_unclassified	4.47	Vag-post	3.90	0.0032
Fusobacteriaceae_unclassified	4.43	Vag-post	3.81	0.0057
Clostridiales_unclassified	4.50	Vag-post	3.81	0.0312
Lachnospiraceae_unclassified	4.40	Vag-post	3.78	0.0012
Intrasporangiaceae_unclassified	4.00	Vag-post	3.67	<0.0001
Glutamicibacter	3.97	Vag-post	3.65	0.0001
Acinetobacter	3.89	Vag-post	3.62	0.0016
Oerskovia	3.80	Vag-post	3.51	<0.0001
Micrococcales_unclassified	3.78	Vag-post	3.46	<0.0001
Chitinophagaceae_unclassified	3.62	Vag-post	3.26	0.0026
Kocuria	3.47	Vag-post	3.21	0.0002
Phocaeicola	3.63	Vag-post	3.19	0.0197
Peptostreptococcus	3.64	Vag-post	3.16	0.0467
Paraburkholderia	3.46	Vag-post	3.12	0.0001
Alphaproteobacteria_unclassified	3.49	Vag-post	3.04	0.0062
Burkholderiales_unclassified	3.41	Vag-post	3.03	0.0001
Betaproteobacteria_unclassified	3.67	Vag-post	3.02	0.0031
Actinobacteria_unclassified	3.70	Vag-post	2.98	0.0113
Rhizobiales_unclassified	3.55	Vag-post	2.94	0.0085
Uruburuella	3.25	Vag-post	2.88	0.0001
Corynebacterium	3.20	Vag-post	2.88	0.0002
Cellulomonadaceae_unclassified	3.12	Vag-post	2.87	<0.0001
Micrococcus	3.06	Vag-post	2.86	0.0001
Tersicoccus	2.98	Vag-post	2.80	0.0004
Lactobacillales_unclassified	3.28	Vag-post	2.79	0.0004
Dietzia	3.02	Vag-post	2.77	0.0013
Burkholderiaceae_unclassified	3.17	Vag-post	2.75	0.0020
Paeniclostridium	3.27	Vag-post	2.75	0.0010
Rhodanobacter	3.17	Vag-post	2.72	0.0007

Clostridium_sensu_stricto	3.33	Vag-post	2.72	0.0366
Paenibacillaceae_1_unclassified	2.95	Vag-post	2.72	0.0006
Pseudomonadaceae_unclassified	3.18	Vag-post	2.72	0.0002
Moraxellaceae_unclassified	2.75	Vag-post	2.69	0.0023
Burkholderia	2.24	Vag-post	2.69	0.0032
Devosia	3.18	Vag-post	2.68	0.0014
Staphylococcaceae_unclassified	2.73	Vag-post	2.67	0.0115
Oceanotoga	2.85	Vag-post	2.66	0.0009
Ihubacter	3.19	Vag-post	2.66	0.0497
Mollicutes_unclassified	2.95	Vag-post	2.62	0.0077
Staphylococcus	3.12	Vag-post	2.62	0.0084
Enterococcaceae_unclassified	2.81	Vag-post	2.62	0.0018
Enterococcus	2.65	Vag-post	2.60	0.0006
Pseudomonas	2.38	Vag-post	2.60	0.0005
Clostridiales_Incertae_Sedis_XIII_uncl	3.13	Vag-post	2.59	0.0189
Opitutaceae_unclassified	3.17	Vag-post	2.59	0.0072
Turicibacter	3.05	Vag-post	2.57	0.0008
Gp6_unclassified	2.77	Vag-post	2.56	0.0132
Brachybacterium	2.50	Vag-post	2.56	0.0005
Romboutsia	3.01	Vag-post	2.55	0.0038
Harryflintia	2.85	Vag-post	2.50	0.0489
Peptostreptococcaceae_unclassified	2.82	Vag-post	2.48	0.0112
Rhizobiaceae_unclassified	2.78	Vag-post	2.48	0.0281
Mycobacteriales_unclassified	2.78	Vag-post	2.46	0.0008
Streptomycetaceae_unclassified	2.71	Vag-post	2.46	0.0079
Anaerotignum	2.89	Vag-post	2.44	0.0179
Sphingopyxis	2.80	Vag-post	2.44	0.0298
Mogibacterium	2.40	Vag-post	2.43	0.0066
Opitutus	2.79	Vag-post	2.42	0.0299
Comamonadaceae_unclassified	2.88	Vag-post	2.38	0.0221
Anaerobutyricum	2.37	Vag-post	2.34	0.0214
Actinobacteria_unclassified	2.75	Vag-post	2.32	0.0060
Myxococcales_unclassified	2.46	Vag-post	2.21	0.0245
Trueperella	4.12	Utr-post	3.79	0.0442
Streptococcus	4.15	Utr-post	3.70	0.0080
Peptoniphilus	4.04	Utr-post	3.55	0.0378
Rhodobacteraceae_unclassified	3.18	Utr-post	2.85	0.0035
Gemmatimonadaceae_unclassified	3.11	Utr-post	2.68	0.0103
Rhodospirillales_unclassified	2.86	Utr-post	2.61	0.0088

