Journal of Microbiology
https://doi.org/10.1007/s12275-023-00039-3

MICROBIAL PATHOGENESIS AND HOST-MICROBE INTERACTION

Vaginal Microbiome Dysbiosis is Associated with the Different Cervical Disease Status

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Received: 4 January 2023 / Revised: 20 February 2023 / Accepted: 6 March 2023

Abstract

Vaginal microbiome composition was demonstrated to be associated with cervical disease. The colonization characteristics of vaginal microbes and their association with the different cervical disease status, especially cervical cancer (CC), are rarely investigated. In this cross-sectional study, we characterized the vaginal microbiome of women with different status of cervical diseases, including 22 NV + (normal tissue with HPV infection), low-grade squamous intraepithelial lesion (LSIL, $n = 45$), high-grade squamous intraepithelial lesion (HSIL, $n = 36$) and CC ($n = 27$) using bacterial 16S DNA sequencing. Thirty HPV-negative women with normal tissue were used as the control group. We found that higher diversity of microbiome with gradual depletion of *Lactobacillus*, especially *L. crispatus,* was associated with the severity of cervical disease. High-risk HPV16 infection was associated with higher microbiome diversity and depletion of *Lactobacillus* in high-grade cervical diseases (i.e. HSIL and CC). The CC group was characterized by higher levels of *Fannyhessea vaginae*, *Prevotella*, *Bacteroides*, *Finegoldia*, *Vibrio*, *Veillonella*, *Peptostreptococcus*, and *Dialister*. Co-occurrence network analyses showed that negative correlations were exclusively observed between *Lactobacillus* and other bacteria, and almost all non-*Lactobacillus* bacteria were positively correlated with each other. In particular, the most diverse and complex co-occurrence network of vaginal bacteria, as well as a complete loss of *L. crispatus*, was observed in women with CC. Logistic regression model identified HPV16 and *Lactobacillus* as significant risk and protective factors for CC, respectively. These results suggest that specific *Lactobacillus* species (e.g. *L. crispatus* and *L. iners*) can be used as important markers to target prevention measures prioritizing HPV16-infected women and other hrHPV-infected women for test, vaccination and treat initiatives.

Keywords Vaginal microbiome · Cervical cancer · Dysbiosis · Disease status · HPV

Introduction

Cervical cancer (CC) is the fourth most common cancer among women, which brings a serious public health problem worldwide. Persistent high-risk human papillomavirus (hrHPV) infections are major risk factors for the development of CC. Other co-factors, such as cervical microenvironment, systemic immune response, and hygiene habits, are believed to be involved in and/or contribute to the progression of cervical cancer.

Cervical microenvironment, including microbiota and the mucosal and immune system, plays an important role in the maintaining of reproductive tract health and the process of the cervical diseases (Mitra et al., 2015). Vaginal microbiome composition varies in different populations, and could be affected by many factors, such as sexual activity, preterm, estrogen and age (Kroon et al., 2018; Torcia, 2019). A healthy vaginal microbiota characterized by *Lactobacillus*

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Published online: 03 April 2023

spp. is an important feature in maintaining a healthy vaginal microenvironment among most women. *Lactobacillus* spp. play a crucial role in maintaining a low pH environment of vagina by producing lactic acid, bacteriocins and biosurfactants, and the acidic microenvironment is essential for the normal function of cervical epithelial barrier to prevent anaerobes colonization or opportunistic infections (Aroutcheva et al., 2001; Boskey et al., 2001). However, under disease status, vaginal microbiome is characterized by increased microbiome diversity and an enrichment of anaerobic bacteria, implying a dysbiosis of the vaginal microbiota. For example, the enrichment of *Sneathia*, *Megasphaera*, *Prevotella*, *Gardnerella* and/or *bacterial vaginosis-associated bacteria* (*BVAB*) *1* and *2* were often associated with HPV infection (especially infection with hrHPV types) (Cheng et al., 2020; Lin et al., 2022; Usyk et al., 2020). According to the relative abundance of bacterial compositions, the vaginal microbiota has been classified into five major community state types (CSTs I-V). CSTs I, II, III, and V were dominated by *Lactobacillus crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*, respectively. CST IV was dominated by anaerobic bacteria (Ravel et al., 2011). Furthermore, vaginal microbiota was characterized by higher levels of *Sneathia sanguinegens*, *Anaerococcus tetradius* and *Peptostreptococcus anaerobius* in patients with HSIL compared to those with high-grade squamous intraepithelial lesions (LSIL) (Audirac-Chalifour et al., 2016; Mitra et al., 2015). In patients with CC, *Fusobacterium* spp.-dominated vaginal microbiota was often observed (Audirac-Chalifour et al., 2016).

Although previous studies reported the altered microbiome in the CC patients, and revealed the association of some vaginal bacteria taxa with certain cervical disease (Audirac-Chalifour et al., 2016; Cheng et al., 2020; Mitra et al., 2015), few studies were conducted to characterize and compare the vaginal microbiome composition among different cervical disease status. Furthermore, little is known about the bacteria-bacteria interaction in different cervical disease status. In this study, we characterized the vaginal microbiome composition and diversity, and investigated the correlations of vaginal microbes in different cervical disease status (LSIL, HSIL, and CC). We concluded that increased vaginal microbiome diversity with the depletion of *Lactobacillus*, as well as HPV16 infection, are associated with high-grade cervical diseases (HSIL and CC).

