RESEARCH ARTICLE

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The vaginal microbiota of healthy pregnant women during their third trimester in Ismailia, Egypt

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Abstract

Maternal and neonatal health outcomes have proven to be primarily dependent on the vaginal microbiota during pregnancy. The aim of the current study was to characterize the vaginal microbiome of Egyptian pregnant women in Ismailia, during the third trimester, using 16S rRNA next-generation sequencing of the V3 - V4 hypervariable region. The genera with the highest relative abundance were *Lactobacillus*, followed by *Gardnerella*, *Prevotella*, and *Ureaplasma*. *Lactobacillus iners* was the sole dominant *Lactobacillus* species. Alpha and beta diversity revealed no significant differences between vaginal microbial communities in respect to parity. LEfSe analysis identified no significant features when comparing primigravida and multigravida. Highly positive correlation pairs were detected between the genera *Gardnerella* and *Ureaplasma*. Other positive correlation cluster pairs included *Staphylococcus-Streptococcus, Staphylococcus-Corynebacterium,* and *Aerococcus-Atopobium*. A large positive polymicrobial correlation cluster was detected between the genera *Anaerococcus, Campylobacter, Corynebacterium, Dialister, Fingoldia, Mobiluncus, Peptoniphilus, Peptostreptococcus, Porphyromonas, Prevotella, Veillonella,* and *WAL_1855D*. Urogenital vaginal pathogens may promote normal pregnancy rather than preterm birth under *Lactobacillus*-dominant vaginal microbial communities. Larger cohorts characterizing the vaginal microbiota in Egypt are warranted.

Keywords: vagina; microbiome; pregnant women; Egypt; pregnancy; parity; preterm birth; *Lactobacillus*.

1. Introduction

Maternal and neonatal health outcomes have proven to be primarily dependent on the vaginal microbiota during pregnancy (**Di Simone et al., 2020; Zheng et al., 2021**). *Lactobacillus*-dominant vaginal ecosystems seem crucial for maintaining vaginal health (**Fettweis et al., 2019; Di Simone et al., 2020; Zheng et al., 2021**). Low vaginal pH induced by dominating *Lactobacillus* species is detrimental to invading urogenital pathogens (**Di Simone et al., 2020; Zheng et al., 2021**).

Lactobacilli interfere with invading urogenital pathogens through several defending mechanisms such as lactic acid production, releasing hydrogen peroxide, antimicrobials, and adhesion-inhibiting bio-surfactants, as well as competitive exclusion from host surfaces (**Ruiz et al., 2009**). *Lactobacillus*-deficient vagina render women at risk for bacterial vaginosis and genital tract infections (**Fettweis et al., 2019**). Pregnant women with *Lactobacillus*-depleted vaginal microbiota are more prone to ascending infections associated with adverse pregnancy outcomes (**Dunlop et al., 2021**).

The vaginal microbiome of pregnant women was found to maintain a stable structure characterized by the domination of a particular *Lactobacillus* species (**Romero et al., 2014b; Freitas et al., 2017**). Previous literature categorized vaginal microbiomes into distinct community state types (CSTs) based on the *Lactobacillus*-dominant species (**Ravel et al., 2011**). These CSTs were further related to women`s health and susceptibility to pregnancy complications. For example, the dominance of *L. crispatus* was reported to be protective against preterm birth (PTB) (**Gudnadottir et al., 2022**).

Moreover, compositional variations of the vaginal microbiomes have also been related to race and geographical residence (**Serrano et al., 2019; Di Simone et al., 2020; Ng et al., 2021**). However, previous reports demonstrated a potential impact of ethnicity on vulnerability to pregnancy complications (**Dunlop et al., 2021**). For instance, PTB and preterm premature rupture of membranes have been linked to vaginal microbiomes which are rich in *Gardnerella* and deficient in *Lactobacillus* species in White populations but not in African American populations (**Romero et al., 2014a; Brown et al., 2016; Nelson et al., 2016; Nasioudis et al., 2017; Stout et al., 2017; Dunlop et al., 2021**). Thus, ethnicity has been proposed as a potential confounder controlling the relationship between the vaginal microbiome and adverse pregnancy outcomes.