The probabilistic Dirichlet multinomial mixtures (DMM) model analysis revealed a minimum Laplace value at K=2 (Figure 6), indicating that this unsupervised analysis segregates the samples into two distinct community types based on their inherent community structures [25]. Of the 80 samples included in the analysis, 34 samples were allocated to one community type (community P1), and 46 samples to the second community (community P2). Interestingly, the vast majority of the samples from the Utr-post group (20/24, 83.3%) were segregated to community type P1, the vast majority of samples from the Vag-pre group (29/32, 90.6%) were segregated to community type P2, while the samples from Vag-post were divided quite evenly between the two (P1: 11/24. 45.8%; P2: 13/24, 54.2%). The first 36 genera that were most responsible for separating the samples into these two community types are presented in Table 5. This list of top genera represents the cumulative 50% proportion of the total abundance. The most prominent difference between the two community types was in the relative abundance of the Trinickia genus, which was much higher in community type P2 (P1: 1.04%, P2: 12.82%). Other notable differences included higher abundances of *Histophilus*, *Helcococcus*, *Fusobacterium*, *Firmicutes_unclassified*, *Ureaplasma*, and *Mycoplasmopsis* in community type P1, while higher levels of *Micrococcaceae_unclassified*, *Intrasporangiaceae_unclassified*, and *Ruminococcaceae_unclassified* characterized the community type P2. Overall, these findings suggested a distinct microbial profile associated with the prepartum vaginal environment (associated mainly with community type P2), a unique postpartum uterine microbial signature (associated mainly with community type P2), and two communities that imply a transitional phase or variability in the postpartum vaginal microbiome.

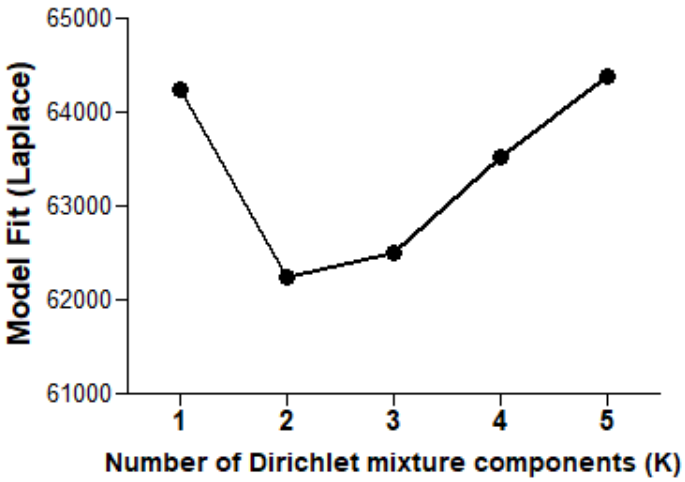


Figure 6. Model fit using the probabilistic Dirichlet Multinomial Mixtures (DMM) model analysis. The Y-axis represents the Laplace values as the number of Dirichlet mixture components K increases.

Table 5. Relative abundance of different genera in two distinct community types (P1 and P2) defined by unsupervised probabilistic Dirichlet Multinomial Mixtures (DMM) model analysis. The DMM analysis segregated most Utr-post samples (83.3%) to community P1, most Vag-pre samples (90.6%) to community P2, while Vag-post samples were divided between P1 (45.8%) and P2 (54.2%). Relative abundances (%) of the first 36 genera most responsible for separating the samples into these two community types are presented (as mean and 95%CI). Data presented also include the relative abundances of all samples before segregation, the difference between P1's and P2's relative abundances, and the cumulative proportion of the total abundance explained by the listed genera. Conditional formatting was applied to mean values to visually represent the data.