Materials and Methods

Study Population

Women with cervical diseases were recruited from those who visited the Obstetrics and Gynecology Hospital of Fudan University for treatment of gynecological diseases during April 2021 and July 2021. Women during pregnancy, menstrual period, using antibiotics during the past two weeks, and having a history of cervical treatment were excluded from this study. Cervical status was firstly diagnosed according to cytology and HPV test results. Colposcopy and biopsy were carried out to confirm the cervical diseases status for women with abnormal cytology and HPV-positive women with normal cytology. As a result, 45 women with LSIL, 36 with HISL, 27 with CC, and 22 NV + (normal tissue with HPV infection) were included in this study. Thirty HPV-negative women with normal cytology were selected as a control group (NV-). Demographic, basic clinical feature and detailed medical history were recorded upon their visits (Table 1).

Sample Collection

A total of 160 mid-vaginal swabs samples were collected by the clinicians, placed in virus transport medium (Yocon) and stored at − 80℃ until use. The samples were previously used for investigating the vaginal virome (Li et al., 2022) and here we investigated vaginal microbiota and its association with cervical diseases. After adding beads to crush the vaginal swabs, the supernatant was used for extraction of the bacterial DNA using TIANGEN bacterial DNA Kit (TIANGEN). DNA extraction and purification can well remove the potential influence of transport medium on nucleic acid amplification and subsequent next generation sequencing (NGS) by Illumina (Li et al., 2022; Xu et al., 2021a, 2021b).HPV infection was detected by BioPerfectus HPV Genotyping Real Time PCR kit (BioPerfectus Technologies), which covers 21 high and low risk HPV subtypes (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -26, -53, -66, -73, -82, -6, -11, and -81)". The V4 hypervariable region (515–806 nt) of bacterial 16S rRNA gene was amplified by two rounds of PCR as previously described (Xu et al., 2021a, 2021b). The amplicon libraries were purified and then subjected to NGS by Illumina MiSeq.

16S rRNA Gene Sequence Analysis

The raw data of NGS were first filtered by Trimmomatic v.0.39 to remove Illumina sequencing adaptors and low-quality sequences (SLIDINGWINDOW:5:20 MINLEN:50) (Bolger et al., 2014). The Dual-sequencing reads were merged using Vsearch v.2.18 and demultiplexed by Fastx_ toolkit, then the barcode and primer sequences were trimmed using Vsearch v.2.18 (Rognes et al., 2016). The DADA2 was used for quality filtering, dereplication and denoising with default settings and the length of sequence was truncated to 250 bp in QIIME 2 platform (https:// qiime2.org) (Caporaso et al., 2010). We finally obtained 2291 amplicon sequence variants (ASVs). The taxonomic classification of ASVs

Table 1 Patient characteristic of 160 patients included in study cohort

Variables		NV- ($n=30$)	NV + ($n=22$)	LSIL ($n=45$)	HSIL ($n=36$)	CC ($n=27$)	Total ($n=160$)	*P* value[a]
Age, years	Median (range)	32.5 (23–63)	43.5 (29–74)	40 (26–69)	34 (23–69)	50.5 (20–75)	39 (20–75)	0.0004
Age of first sexual intercourse	Median (range)	23.5 (16–31)	22.5 (18–26)	22 (20–27)	21 (16–31)	22 (15–28)	22 (15–31)	0.6904
Current/previous vaginal douching, *n* (%)	Yes	2 (6.7)	5 (22.7)	3 (6.7)	5 (13.9)	3 (11.1)	18 (11.2)	0.343
	No	28 (93.3)	17 (77.3)	42 (93.3)	31 (86.1)	20 (74.1)	138 (86.3)	
	Unknown	0 (0)	0 (0)	0 (0)	0 (0)	4 (14.8)	4 (2.5)	
Contraception, *n* (%)	Nil	13 (43.3)	12 (54.5)	15 (33.3)	11 (30.6)	12 (44.4)	63 (39.4)	0.237
	Condoms	16 (53.3)	6 (27.3)	20 (44.4)	17 (47.2)	7 (25.9)	66 (41.3)	
	Copper IUD + Ligation	1 (3.4)	3 (13.6)	9 (20)	7 (19.4)	6 (22.2)	26 (16.3)	
	Oral hormonal contraception	0 (0)	0 (0)	1 (2.2)	1 (2.8)	2 (7.4)	4 (2.5)	
	Unknown	0 (0)	1 (4.6)	0 (0)	0 (0)	0 (0)	1 (0.6)	
Number of abortion	Median (range)	1 (0–5)	1 (0–5)	1 (0–4)	1 (0–11)	1 (0–4)	1 (0–11)	0.3171
Number of sexual partners	Median (range)	1 (1–24)	1 (1–4)	1 (1–8)	2 (1–8)	1 (1–24)	1 (1–24)	0.2712
Menopause, *n* (%)	Yes	3 (10.0)	6 (27.3)	7 (15.6)	4 (11.1)	12 (44.4)	32 (20.0)	0.008
	No	27 (90.0)	16 (72.7)	38 (84.4)	32 (88.9)	15 (55.6)	128 (80.0)	
HPV status, *n* (%)	Positive	0 (0)	22 (100)	41 (91.1)	34 (94.4)	21 (77.8)	118 (73.8)	0.654
	Negative	30 (100)	0 (0)	3 (6.7)	1 (2.8)	2 (7.4)	36 (22.5)	
	ND	0 (0)	0 (0)	1 (2.2)	1 (2.8)	4 (14.8)	6 (3.8)	
High/Low-risk HPV status	hrHPV		19 (86.4)	37 (90.2)	31 (91.2)	20 (95.2)	107 (90.7)	0.810
	lrHPV		3 (13.6)	4 (9.8)	3 (8.8)	1 (4.8)	11 (9.3)	
HPV16 status	Positive		3 (13.6)	6 (14.6)	20 (58.8)	13 (61.9)	42 (35.6)	0.000
	Negative		19 (86.4)	35 (85.4)	14 (41.2)	8 (38.1)	76 (64.4)	
HPV18 status	Positive		3 (13.6)	5 (12.2)	3 (8.8)	4 (19)	15 (12.7)	0.720
	Negative		19 (86.4)	36 (87.8)	31 (91.2)	17 (81)	103 (87.3)	
Number of HPV types	Single		13 (59.0)	27 (65.9)	22 (64.7)	11 (52.4)	73 (61.9)	0.745
	Two or more		9 (41.0)	14 (34.1)	12 (35.3)	10 (47.6)	45 (38.1)	

[a]Fisher's exact test and Kruskal–Wallis test were used for the comparison of five groups (NV-, NV + , LSIL, HSIL, and CC).