However, studies characterizing the Egyptian vaginal microbiota are limited. Only a few reports investigated the vaginal microbial profiles in Egyptian cohorts (**Mohamed et al., 2020; Shabayek et al., 2022; Amin et al., 2023**). The current study aimed to characterize the vaginal microbiome of Egyptian pregnant women in Ismailia, sampled at late pregnancy, using 16S rRNA next-generation sequencing technology. We further evaluated potential correlations between the microbiota comprising the vaginal microenvironment.

2. Methods

2.1 Ethics statement

All study procedures involving human subjects were reviewed and approved by the

Research Ethics Committee at the Faculty of Pharmacy, Suez Canal University, Egypt (Reference number 201811RH2). The study was conducted in accordance with all applicable ethical regulations. All participants provided their consent with knowledge.

2.2 Participants and recruitment

A cross-sectional study was conducted at the Gynecological Clinic of the Suez Canal University Hospital in the period from December 2018 to February 2019. A total of 44 healthy pregnant women during the third trimester were enrolled in the study. One vaginal swab was collected from the vaginal introitus. The inclusion and exclusion criteria were as described before (**Aagaard et al., 2012; Serrano et al., 2019**).

2.3 DNA extraction and 16S rRNA next-generation sequencing

Microbial DNA extraction was done by DNeasy PowerSoil Kit cat no. 12888-100 (Qiagen, Valencia, CA) according to the manufacturer's instructions. The Illumina MiSeq standard protocol of 16S rRNA gene amplicons (https://support.illumina.com/documentation.html) was followed. Amplification of the V3–V4 regions of 16S rRNA was done using the following primer pair: (Forward 5'-TCGTCGGCAGCGTCAGATGTGTATAA GAGACAGCCTACGGGNGGCWGCAG – 3′) and (Reverse 5'-GTCTCGTGGGCTCGGAGATGTGTATA AGAGACAGGACTACHVGGGTATCTA ATCC – 3′). Negative controls including an empty swab and extraction reagents were also amplified to reduce the likelihood of contamination. Library preparation and sequencing on Illumina MiSeq Platform (Illumina, San Diego, CA) in 300 bp paired-end mode was done at IGA Technology Services (Udine, Italy).

2.4 Data processing and statistical analysis

FastQC was applied to check sequence quality (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Illumina MiSeq sequences were prepared for 16S rRNA microbiome analysis using Quantitative Insights into Microbial Ecology (QIIME, version 1.9.1) pipeline (**Caporaso et al., 2010**). Sequences showing poor quality were trimmed with the QIIME script split_liberaries.py. All sequences displayed more than 1000 high-quality amplicon reads. The databases Greengenes (**DeSantis et al., 2006**) version 13.8 and SILVA (**Quast et al., 2013**) version 138 were used for the taxonomic assignment of Operational Taxonomic Units (OTUs) with 97% sequence homology. Rarefaction, alpha-diversity, beta-diversity, core microbiome, taxa abundance, and Linear discriminant analysis (LDA) effect size (LEfSe) were performed using Microbiomeanalyst (**Dhariwal et al., 2017; Chong et al., 2020**) platform (https://www.microbiomeanalyst.ca).

Statistical significance was evaluated using Kruskal Wallis rank-sum test and Wilcoxon rank-sum test. FDR-adjusted p values < 0.05 were considered statistically significant. For LEfSe analysis, p values < 0.05 and LDA scores greater than > 2 were considered statistically significant. Correlation analysis was done in R on the most abundant taxa. Spearman correlation was performed using the R package "Hmisc". Taxa correlations

showing r values ≥ 0.6 and p values < 0.05 were considered statistically significant.

2.5 Data availability

Raw sequences are available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA833599 under the Bio project accession number PRJNA833599 in the NCBI database with SRA.

3. Results

3.1 Characteristics of 16S rRNA sequence

Sequence processing yielded 3,299,595 reads with an average of 74, 990 per sample. These resulted in 1793 and 2215 OTUs as revealed by Greengenes and SILVA respectively. Sufficient sequencing depth was achieved as revealed by alpha-rarefaction.

3.2 The core microbiome

The databases Greengenes and SILVA revealed comparable taxonomic assignments. However, taxa assignation to the species level was more powerful using SILVA. The results obtained using these two databases could be integrated with no obvious discrepancies. Only the results for SILVA database are illustrated in the provided figures within the main text.