Phylum	**All samples**	**Community Type P1**	**Community Type P2**	**Difference**	**Cumulative**
	mean	mean (95%CI)	mean (95%CI)	(P1 vs.P2)	**Fraction**
Trinickia	2.89	1.04 (0.71-1.51)	12.82 (11.8-13.9)	11.78	0.16
Histophilus	1.29	2.73 (2.01-3.71)	0.67 (0.52-0.86)	2.06	0.18
Micrococcaceae_unclassified	1.09	0.67 (0.43-1.03)	2.56 (2.19-3.01)	1.9	0.21
Helcococcus	1.53	2.77 (2.01-3.82)	0.90 (0.72-1.13)	1.87	0.24
Fusobacterium	4.55	5.34 (4.08-6.98)	4.01 (3.52-4.56)	1.33	0.25
Firmicutes_unclassified	1.39	2.23 (1.64-3.04)	0.95 (0.76-1.18)	1.29	0.27
Intrasporangiaceae_unclassified	0.82	0.54 (0.34-0.85)	1.61 (1.33-1.94)	1.07	0.28
Ureaplasma	1.01	1.57 (1.09-2.26)	0.64 (0.50-0.82)	0.93	0.30
Mycoplasmopsis	1.19	1.69 (1.16-2.46)	0.83 (0.66-1.05)	0.87	0.31
Ruminococcaceae_unclassified	1.40	1.21 (0.84-1.74)	2.07 (1.75-2.46)	0.86	0.32
Fusobacteriaceae_unclassified	1.46	1.98 (1.42-2.75)	1.16 (0.94-1.42)	0.82	0.33
Lachnospiraceae_unclassified	1.22	1.00 (0.67-1.47)	1.81 (1.52-2.17)	0.82	0.34
Fusobacteriales_unclassified	0.63	1.16 (0.81-1.67)	0.34 (0.26-0.45)	0.82	0.35
Bacteroidales_unclassified	1.08	1.55 (1.09-2.20)	0.79 (0.63-1.00)	0.76	0.36
Bacteria_unclassified	5.55	6.12 (4.78-7.83)	5.41 (4.83-6.06)	0.71	0.37
Betaproteobacteria_unclassified	0.82	0.65 (0.43-0.98)	1.33 (1.09-1.62)	0.68	0.38
Peptoniphilus	0.85	1.25 (0.88-1.78)	0.62 (0.48-0.80)	0.63	0.39
Parvimonas	0.49	0.89 (0.58-1.35)	0.27 (0.2-0.38)	0.61	0.40
Caviibacter	0.25	0.67 (0.44-1.01)	0.09 (0.06-0.15)	0.58	0.41
Bacteroidetes_unclassified	1.43	1.77 (1.27-2.48)	1.27 (1.04-1.55)	0.5	0.41
Bacteroides	0.51	0.82 (0.54-1.23)	0.32 (0.23-0.43)	0.5	0.42
Paraburkholderia	0.56	0.35 (0.20-0.59)	0.83 (0.66-1.05)	0.48	0.43
Erysipelotrichaceae_unclassified	0.57	0.86 (0.57-1.30)	0.38 (0.28-0.51)	0.48	0.43
Clostridia_unclassified	0.38	0.69 (0.47-1.02)	0.22 (0.15-0.30)	0.48	0.44
Mediterranea	0.30	0.62 (0.41-0.95)	0.15 (0.10-0.22)	0.47	0.44
Leptotrichiaceae_unclassified	1.00	1.29 (0.90-1.85)	0.82 (0.65-1.04)	0.47	0.45
Gemella	0.85	1.11 (0.74-1.66)	0.64 (0.50-0.83)	0.47	0.46
Peptoniphilaceae_unclassified	0.24	0.54 (0.35-0.85)	0.11 (0.07-0.16)	0.44	0.46
Micrococcales_unclassified	0.71	0.58 (0.37-0.90)	1.00 (0.80-1.25)	0.42	0.47
Pasteurellaceae_unclassified	0.34	0.57 (0.36-0.90)	0.19 (0.14-0.27)	0.38	0.47
Porphyromonas	1.09	1.33 (0.91-1.94)	0.96 (0.76-1.21)	0.37	0.48
Burkholderiales_unclassified	0.61	0.47 (0.29-0.74)	0.83 (0.66-1.04)	0.36	0.48
Mollicutes_unclassified	0.18	0.44 (0.27-0.71)	0.08 (0.05-0.13)	0.36	0.49
Prevotella	0.27	0.50 (0.31-0.80)	0.14 (0.10-0.21)	0.35	0.49
Clostridiales_unclassified	2.04	2.37 (1.74-3.24)	2.02 (1.71-2.40)	0.35	0.50
Acinetobacter	0.54	0.40 (0.25-0.66)	0.74 (0.58-0.94)	0.33	0.50