IUD intrauterine-device, *ND* not detected

representative sequences was performed using the Naive Bayesian Classifier algorithm based on the Silva database at genus level (Quast et al., 2013). To eliminate the sequences bias among samples, the ASVs table were randomly subsampled to the lowest read count of 5295, providing a minimum coverage of 98.16% for all samples.

Characteristics of Vaginal Microbial Composition

The vaginal microbiome composition at the genus level was analyzed using the Statistical Analysis of Metagenomic Profiles (STAMP) package (v.2.1.3). The vaginal microbiota composition at the species level was classified based on the previously described vaginal community state types (CSTs) I–V (Ravel et al., 2011). Shannon index, Inverse Simpson Index and Sobs (species observed) were calculated using the Vegan package in RStudio v.3.8. Welch's *t* test was used to compare the relative abundance of specific species among Normal-LSIL, HSIL and CC groups. Lefse analysis was performed to identity discriminative vaginal microbiota features. The linear discriminant analysis (LDA) was used to analyze specific taxa among Normal-LSIL, HSIL and CC groups based on the relative abundance of each taxon (Segata et al., 2011). The microbial genera appearing in at least 30% of samples were selected as core microbiome composition for microbial co-occurrence network analysis. The Spearman's "r" higher than 0.5 or lower than − 0.5 with *P* value that was below 0.05 after the FDR adjustment was considered as a significant correlation. Co-occurrence network of correlated bacterial genus pairs was visualized using Gephi v.0.9.2.

Statistics

Differences between groups were compared with Fisher's exact test, chi-squared test, non-parametric Kruskal–Wallis Test or *T* Test implemented in SPSS 26.0 and Graphpad Prism 6.0. A *P* value less than 0.05 was considered statistically significant. A stepwise logistic regression model with backward elimination was used to determine the risk factors of CC. Eleven variables, including age, menopause, HPV16 status, and the microbes (*Lactobacillus*, *Vibrio*, *Bacteroides*, *Finegoldia*, *Prevotella*, *Atopobium*, *Streptococcus*, and *Gardnerella*) with an abundance difference at the significance level of less than 0.05 between CC and non-CC groups were subjected to the logistic regression analysis.

Results

Study Cohort and HPV Types with Different Cervical Disease Status

A total of 160 women were classified into five groups: NV- ($n=30$), NV + ($n=22$), LSIL ($n=45$), HSIL ($n=36$), and CC ($n=27$). The demographic characteristics of each group was detailed in Table 1. Of twelve variables, age, menopause and HPV16 infection status were significantly different among groups. The median age of the CC group (50.5 years old) was higher than other four groups (32.5–43.5 years old). Compared to the NV-, NV + and LSIL groups, HPV16 was more prevalent in both the HSIL and CC groups, accounting for 58.8% and 61.9%, respectively (Table 1). In total, 20 HPV types were detected. 118 (73.8%) women were found to be positive for at least one HPV type (Table 1), and 45 (38.1%) of them were co-infected with at least two different types. HPV16 was the most prevalent (24.6%) type among all subjects, followed by HPV52 (11.4%), HPV58 (9.0%), and HPV18 (8.4%) (Fig. 1A). Among all hrHPV types, HPV16 infection percentage was significantly higher in the HSIL (45.0%) and CC (50.0%) groups than the NV + (12.0%) and LSIL (16.3%) groups ($P=0.001$) (Fig. 1B, C and Table S1).

The Vaginal Microbiome Composition was Related with Cervical Disease Severity

In total, 20,691,742 reads were obtained from 160 samples with an average of 129,323 reads per sample. The 16SrRNA gene sequences were resolved into 2291 amplicon sequence variants (ASVs), representing 20 known phyla and 265 known genera. The top 16 genera that accounted for 93.7% of the total sequence reads were used in the subsequent analyses, and other 249 genera were collapsed as "other". We identified two major groups of the bacterial community composition at the genus level using the hierarchical cluster analysis. The vaginal microbiome with *Lactobacillus* relative abundance of > 65.7% was classified as *Lactobacillus*-dominant, and the microbiome with *Lactobacillus* relative abundance of < 65.7% was classified as *Lactobacillus*-depleted (Fig. 2A). *Lactobacillus*-dominant and *Lactobacillus*-depleted microbiomes accounted for 58.8% (94/160) and 41.2% (66/160) of all subjects, respectively. The proportion of *Lactobacillus*-dominant subjects was more than 60% in NV-, NV + , LSIL and HSIL groups, and did not significantly