Firmicutes was the most abundant phylum dominating the vaginal microbiome of pregnant women during the third trimester. This was followed by the phyla *Actinobacteria* then *Proteobacteria*, *Bacteriodetes*, and *Tenericutes*. The most predominant families were *Lactobacillaceae* and *Bifidobacteriaceae*. The families *Enterobacteriaceae*, *Veillonellaceae*, *Prevotellaceae*, and *Mycoplasmataceae*

appeared as minorities. The genera with the highest relative abundance were *Lactobacillus*, followed by *Gardnerella*, *Prevotella*, and *Ureaplasma*. The species *L. iners* was the single predominant *Lactobacillus* species. According to LEfSe analysis, no significant features were identified in respect to parity when comparing primigravida and multigravida. Vaginal bacterial community composition on phylum, genus, and species levels for individual samples is shown in panels A and B of Figures 1, 2, and 3.

3.3 Alpha-diversity and Beta-diversity

There were no significant differences within vaginal microbial communities of pregnant women during the third trimester in respect to parity as revealed by the alpha-diversity indices Simpson ($p = 0.71$), Shannon ($p = 0.71$), and Chao1 ($p = 0.48$) (Figure 4 panels A, B, and C). Beta-diversity showed no statistically significant differences in the composition of the vaginal microbial communities of pregnant women during the third trimester as revealed by the Bray-Curtis ($p = 0.78$) and Jaccard ($p = 0.62$) dissimilarity indices. No significant differences in the microbiota profiles were found between primigravida and multigravida (Figure 4 panels D and E).

3.3 Correlation analysis

(A)

(B)
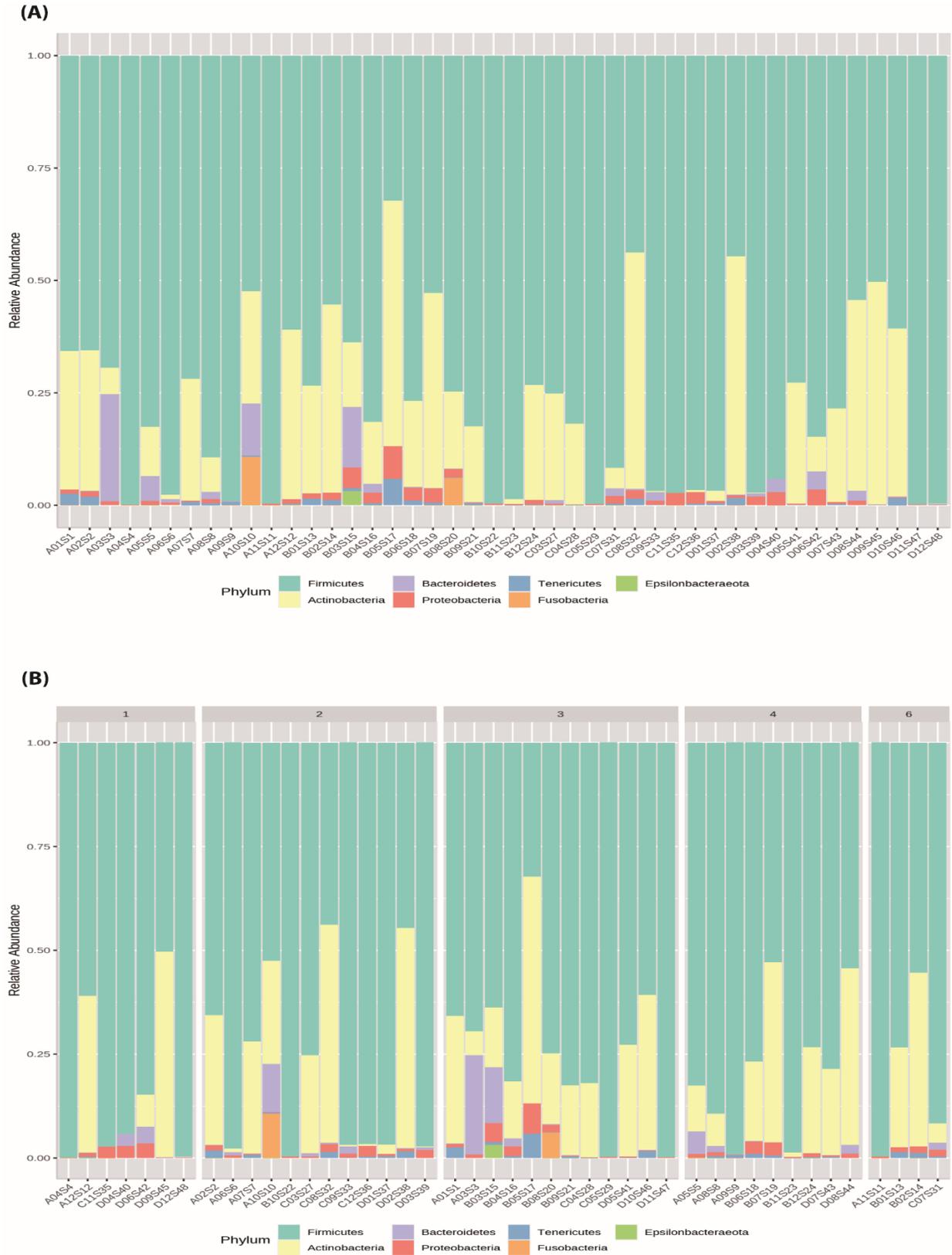
Figure 1: Phyla relative abundance in the vagina of pregnant Egyptian women during the third trimester. **(A)** Stacked bar charts represent relative proportions of the most abundant phyla. **(B)** Stacked bar charts represent relative proportions of the most abundant phyla in respect to parity. Each bar represents one sample.