3. Discussion

The results of this study highlight the distinct transition of microbial communities in the reproductive tract of first-pregnancy heifers, from late pregnancy to early postpartum, focusing on the vagina and uterus. The study addressed a significant gap in understanding the dynamics of microbial colonization during this critical period, as the uterus is substantially contaminated. This is particularly interesting in primiparous cows, as the first parturition is a once-in-a-lifetime event, most likely characterized by the first and most massive microbial invasion into the relatively naïve uterus. Furthermore, our study may provide insights into the increased susceptibility of primiparous females to uterine inflammatory diseases. By employing next-generation sequencing of 16S-rDNA amplicon libraries, we aimed to comprehensively characterize the bacterial communities and shed light on their changes across anatomical sites and time points. Our results revealed notable shifts in microbial composition between the prepartum vagina (Vag-pre), postpartum vagina (Vag-post), and postpartum uterus (Utr-post). Notably, the postpartum uterus exhibited lower richness and diversity compared to the prepartum and postpartum vagina, indicating a dynamic microbial landscape associated with parturition. The lower diversity and richness in the Utr-post group suggest a selective environment in the postpartum uterus, possibly due to the luminal secretions, immune response, or other physiological changes occurring during and after parturition. These findings align with previous findings in dairy cows and women, suggesting an impact of delivery on uterine microbial colonization [2, 7, 9].

The meticulous data preparation and sequence quality control steps done via Mothur were crucial for ensuring the reliability of our results [1]. By applying rigorous screening and denoising procedures, we reduced potential biases introduced by sequencing and PCR errors. Also, it enabled us to recognize and exclude samples whose data was likely to be unrepresentative, more commonly among postpartum samples. The presence of low-quality samples in both postpartum vagina and uterus, while scarce in the prepartum vagina, could be influenced by several factors. The increased difficulty in obtaining high-quality samples from the uterus, attributed to its internal location and potential for contamination during collection, may contribute to the higher incidence of low-quality uterine samples. Postpartum vaginal and uterine samples might also face challenges during collection, possibly due to residual fluids or tissues affecting DNA extraction. The relatively low occurrence of low-quality prepartum vaginal samples could be associated with less invasive collection methods and a cleaner environment compared to the postpartum period. Additionally, hormonal and physiological changes during and after parturition might influence the microbial composition and DNA quality, further impacting the sample quality in these reproductive tract compartments. Overall, quality control measures enhanced the robustness of our findings and allowed for more accurate interpretations of the observed microbial dynamics.

The analysis of phylum-level relative abundance provided insights into the major players in the microbial communities. Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Fusobacteria emerged as the dominant phyla, consistent with previous studies in the bovine reproductive tract [3, 9, 26]. Importantly, significant differences between Vag-pre and Utr-post groups, particularly in Proteobacteria, Bacteroidetes, and Fusobacteria, suggest a distinct uterine microbial signature postpartum, as also observed in the genus-level analyses.

Alpha diversity metrics, including sample coverage, observed OTUs, and Inverse Simpson diversity estimates, provided valuable insights into community richness and diversity [27]. The lower diversity in the Utr-post group indicated a shift in the uterine microbial landscape, potentially influenced by postpartum physiological changes and the initiation of uterine involution. These findings underscore the importance of considering both anatomical location and postpartum status when studying microbial diversity. The NMDS ordination plot illustrated spatial separation among Vag-pre, Vag-post, and Utr-post samples, emphasizing the distinct community structures associated with each anatomical niche. AMOVA results further supported significant differences between the groups, highlighting the impact of anatomical location on bacterial community variation. The LEfSe analysis identified discriminative genera, providing a finer resolution of microbial differences between groups. Key genera, such as *Trinickia*, *Micrococcaceae_unclassified*, and *Streptococcus*, were more abundant in Vag-pre samples, while *Gemella* and *Trueperella* were enriched in Utr-post samples. The DMM model analysis revealed two distinct community types, P1 and P2, segregating the samples. Notably, the majority of Utr-post samples clustered in P1, suggesting a unique postpartum uterine microbial profile. The differential abundance of genera, including *Trinickia*, *Histophilus*, and *Fusobacterium*, further characterized the distinct microbial dynamics associated with each community type.