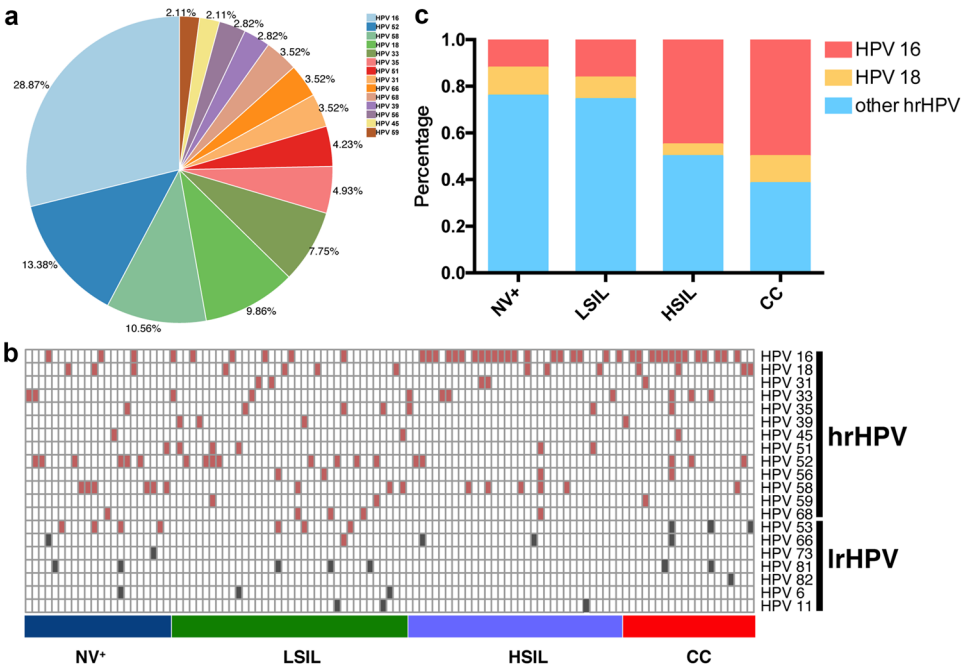

Fig. 1 The distribution of HPV types. **A** The pie chart shows the percentage of hrHPV type. **B** The heatmap showed the presence of hrHPV and lrHPV in four groups (NV + , LSIL, HSIL, and CC). **C** The relative percentage of HPV16, HPV18 and other hrHPV in four groups. The original data of the panel c are shown in Table S1

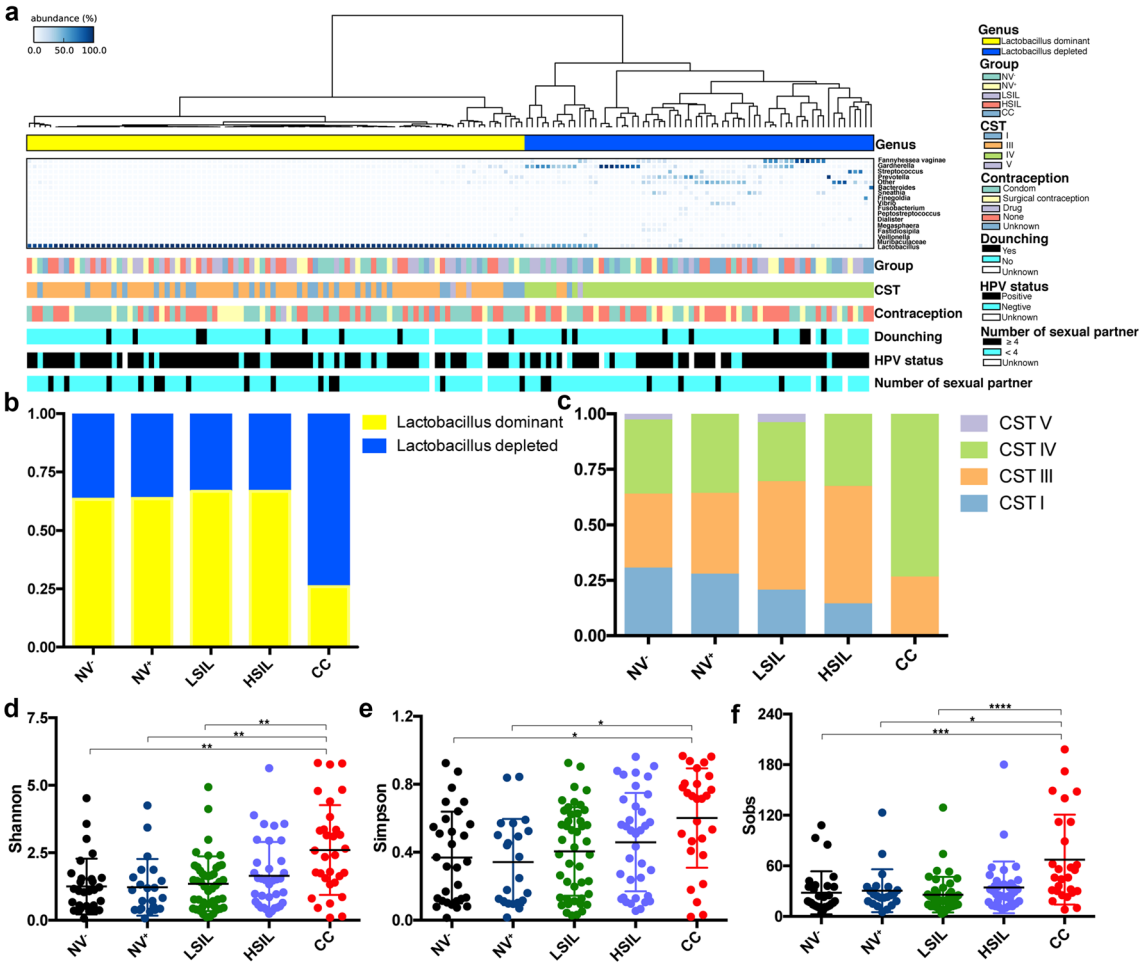

Fig. 2 The vaginal microbiome and diversity in five groups with different cervical disease status. **A** The heatmap shows the vaginal microbiome composition of 160 women in five groups. Top 16 genera are shown, with the rest genera to be defined as "other". The proportion of *Lactobacillus*-dominant and -depleted subjects **B** and CSTs **C** are shown in the groups with different cervical disease status. CST Community state type, CST I (*Lactobacillus crispatus*-dominant), CST III (*Lactobacillus iners*-dominant), CST V (*Lactobacillus jensenii*-dominant), CST IV (*Lactobacillus* spp.-depleted). Diversity and richness indices are shown by Shannon index **D**, Inverse Simpson **E** and the number of species observed (Sobs) **F**. Comparisons were performed using the Kruskal–Wallis test. $*P<0.05$; $**P<0.01$; $***P<0.001$. The original data of panel b and c are shown in Table S2