(A)

(B)

Figure 2: Genera relative abundance in the vagina of pregnant Egyptian women during the third trimester. **(A)** Stacked bar charts represent relative proportions of the most abundant genera. **(B)** Stacked bar charts represent relative proportions of the most abundant genera in respect to parity. Each bar represents one sample.

Figure 3: Species relative abundance in the vagina of pregnant Egyptian women during the third trimester. **(A)** Stacked bar charts represent relative proportions of the 10 most predominant species. **(B)** Stacked bar charts represent relative proportions of the 10 most predominant species in respect to parity. Each bar represents one sample.
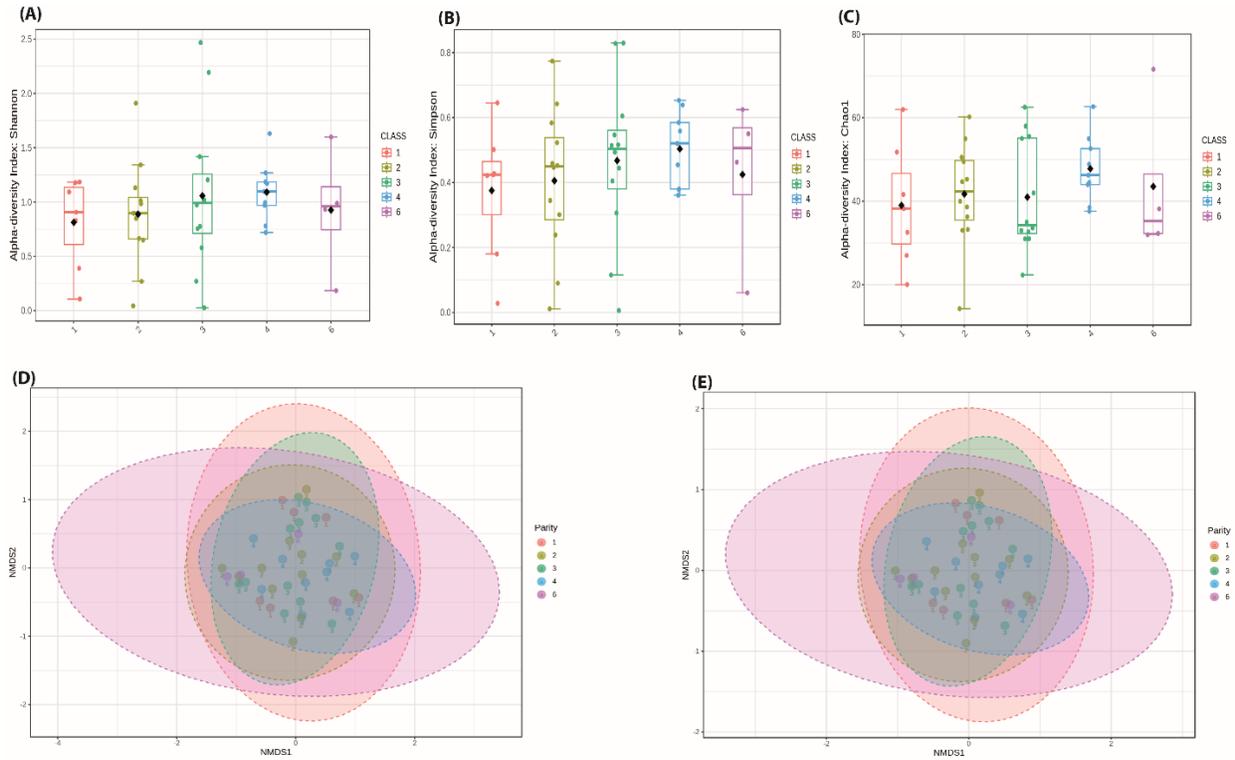