As detailed above, our findings offer specific microbial markers associated with anatomical and temporal transitions. Interestingly, *Trinickia* is a genus of gram-negative, aerobic, and rod-shaped bacteria in the phylum Firmicutes. In this study, it was identified with high abundance in the prepartum vagina of first-pregnant heifers. To the best of our knowledge, it has not been reported in the bovine reproductive tract; it is a relatively novel recognized genus with limited information available [28, 29]. *Histophilus* has been reported in the cow reproductive tract and was associated with high abundances in cows diagnosed with purulent vaginal discharge and metritis [26, 30]. The genera *Fusobacterium* and *Trueperella* have been reported extensively in the bovine uterus and have been associated with postpartum uterine diseases [9, 30]. A high prevalence of the genus *Gemella* has been reported in the vagina of pregnant women and was associated with alteration of the immune response, preterm delivery, and postpartum uterine complications [31, 32]. To our best knowledge, it was not reported in the cow reproductive tract, but has been recognized recently in high abundance in the seminal microbiota of bull's semen [33]

Based on the observed differences in bacterial composition among the Vag-pre, Vag-post, and Utr-post samples, it is plausible that the postpartum uterine bacterial composition results from both vaginal bacterial invasion and potential environmental sources. Significant shifts in microbial profiles from the late-pregnancy vaginal state to the postpartum uterine state suggest that vaginal microbiota may seed the uterine microbiome. The close anatomical proximity between the vagina and uterus supports bacterial migration from the lower to upper reproductive tract. However, some unique microbial signatures in postpartum uterine samples, differing from the vaginal microbiota, indicate that environmental exposures during and after delivery may also shape the uterine microbiota. Additionally, uterine contamination, bacterial community establishment, and involution processes influence uterine secretions, likely affecting postpartum vaginal bacterial dynamics. These factors may explain changes in postpartum vaginal microbiota compared to late pregnancy, while some pre-calving vaginal

characteristics remain, albeit modified. Thus, reproductive bacterial compositions are temporally and spatially affected by the unique characteristics of each niche, with different niches (vagina, uterus, environment) likely influencing each other. The relative prominence of each scenario is challenging to determine definitively without detailed metagenomic analyses (such as Shotgun Metagenomic Sequencing, or Long Read Sequencing), additional functional data, and well-planned environmental sampling [34, 35]. Future studies employing advanced sequencing techniques and comprehensive environmental monitoring may further elucidate postpartum uterine bacterial colonization dynamics and clarify the contributions of vaginal and environmental sources.

The implementation of Next-Generation Sequencing technology and analytical methods has advanced our understanding of microbial communities within the reproductive tract of first-pregnancy heifers. The resolution nature of our approach allowed for the identification of subtle changes in community composition, offering a detailed portrayal of the dynamics within these intricate ecosystems. The assignment of OTUs to phylotypes, as conducted in our study, provides a taxonomic framework that aids in discerning the diversity and relationships among microbial populations. This method allows for a more nuanced analysis compared to calculating pairwise distances between aligned DNA sequences and clustering OTUs accordingly, as it may account for phylogenetic relatedness, potentially providing a more biologically meaningful representation of microbial diversity. However, this approach has some disadvantages. Assigning OTUs by phylotypes typically relies on sequence similarity thresholds for clustering, which may lead to overestimation or underestimation of species diversity based on the chosen threshold. This can introduce biases and affect the accuracy of taxonomic assignments. In contrast, calculating uncorrected pairwise distances provides a more continuous and quantitative measure of sequence similarity, allowing for a more focused assessment of genetic relationships between organisms [36]. The use of phylotype-based OTU assignment may result in splitting single taxa into multiple OTUs, depending on the chosen threshold, which might have occurred in our study, at least to some degree. This lack of resolution can limit the precision of microbial community analysis, especially in environments with high species diversity or closely related species. On the other hand, clustering OTUs based on sequence distances allows for a more flexible and data-driven approach, capturing a finer-scale representation of microbial diversity. However, it is essential to consider the computational demands and potential biases associated with different distance metrics when employing this approach. The choice between phylotype-based OTU assignment and sequence distance-based clustering also depends on the research objectives, the nature of the microbial community under investigation, and the available computational resources; all were part of our consideration when the data was analyzed.

There are other inherent limitations associated with our study approach. Potential biases introduced during sample collection and DNA extraction processes may impact the accuracy of our results. Variability in sampling techniques or the DNA extraction process could introduce biases in representing certain taxa, potentially influencing the observed microbial composition. Moreover, while our study contributes valuable insights into the taxonomic aspects of microbial dynamics, further investigations employing functional analyses would be instrumental in elucidating the ecological roles of these microbial communities [34, 35]. Future studies should also compare the reproductive tract microbiota before and after calving in both first-pregnancy heifers and

multiparous cows and explore how their dynamics may be associated with postpartum uterine diseases. Integrating metagenomic or metatranscriptomic approaches could unveil functional attributes, shedding light on the potential contributions of these microbes to reproductive health and overall host physiology.