different among these groups (Fig. 2B, Table S2). Importantly, however, only 25.9% of the subjects in the CC group had a *Lactobacillus*-dominant microbiome. The depletion of *Lactobacillus* among the CC subjects was accompanied by an increase of other bacterial taxa, including *Prevotella*, *Bacteroides*, *Vibrio*, and *Finegoldia* (Fig. S1). In addition, a slight increase in the percentage of *Lactobacillus*-depleted subjects was observed in HPV16 positive women compared to negative individuals (Fig. S2B).

The distributions of different CSTs (I, III, V, and IV) among different groups were also analyzed. The percentage of CST I (*Lactobacillus crispatus*-dominant) gradually decreased as the cervical disease severity increased from 30% in the NV- group to zero in the CC group (Fig. 2C, Table S2). On the contrary, the percentage of CST III (*Lactobacillus iners*-dominant) gradually increased from 33.3% in the NV- group to 52.8% in the HSIL group. Only CST III and CST IV were detected in the CC group. The percentage of CST III was lower (25.9% vs. 33.3%-52.8%), whereas and the percentage of CST IV was higher (74.1% vs. 26.7%-36.4%) compared to other groups (Fig. 2C, Table S2). Compared to the HPV16 negative group, HPV16 positive group had a slightly higher CST IV and lower CST I percentages (Fig. S2B). No significant association was observed between other factors (including contraception methods, douching habits, and the sexual partner number) and vaginal microbiome composition (Fig. 2A).

Further analyses were performed to determine the vaginal bacterial diversity among the groups with different disease status. A similar trend was observed for Shannon

index, Inverse Simpson index and the number of the species observed (Sobs) (Fig. 2D, E, F). No significant difference was observed between the NV- and NV + groups (Shannon: $P > 0.05$; Simpsons: $P > 0.05$; Sobs: $P > 0.05$). Importantly, however, the bacterial diversity was significantly higher in HPV16 infection group than non-HPV16 infection group (Shannon: $P < 0.05$; Simpsons: $P < 0.05$; Sobs: $P < 0.05$) (Fig. S2A), and it appeared to increase along with the severity of cervical diseases. Women in the HSIL and CC groups had higher vaginal microbiome richness and diversity than those in NV + and LSIL groups. In particular, the CC group showed the highest richness and diversity ($P < 0.01$) (Fig. 2D, E, F). Similarly, the alpha diversity (Observed OUT number and Shannon index) was higher in the CC group than other groups in the rarefaction curve analyses (Fig. S3). This result matched with above observation that the CC group had the highest proportion of CST IV, which was characterized by the depletion of *Lactobacillus* and the presence of diverse anaerobic bacteria (e.g. *Fannyhessea vaginae*, *Gardnerella*, *Streptococcus*, *Prevotella*, and *Bacteroides*) (Fig. 2A).

Identification of the Vaginal Bacterial Markers

In order to identify the specific bacterial taxa associated with high-grade cervical diseases (HSIL and CC), we collapsed the NV-, NV + and the low-grade disease group LSIL as one group (Normal-LSIL) to perform further analysis. The CC group was characterized by significantly higher abundance of *Fannyhessea vaginae* (CC vs. Normal-LSIL, $P < 0.01$), *Prevotella* (CC vs. HSIL, $P < 0.05$; CC vs. Normal-LSIL, $P < 0.01$), *Bacteroides* (CC vs. HSIL, $P < 0.001$; CC vs. Normal-LSIL, $P < 0.0001$), *Finegoldia* (CC vs. HSIL, $P < 0.01$; CC vs. Normal-LSIL, $P < 0.05$), *Vibrio* (CC vs. HSIL, $P < 0.0001$; CC vs. Normal-LSIL, $P < 0.0001$), *Veillonella* (CC vs. Normal-LSIL, $P < 0.001$), *Peptostreptococcus* (CC vs. Normal-LSIL, $P < 0.01$), *Dialister* (CC vs. HSIL, $P < 0.05$; CC vs. Normal-LSIL, $P < 0.01$), *Sneathia* (CC vs. Normal-LSIL, $P < 0.01$), and *Megaphaera* (CC vs. Normal-LSIL, $P < 0.001$) (Fig. 3A, Mann–Whitney *U* test). The relative abundances of *Prevotella* (CC vs. HSIL, $P < 0.05$; CC vs. Normal-LSIL, $P < 0.01$), *Bacteroides* (CC vs. HSIL, $P < 0.001$; CC vs. Normal-LSIL, $P < 0.0001$), *Finegoldia* (CC vs. HSIL, $P < 0.01$; CC vs. Normal-LSIL, $P < 0.05$), *Vibrio* (CC vs. HSIL, $P < 0.0001$; CC vs. Normal-LSIL, $P < 0.0001$) and *Dialister* (CC vs. HSIL, $P < 0.05$; CC vs. Normal-LSIL, $P < 0.01$) were significantly higher in the CC group than the Normal-LSIL group and HSIL group, whereas the relative abundance of *Lactobacillus* (CC vs. HSIL, $P < 0.01$; CC vs. Normal-LSIL, $P < 0.001$) was significantly lower in the CC group than the HSIL and Normal-LSIL groups. Further, Lefse analysis supported that the CC group was associated with the enrichment of *Fannyhessea vaginae*, *Prevotella*, *Bacteroides*, *Finegoldia*, *Vibrio*, *Veillonella*, *Peptostreptococcus*, and *Dialister* genera.