Figure 4: Alpha and Beta diversity indices in vagina of pregnant Egyptian women during the third trimester in respect to parity. **(A)** Box plot of Shannon alpha-diversity index. **(B)** Box plot of Simpson alpha-diversity index. **(C)** Box plot of Chao1-alpha diversity index. **(D)** 2D PCoA clustering plot as revealed by Bray-Curtis beta-diversity dissimilarity index. **(E)** 2D PCoA clustering plot as revealed by Jaccard beta-diversity dissimilarity index. Each dot represents one sample.
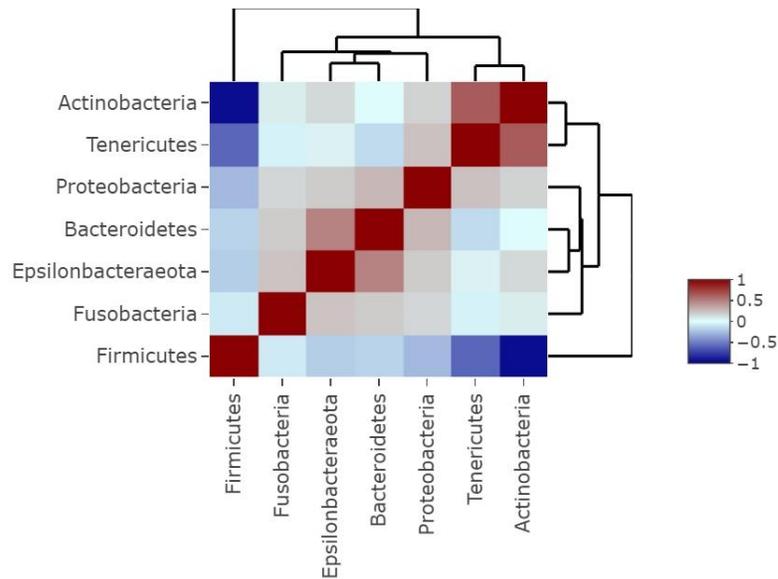
Figure 5: Correlation plot of dominant phyla in the vagina of pregnant Egyptian women during the third trimester. Red squares represent positive correlations while blue squares represent negative correlations. The plot was generated based on Spearman correlation coefficient.