In summary, our study highlights the distinct transition of microbial communities in the reproductive tract of first-pregnancy heifers from late pregnancy to early postpartum, focusing on the vagina and uterus. We identified significant shifts in microbial composition by utilizing next-generation sequencing of 16S-rDNA amplicon libraries. Notably, the microbial profiles of the vaginal samples at late gestation and the uterine samples at early postpartum were the most distinct, with the postpartum vaginal samples serving as an intermediary link between them. Additionally, the study revealed a substantial reduction in bacterial richness and diversity in the postpartum uterus compared to the vagina. These findings underscore the importance of considering anatomical and temporal factors when studying reproductive tract microbiota in primigravid females. The results also suggest that postpartum uterine bacterial composition is likely to be influenced by the selective environment of the postpartum uterus, as well as the vaginal microbiota and environmental microorganisms. Overall, our study contributes to a broader understanding of uterine health in primiparous females and emphasizes the need for further research using advanced sequencing techniques to fully understand these microbial dynamics, their implications for uterine health, and the pathophysiology of postpartum uterine inflammatory diseases.

4. Materials and Methods

4.1. Animals and sampling procedures

All animal handling and sampling procedures were performed by a certified veterinarian according to the ethical approval obtained from the Hebrew University Institute Animal Care and Use Committee (IACUC MD-13-13807-2). Three swab samples were collected from first-pregnancy Holstein-Friesian heifers, as detailed below; one from the vagina at gestation day 258±4 ('Vag-pre'), and two additional samples from the vagina ('Vag-post') and uterus ('Utr-post') at postpartum day 8±3. Heifers included in the study were from a commercial dairy farm in Israel. They were housed in loose housing systems in large, completely covered open sheds, and fed a total mixed ration, according to the Nutrient Requirement of Dairy Cattle recommendations. All pregnancies were achieved following artificial inseminating with thawed frozen semen (fAI) while the mature heifer was on spontaneous estrus, and pregnancy diagnosis was performed by trans-rectal palpation of the uterus 40-50 days after the fAI date (i.e., indicated as the first gestation day) and reconfirmed before the first sampling at late gestation. Individual cow health and reproduction data were recorded using the herd management computer program NOA (Israeli Cattle Breeders Association, Caesarea, Israel). Heifers were excluded from the study if they had a history of pregnancy-related illness during gestation, if treated with antibiotics during the six weeks before the first sampling, or until three swab samples were collected. Furthermore, heifers were excluded from the study if they had difficult assisted calving, twin pregnancy, postpartum retained fetal membranes, or systemic clinical signs indicating postpartum septic metritis. Accordingly, the study included data from 99 swab samples collected from 33 first-pregnancy Holstein-Friesian heifers.

Swab samples were collected using acceptable methods [9, 37]. Before sampling, the vulva was cleaned with a brush and soap water, followed by wipes containing chlorhexidine gluconate and isopropyl alcohol. Sampling was performed using a sterile, disposable, double-guarded endometrial swab (76 cm long; Equivet, Denmark Cat # 290955) inserted into the vagina and managed by one hand while it was directed to the sampling location trans-rectally by the other hand. For vaginal sampling, the swab was inserted into the cranial vagina, where the inner protective sheath and the swab gradually extruded, and the protruded swab was used to sample the most cranial vagina and the external os of the cervix for approximately 10 seconds. For uterine sampling, the protected swab was guided transcervicaly into the uterine body, where the inner sheath and the swab gradually extruded, and the swab was pressed and rotated against the endometrium for approximately 10 seconds. Before retraction, the inner swab was inserted back into its protective sheathes to avoid contamination while withdrawing it from the reproductive tract. Outside the reproductive tract, the inner swab tip was kept in its designated sterile sheath and transported at 4ºC to the lab. In the lab, the tip of the swab was placed into a sterile vial containing 1ml sterile PBS; the vial was vortexed (2 min, maximum speed, twice, 10 min apart) to release the biological material, the tip was discarded, and the fluid was kept frozen at -80ºC until DNA extraction.

4.2. DNA Extraction

DNA extraction was done using the DNeasy® Blood & Tissue kit (catalog number 69504, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted DNA was quantified in a Qubit 4 fluorometer using the dsDNA HS and the dsDNA BR assay kits (Thermo Scientific, MA, USA). Additional quality evaluation included NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA) absorbance curve inspection and 260/280 and 260/230 ratio. DNA samples were kept at -20 ºC until use.