The HSIL group had significant over-representation of *Sneathia*, *Fastidiosipila*, and *Megasphaera* genera, whereas the Normal-LSIL group had significant over-representation of *Lactobacillus* genus (Fig. 3B, C). To determine which factor contribute to the occurrence of CC, we performed a multivariate logistic regression analysis using representative bacteria taxa, age, contraception and HPV16 infection status. Age ($P = 0.012$, OR = 1.957) and HPV16 infection status ($P = 0.031$, OR 2.867) were identified as important risk factors for CC, whereas the *Lactobacillus* composition ($P = 0.006$, OR 0.197) appeared to be a protective factor to prevent the occurrence of CC (Table S3).

Co-occurrence Networks of the Vaginal Microbes in Different Cervical Disease Status

To investigate the bacteria-bacteria interaction in vaginal tract, we first performed a co-occurrence network analysis using all data. *Prevotella* and *Sneathia* appeared to be the main hubs connecting with other bacteria in the vaginal tract and most bacteria showed positive correlations with each other, expect *Lactobacillus* that exclusively displayed negative correlations with the anaerobic bacteria (e.g. *Gardnerella*, *Fannyhessea vaginae*, *Prevotella*, and *Dialister*) (Fig. S4).

To reveal the bacteria-bacteria interaction in different cervical disease status, we further analyzed the co-occurrence network of the vaginal bacteria in five groups. A more complex bacteria-bacteria co-occurrence network was observed in women with high-grade cervical diseases (HSIL and CC) compared to the control groups (NV- and NV +) and the LSIL group (Fig. 4). *Lactobacillus* was negatively correlated with several bacteria, and almost all non-*Lactobacillus* bacteria were linked by positive correlation. In the NV- group, *Lactobacillus*, *Gardnerella*, *Prevotella*, and *Fannyhessea vaginae* were the main bacterial taxa, and *Lactobacillus* was negatively correlated with *Gardnerella*. In the NV + and LSIL groups, more bacteria, such as *Sneathia* and *Dialister*, joined in the network and *Lactobacillus* was negatively correlated with two to three additional bacteria. Increased negative interactions between *Lactobacillus* and other bacteria were observed in the HSIL group. In the CC group, the bacteria-bacteria interaction further developed to a highly connected complex network with more non-*Lactobacillus* bacterias but no *Lactobacillus* involved. These results suggested that increased dysbiosis of vaginal microbiome was associated with high-grade cervical lesions.