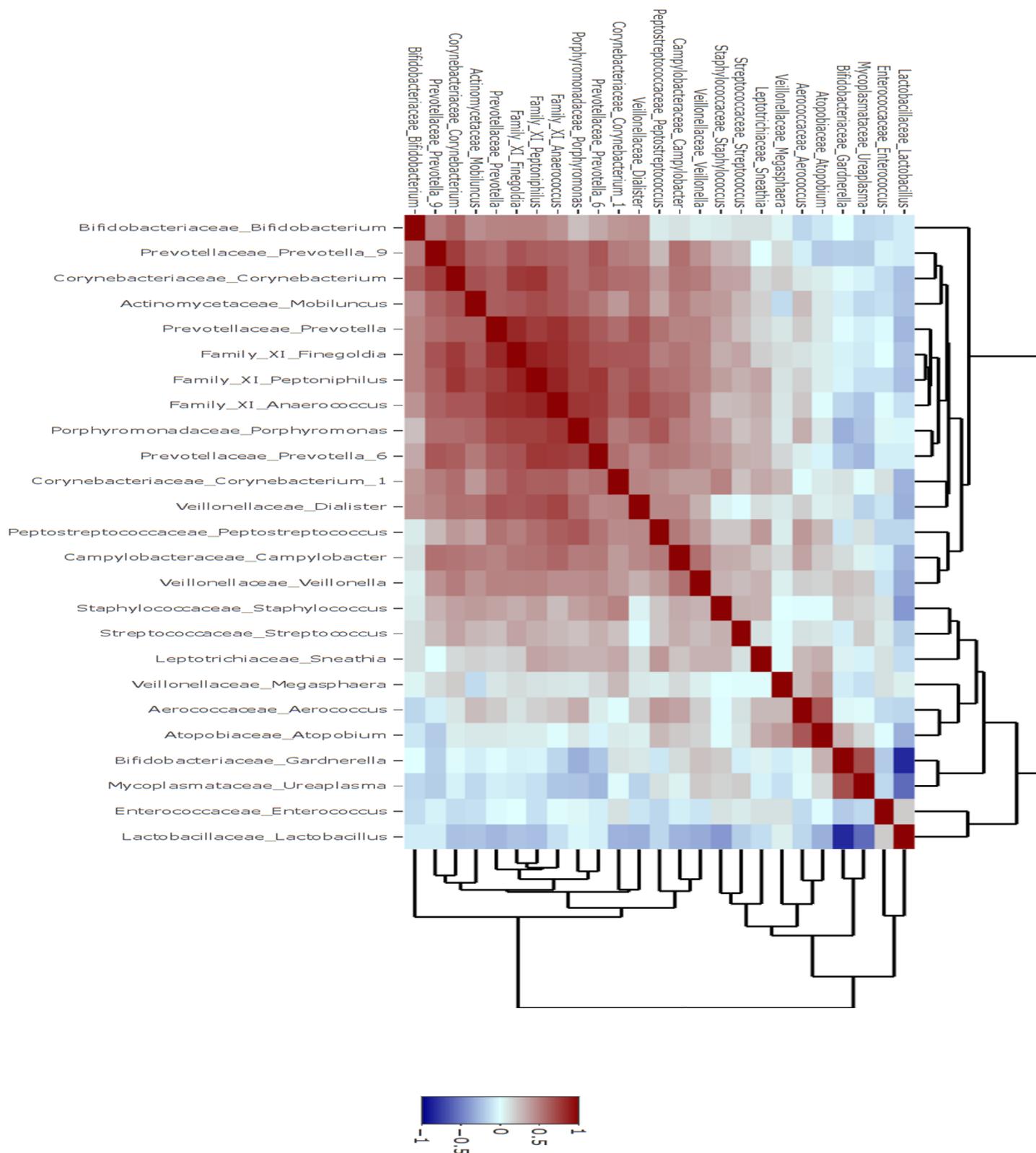
Figure 6: Correlation plot of dominant genera in the vagina of pregnant Egyptian women during the third trimester. Red squares represent positive correlations while blue squares represent negative correlations. The plot was generated based on Spearman correlation coefficient.
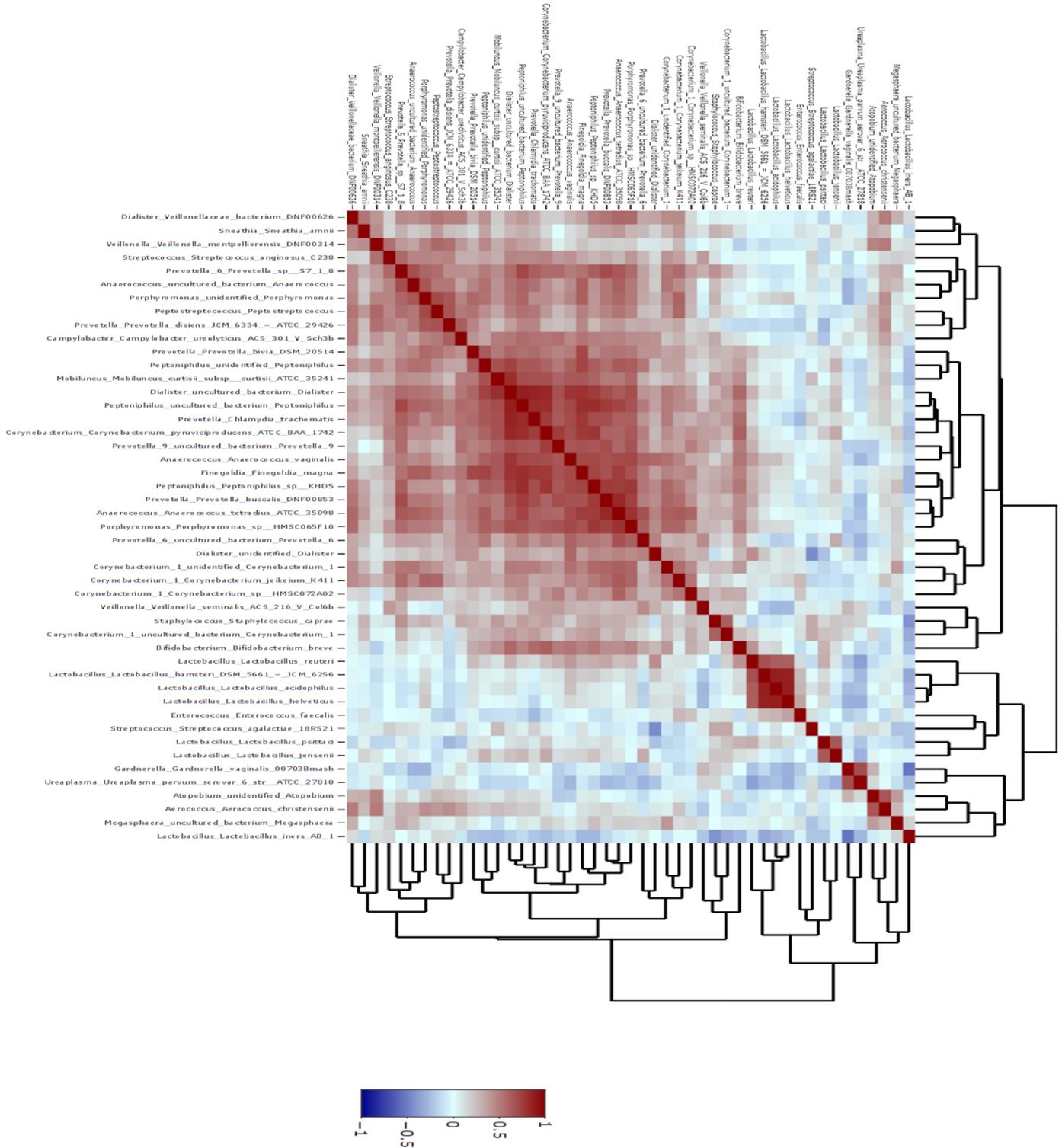
Figure 7: Correlation plot of dominant species in the vagina of pregnant Egyptian women during the third trimester. Red squares represent positive correlations while blue squares represent negative correlations. The plot was generated based on Spearman correlation coefficient.