4.3. 16S rRNA Gene Amplicon Libraries Preparation and NGS Sequencing

Genomic DNA was subjected to polymerase chain reaction (PCR) amplification using the 515F and 806R primers, targeting the V4 hypervariable region of the bacterial and archaeal 16S rRNA gene [21, 22]. The primers, CS1_515F (ACACTGACGACATGGTTCTACAGTGCCAGCMGCCGCGGTAA) and CS2_806R (TACGGTAGCAGAGACTTGGTCTGGACTACHVGGGTWTCTAAT), were designed with common sequences 1 (CS1) and 2 (CS2) at their 5' ends, as previously described [23]. Primers were purchased from Integrated DNA Technologies as standard primers and diluted to 10 μM concentration. DNA samples were adjusted to a 10-20 ng/μl concentration before the amplification process. Amplification reactions were conducted in 96-well plates with a 20 μl reaction volume. The master mix for the entire plate included 10 μl of OneTaq Quick-Load 2X Master Mix with standard buffer (New England Biolabs, MA, USA), 0.5 μl of each primer (10 μM), and 8 μl of nuclease-free water. One microliter of template DNA was added to each well. The cycling conditions consisted of an initial denaturation at 95˚C for 5 minutes, followed by 35 cycles of denaturation at 95˚C for 30 seconds, annealing at 56˚C for 45 seconds, and extension at 72˚C for 30 seconds. A final extension step of 3 minutes at 72˚C was performed. Each plate included a positive control containing E. coli DNA and a negative control (double-distilled water, DDW). Visible amplification was confirmed through 1.7% agarose gel electrophoresis. Subsequently, PCR

products were purified and normalized using the SequalPrep normalization plate kit (Thermo Scientific, MA, USA) following the manufacturer's instructions. Size selection was achieved using Pippin Prep (Sage Science, MA, USA).

Following the normalization and size selection steps, a second round of PCR amplification was conducted in 10 μL reactions within 96-well plates. The master mix for the entire plate was prepared, with the composition of each reaction as follows: 5 μL of 2X AccuPrime SuperMix II (Life Technologies, MD, USA), 2 μL of double-distilled water (DDW), 1 μL of each primer (4 μM), and 1 μL of the PCR product obtained from the previous stage. Each reaction employed a distinct primer pair sourced from the Access Array Barcode Library for Illumina Sequences. Each primer pair featured a unique 10-base barcode (Fluidigm, South San Francisco, CA; Cat # 100-4876). Reactions with unique barcodes were designated for the positive control, no-template control (reaction 1), and a second no-template control reaction containing only Access Array Barcode library primers.

The cycling conditions were programmed as follows: an initial denaturation at 95°C for 5 minutes, followed by 8 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 68°C for 30 seconds. A final elongation step of 7 minutes at 68°C concluded the PCR. Validation of the PCR yield for positive and negative controls, along with select samples, was performed using Qubit analysis and size quantification utilizing an Agilent TapeStation 2200 device with D1000 ScreenTape (Agilent Technologies, Santa Clara, CA). Subsequently, samples were pooled in equal volumes and subjected to purification using solid-phase reversible immobilization (SPRI) cleanup with AMPure XP beads at a ratio of 0.6X (v/v) SPRI solution to sample. Quality control was performed using TapeStation2200 and Qubit analysis, and the pool was diluted to 6.5 pM for emulsion PCR. Pooled libraries were then sequenced on the Illumina MiSeq platform using V2 reagent kit (150 bp paired-end; UIC Research Resources Center, University of Illinois) [21].

4.4. Bioinformatic Analysis

The bioinformatic analysis was performed by using the Mothur program (v.1.48.0) in an interactive mode via Linux terminal, based on the Mothur MiSeq SOP (https://mothur.org/wiki/miseq_sop/) [1]. Paired-end fastq files (R1, R2) from 99 samples obtained from 33 heifers were included in the metagenetic analysis. Data preparation and initial sequence quality control steps to minimize sequencing and PCR errors included reads concatenation, contig formation, and length screening. Throughout the process, sequence numbers and their lengths were assessed, and accordingly, screening parameters were adjusted to remove sequences based on length criteria. This included the removal sequences in the upper and lower 2.5%-tile, and adjustment of the remaining sequence length to ≤ 293 bp, which is suitable for the V4 region of the 16S rRNA gene. Thereafter, a sequence uniquefication step was performed to reduce redundancy (duplications merging) and streamline downstream analyses. The unique sequences were aligned to the SILVA reference database (Version 132), which was customized to target the V4 region. Coordinates for the V4 region (start=11895, end=25318) were set based on a parallel analysis done via Mothur by aligning the E. coli rrsB 16S rRNA gene sequence (NCBI Gene ID 948466) against the reference alignment. An additional screening step was applied to remove sequences in the upper and lower 2.5%-tile in order to maintain sequences within the desired region. Sequences were then filtered