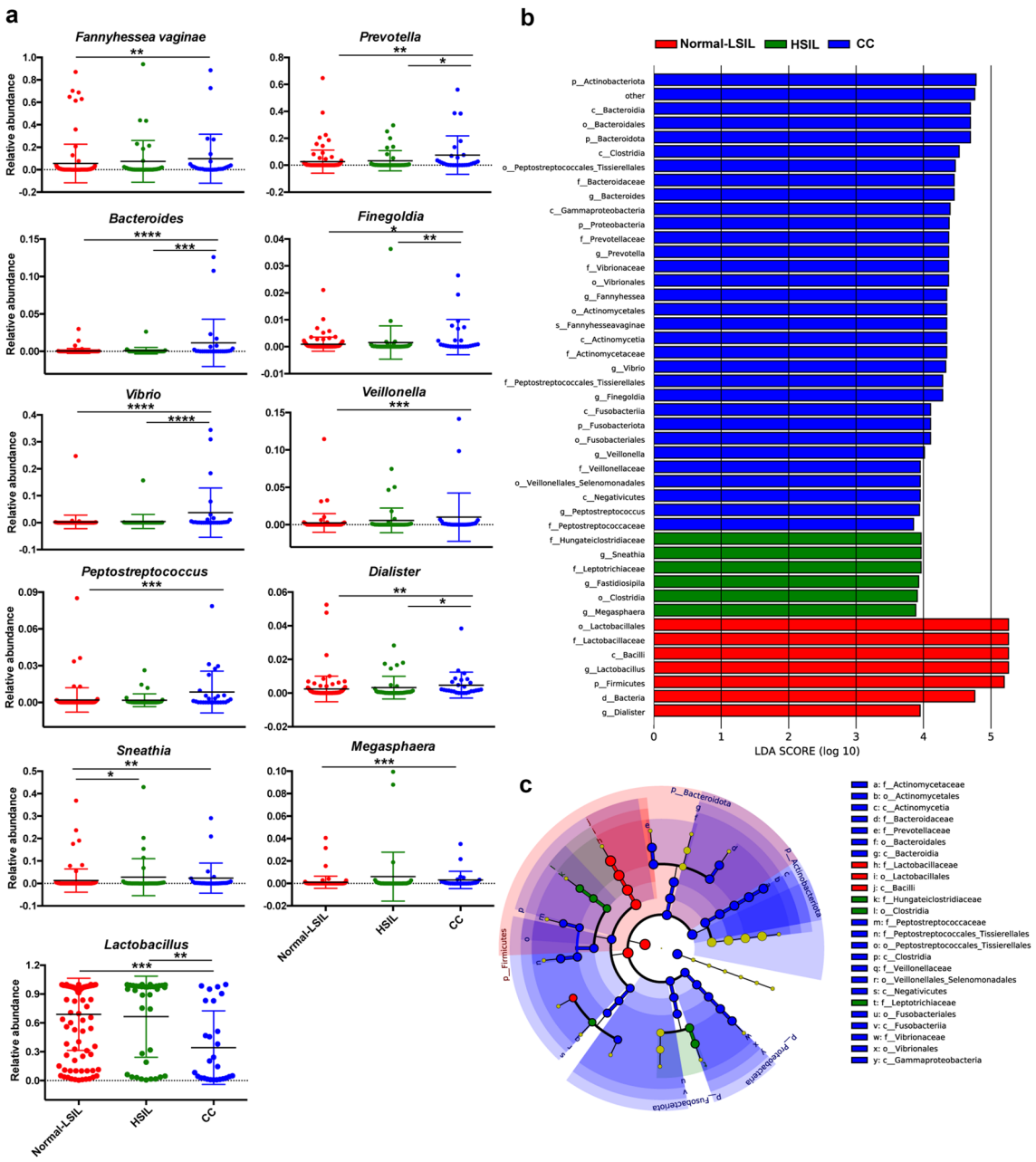

Fig. 3 Identification of vaginal microbiota biomarkers among Normal-LSIL, HSIL and CC. **A** Comparison of relative abundance of different bacterial genera. The three groups (NV-, NV+, and LSIL) were collapsed and redefined as Normal-LSIL group. Comparisons were performed using the Mann–Whitney *U* test. $*P<0.05$; $**P<0.01$; $***P<0.001$. **B** Histogram of the LDA scores were calculated for features that had differentially abundant among Normal-LSIL, HSIL and CC groups. **C** Cladogram representing taxa with different abundance according to different cervical disease status. Size of circle is proportionate to the relative abundance of each taxon

Discussion

Our results showed that higher diversity of microbiome with gradual depletion of *Lactobacillus*, especially *L. crispatus,* was associated with the severity of cervical disease. In addition, HPV16 infection was found to be highly associated with higher vaginal microbiome diversity and the depletion of *Lactobacillus*. This result further strengthens HPV16 infection to be the most important marker to target early screening and prevention (vaccination) measures to women with high-grade cervical diseases. We also found that more diverse and complex co-occurrence networks of vaginal microbes in high-grade disease status, especially CC, represent a result of synergistic effects of various microbes by bacteria-bacteria interactions, and indicate the destruction of a

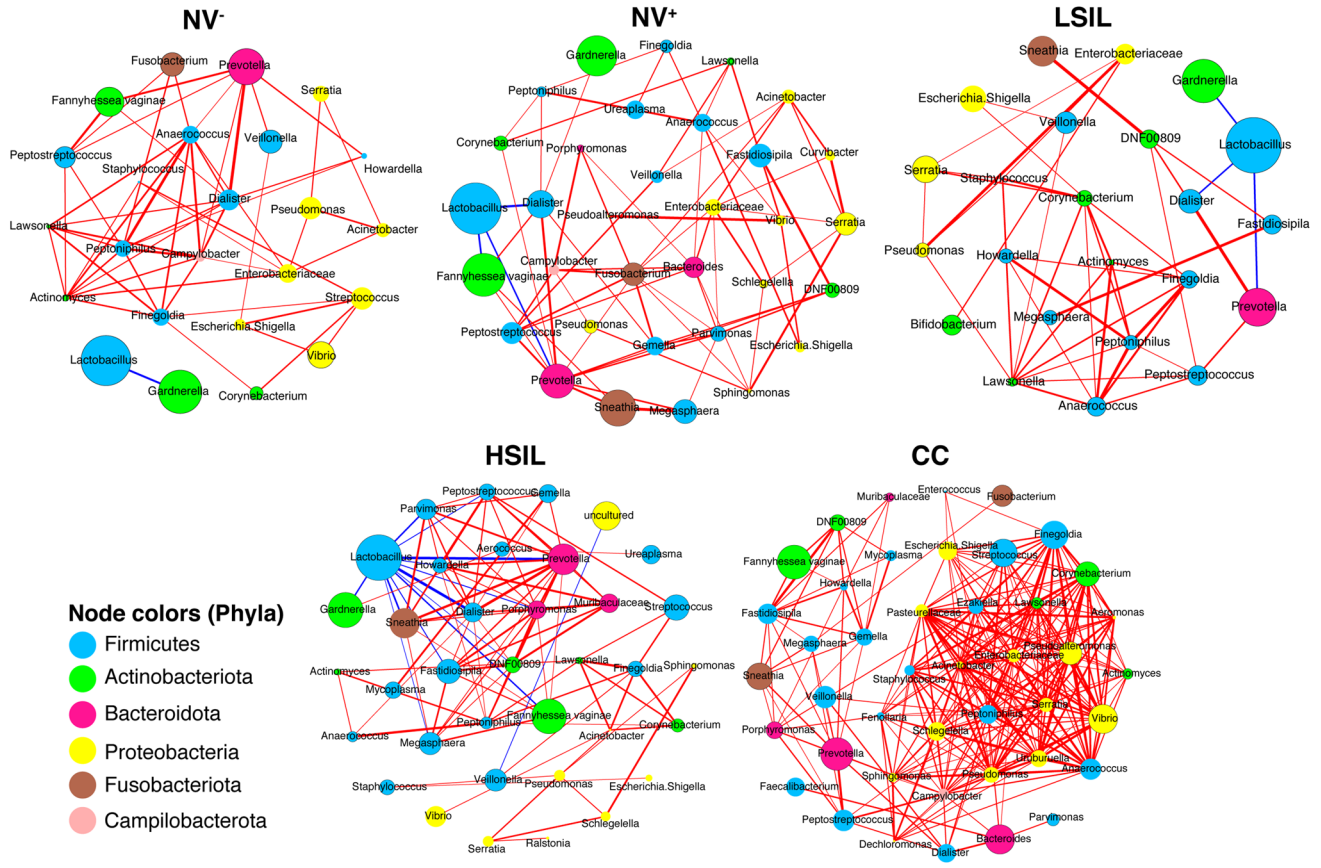

Fig. 4 Co-occurrence networks of vaginal bacteria in five groups with different cervical disease severity status. Bacteria genera appearing in at least 30% of samples were selected to perform Spearman correlation analysis. The relative abundance was normalized by the centered log ratio transformation, and correlated bacteria pairs with both Spearman correlation r values higher than 0.5 or lower than − 0.5 with FDR-adjusted P values lower than 0.05 are shown. The line thickness is proportional to the strength of the correlation. The red lines represent for positive correlations, and the blue lines represent for the negative correlations. The size of circle represents the relative abundance of the bacteria

healthy vaginal tract and the formation of a tumor-favoring microenvironment.