The phylum *Fimicutes*, the genus *Lactobacillus* and the species *L. iners* were negatively correlated with almost all other taxa. However, a highly significant positive correlation cluster was detected between the species *L. acidophilus*, *L. hamster*, *L. helveticus*, and *L. reuteri*. In addition, a highly significant correlation cluster pair was detected between *L. jensenii* and *L. psittaci*.

Strong positive correlation pairs were identified between the phyla *Actinobacteria* and *Tenericutes*. Further highly positive correlation pairs were detected between the genera *Gardnerella* and *Ureaplasma*. This was confirmed by the significant correlation between the species *G. vaginalis* and *U. parvum*. Other positive correlation cluster pairs included *Staphylococcus-Streptococcus*, *Staphylococcus-Corynebacterium*, and *Aerococcus-Atopobium*. The genus *Megasphera* was positively correlated with the *Aerococcus-Atopobium* cluster pair. A large positive polymicrobial correlation cluster was detected between the genera *Anaerococcus, Campylobacter, Corynebacterium, Dialister, Fingoldia, Mobiluncus, Peptoniphilus, Peptostreptococcus, Porphyromonas, Prevotella, Veillonella,* and *WAL_1855D*. All these clusters were further confirmed on the species level. Correlations between the most abundant taxa are illustrated in Figures 5, 6, and 7.