to remove overhangs at both ends, and an additional uniquefication step was performed to remove redundancy. Denoising was done by pre-clustering using a pseudo-single linkage algorithm developed by Huse et al. (2010), allowing up to two differences between sequences, aiming to remove sequences likely due to pyrosequencing errors [38]. Chimeras were removed by using the VSEARCH algorithm [39]. The sequences were then classified taxonomically using a Bayesian classifier with the mother-formatted RDP training set (Version 18), and non-bacteria reads, such as 16S rRNA from chloroplasts, mitochondria, Archaea, 18S rRNA, and random sequences, were excluded from further analyses. The remaining high-quality sequences were summarized taxonomically and were used for downstream analyses.

The generation of operational taxonomic units (OTUs) was performed from phylotypes, which were classified by genus. Relative abundances were calculated by dividing the read abundance value of an OTU in a specific sample by the total number of reads for this sample. The subsequent steps involved the calculation of alpha diversity metrics and constructing rarefaction curves, which were used to identify unrepresentative samples that should be excluded from further analyses. The number of sequences, the sample coverage, the number of observed OTUs, and the Inverse Simpson diversity estimate were calculated and compared among the groups (Vag-pre; Vag-post; Utr-post).

Beta diversity was assessed using the Bray-Curtis dissimilarity index, and a phylip-formatted distance matrix was used to generate an output file for a non-metric multidimensional scaling (NMDS) plot (done with 3-dimensions to achieve a stress value below 0.2) [40]. To explore the spatial separation of community structures observed in the NMDS plot, the molecular variance analysis (AMOVA test) was applied, indicating whether the centers of the clouds representing a group are more separated than the variation among samples of the same treatment. Furthermore, a homogeneity of molecular variance (HOMOVA test) was evaluated to statistically compare variations among the Vag-pre, Vag-post, and Utr-post groups.

The non-parametric LEfSe analysis was conducted to provide additional insights into community composition and identify key microbial taxa distinguishing the different groups [41]. The analysis was performed with a threshold value of 2 on the logarithmic score for the Linear Discriminant Analysis (LDA). Furthermore, the Dirichlet multinomial mixtures model was used to calculate Laplace values that were utilized in an unsupervised evaluation of the partitioning of samples into distinct community types [25]. This analysis was done to reveal inherent community structures (sample segregation and significant OTUs driving community segregation) without specifying sample groupings.

4.5. Additional Statistical Analysis & Plotting

Most statistical analyses were performed using Mothur, as detailed above [1]. However, when applicable, additional statistical analyses were conducted using Statistix 10 software (Analytical Software, Tallahassee, FL, USA) and Prism 5.01 (GraphPad Software, San Diego, CA, USA). The choice of statistical tests was guided by the nature of the parameters analyzed to ensure adherence to the assumptions of each test. For non-normally distributed variables, a non-parametric Kruskal–Wallis one-way analysis of variance (ANOVA) test was employed, complemented by Dunn's all-pairwise comparisons test when relevant. Repeated Measures ANOVA

or a mixed model was applied to account for dependency when applicable. Proportional data were compared using Fisher's exact test. All statistical tests were based on two-tailed hypotheses, with significance established at $P < 0.05$ and adjustments made for multiple comparisons. Tabular presentations were created using Microsoft Excel, while graphical visualizations were generated using Prism 5.01 (GraphPad Software) or Python (Python Software Foundation, Python Language Reference, version 3.10.12). When applicable, data analysis and visualization were performed using in-house custom Python scripts, leveraging the Pandas library for data manipulation, the Matplotlib library for 2D visualizations, and the mplot3d toolkit for generating 3D plots, such as the NMDS plot.

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Author Contributions

Conceptualization, S.A.D., S.S and T.R.; methodology, S.A.D., R.S., S.S, and T.R.; software, T.R.; validation, T.R.; formal analysis, T.R.; investigation, S.A.D., R.S., S.R., and S.S; resources, T.R.; data curation, T.R.; writing—original draft preparation, T.R.; writing—review and editing, all authors.; visualization, T.R.; supervision, T.R.; project administration, S.S., T.R.; funding acquisition, T.R. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

All animal handling and sampling procedures were performed by a certified veterinarian according to the ethical approval obtained from the Hebrew University Institute Animal Care and Use Committee (IACUC MD-13-13807-2).

Data Availability

Sequencing data generated during this study will be deposited in the MG-RAST database and will be publicly available upon acceptance of this manuscript for publication.

Conflicts of Interest

The authors declare no conflicts of interest.

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