Our results highlight the crucial protective role of *Lactobacillus* in vaginal and cervical health, which is consistent with previous study (Norenhag et al., 2019; So et al., 2020). However, the *Lactobacillus* genus contains various species and not all species are beneficial to cervical health (Amabebe & Anumba, 2018; Witkin & Linhares, 2017). *Lactobacillus iners* is not only unable to produce D-lactic acid and H2O2, but also generates inerolysin, forms pores in vaginal epithelium to help virus infections (Curty et al., 2019; Rampersaud et al., 2011). A gradually decreasing proportion of CST I (*L. crispatus*-dominated) accompanied with a gradually growing proportion of CST III (*L. iners*-dominated) was observed from the control groups (NV- and NV +) to LISL, and further to HISL group. Therefore, we speculated that women with the depletion of CST I and expansion of CST III, no matter which group they belong to, have a high risk for deterioration of cervical diseases, and even the development of CC, which was featured by the complete loss of CST I together with the tremendous blooms of anaerobic and/or pathogenic bacteria. Therefore, routine cervical screening and monitoring of specific *Lactobacillus* species in clinic may be helpful for clinical evaluation of the CC risk. Meantime, HPV16 positive rate was significantly higher in the HSIL and CC groups than the Normal-LSIL group, which supported that HPV16 as highest risk factor for CC (Graham, 2017). Therefore, increased knowledge on the mechanisms about how different *Lactobacillus* species would maintain a healthy vaginal microenvironment will provide a basis for the use of probiotic as an adjuvant therapy of hrHPV (especially HPV16) infection and CIN in clinical practice to prevent from CC.

A previous cross-sectional study reported that several microbial taxa, including *Fannyhessea vaginae*, *Porphyromonas*, *Dialister*, *Peptoniphilus*, and *Bacteroides* enriched in tissue hyperplasia and cancer compared with benign uterine (Walther-António et al. 2016). Similarly, three of

above microbial taxa (*Fannyhessea vaginae*, *Dialister*, *Bacteroides*) were also found to be enriched in the CC group in this study. Several mechanisms are currently proposed for the role of vaginal microbes in cervical lesion. The blooms of anaerobes could increase the pro-inflammatory cytokine levels through several cellular pathways, promote active and persistent oncogenic virus (e.g. hrHPV, Epstein Barr virus and hepatitis C virus) infection, and exacerbate cervical disease (Akram et al., 2017; Anahtar et al., 2015; Carrero et al., 2015). In addition, some bacteria were reported to produce compounds such as colibactin and cytolethal distending toxins that can induce DNA damage and/or cell proliferation, and facilitate cancer development (Curty et al., 2019; Garrett, 2015; Schwabe & Jobin, 2013). However, the blooms of vaginal microbiota in different grades of cervical disease favor specific bacterial taxa and vary in different studies. The mechanisms of various bacteria alone or synergistically to exacerbate cervical disease need further studies (Garrett, 2015; Łaniewski et al. 2020).

The strengths of our study include that the gradual depletion of *Lactobacillus*, especially *L. crispatus*, and blooms of anaerobes are associated with the severity of the cervical lesions. However, this study is a cross-sectional investigation, rather than a longitudinal study, which is one of the limitations. Longitudinal samples from each woman would be very helpful in the understanding of the relationship among HPV infection, vaginal microbiome and the development of CC by providing dynamic information of various factors during the disease course, and deserves to be collected and investigated in future studies. Lack of clinical diagnosis on BV infection is another limitation, which makes it more complicated to interpret any associations of bacteria change with the cervical disease severity.

Future longitudinal cohorts, as well as multicenter investigations, would help to better understand the causality and diagnostic potential of vaginal microbiome, and HPV16 infection in the cervical cancer progression. Also, the establishment of corresponding predictive models and/or intervention measures will facilitate better prevention and treatment of CC.

Conclusion

In summary, we investigated the vaginal microbiome change in different status of cervical diseases, and concluded that the gradual depletion of *Lactobacillus*, especially *L. crispatus*, and blooms of anaerobes are associated with the severity of the cervical lesions. HPV16 infection was highly associated with higher vaginal microbiome diversity and the depletion of *Lactobacillus*. These results suggest specific *Lactobacillus* species (e.g. *L. crispatus* and *L. iners*) can be used as important markers to target prevention measures prioritizing HPV16-infected women and other high-risk women for test, vaccination and treat initiatives.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12275-023-00039-3.

Data Availability The raw data of 16S rRNA gene sequences were deposited in the CNSA (https://db.cngb.org/cnsa/) of CNGBdb (project number CNP0002796) under the accession numbers CNS0527767- CNS0527926.

Declarations

Conflict of Interest The authors declare no competing interests.

Ethical Statements The collection of samples was approved by the Ethics Committees of Obstetrics and Gynecology Hospital of Fudan University (No. 2021–103). Written informed consents were obtained from all patients before sample collection.

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