4. Discussion

In the present study, we investigated the vaginal microbiome of pregnant women during their third trimester in Ismailia, Egypt using 16S rRNA next-generation sequencing

of the V3-V4 region. Previous reports (**Graspeuntner et al., 2018**) demonstrated this region is powerful in providing intense bacterial discrimination of vaginal microbiota.

L. iners was the most abundant *Lactobacillus* species comprising vaginal microbial communities. *L. iners*-dominant vaginal microbiomes have been consistently reported as a notable characteristic feature of healthy African women (**Gautam et al., 2015; Jespers et al., 2015; Serrano et al., 2019; Juliana et al., 2021; Shabayek et al., 2022; Amin et al., 2023**) compared to *L. crispatus*-dominant vaginal microbiomes among healthy European women (**Di Simone et al., 2020**). However, *L. iners* has also been reported as a predominant biomarker for vaginal dysbiosis or less stable vaginal ecosystems (**Zheng et al., 2021**) whereas *L. crispatus* was associated with good vaginal health (**Zheng et al., 2021**). Hence, the role of *L. iners* in promoting vaginal health remains controversial. Other features associated with African vagitypes included the common relative abundance of *Ca. Lachnocurva vaginae* (previously named BVAB1) (**Ravel et al., 2011; Fettweis et al., 2014**). Previous literature demonstrated *Ca. Lachnocurva vaginae* is significantly associated with adverse pregnancy outcomes (**Fettweis et al., 2019; Serrano et al., 2019**). However, such species was neither detected by Greengenes nor by SILVA databases in our study. This was consistent with a recent report investigating the longitudinal compositional changes of the vaginal microbiome of pregnant women in Ismailia, Egypt as well (**Amin et al., 2023**). Regional differences and ethnicity seem to have a

significant impact on the structure of the vaginal microbiome.

In consistency with (**Ravel et al., 2011; Ng et al., 2021; Amin et al., 2023**), *L. iners* was negatively correlated with almost all vaginal co-colonizers. The same scenario was found for the genus *Lactobacillus* and the corresponding phylum *Firmicutes*. This confirms the inhibitory role of *Lactobacillus* in preventing the expansion of invading or opportunistic vaginal pathobionts. A similar inhibitory feature was demonstrated for *L. crispatus*-dominant vagitypes (**Ng et al., 2021**).

On the other hand, we found several significant positive mutualistic relationships between non-*Lactobacillus* members. These included significant positive correlations between *Gardnerella* and *Ureaplasma*. Furthermore, a highly significant positively correlated polymicrobial cluster was detected between the genera *Anaerococcus*, *Campylobacter, Corynebacterium, Dialister, Fingoldia, Mobiluncus, Peptoniphilus, Peptostreptococcus, Porphyromonas, Prevotella, Veillonella*, and *WAL_1855D*. Most of these genera were shown to be associated with pregnancy complications (**Romero et al., 2014a; Brown et al., 2016; Nelson et al., 2016; Nasioudis et al., 2017; Dunlop et al., 2021; Liu et al., 2022; Park et al., 2022**). However, the adverse effects of co-existing vaginal pathogens have been related to the relative abundance of *Lactobacillus*. Depleted *Lactobacillus*-vaginal ecosystems were highly prone to adverse pregnancy complications like PTB (**Fettweis et al., 2019; Park et al., 2022**). In contrast, under *Lactobacillus*-dominant vaginal ecosystems, co-existing genera were suggested to promote normal pregnancy and full-term birth (**Park et al., 2022**). Moreover, it has been proposed that microbial vaginal colonization, in terms of presence and absence, is not enough to induce infection or pregnancy complications (**Park et al., 2022**). Dominant bacterial taxa seem crucial determinants of vaginal health and pregnancy outcomes.

5. Conclusion

L. iners-dominant vaginal microbiomes were the most prevalent among pregnant women during the third trimester in Ismailia, Egypt. The phylum *Firmicutes*, the genus *Lactobacillus* and the species *L. iners* appear as competitive inhibitors of co-existing vaginal taxa. Urogenital vaginal pathogens seem to promote normal pregnancy rather than PTB under *Lactobacillus*-dominant vaginal microbial communities. Larger cohorts characterizing the vaginal microbiota in Egypt are warranted.

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